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A Novel Approach to Detect Donor/Recipent Immune Responses Between HLA-Identical Pairs

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ABSTRACT: Renal transplant rejection and graft versus host reactions between HLA genetically-identical sibling (HLA*gi*) donor/recipient (D/R) pairs are thought to result from minor histocompatibility antigen (mHAg) disparities. We have compared two methods of measuring HLA*gi* D/R T lymphocyte responses to "matured" dendritic cells: 1.) a modified Cylex assay of $CD4^+$ ATP levels (MLDC-ATP) versus 2.) ^{3}H-thymidine uptake (MLDC-^{3}H). The MLDC-ATP kinetics peaked at 48 hours versus the MLDC-^{3}H at 7 days, and appeared more sensitive. We tested HLA*gi* (normal) volunteer siblings (NLs), and D/R sibling pairs before and after renal transplantation (pre-Tx and post-Tx). The overall frequencies of positive responses in the MLDC-ATP for HLA*gi* NLs, pre-Tx, and post-Tx D/R pairs were 63%, 50%, and 42%, respectively. The percentage with reciprocal responses was 37.5%, 20%, and 22.22%, respectively. In one set of three HLA*gi* (NLs) siblings (two males and one female), there was a nongender-associated differential response. There was no MLDC correlation with class I MHC-associated mHAg (SSP) incompatibility, nor could some MLDC positive reactive pairs theoretically process the necessary HLA-class I restriction molecules for presentation of known (nanomeric) mHAg peptides. Speculatively, the MLDC reflects class II MHC-restricted mHAg reactions (not yet definable), with possible effects of other polymorphic (nonhistocompatibility) immune response genes, and thereby may be a useful measurement of $CD4^+$ T-cell HLA*gi* transplantation immunity. *Human Immunology 68, 350–361 (2007).* © American Society for Histocompatibility and Immunogenetics, 2007. Published by Elsevier Inc.

KEYWORDS: HLA identical renal transplant donor and recipient; cylex assay; mature dendritic cells; cytokine gene polymorphisms; minor antigen; single nucleotide polymorphisms

ABBREVIATIONS

HLAgi renal transplant D/R pairs	HLA genetically identical renal transplant donor/recipient pairs
mDC	mature dendritic cells
mHag	minor histocompatibility antigen

INTRODUCTION

Although less common than in other donor/recipient (D/R) combinations, rejection of renal transplants does occur between HLA genetically-identical sibling (HLA*gi*) pairs in whom 10-year graft (including patient) survival has hovered at 68–70% in recent analysis [1]. Graft versus host disease (GvHD) also occurs in HLA*gi* bone marrow transplantation. In both instances, this has been thought to result from an immune response against minor histocompatibility antigens (mHAgs) [2–4]. Procedures to measure *ex vivo* HLA*gi* sensitization, both pre-and post-transplant via either the $CD4^+$ or $CD8^+$ immune pathways would provide valuable clinical monitoring tools.

The weak immunogenicity of mHAgs has been associated with the inability to elicit a clearcut primary immune response *in vitro* using peripheral blood mononuclear cells (PBMC). Classical primary mixed lymphocyte reactions (MLR) and cytotoxic T lymphocyte assays

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(CTL) have not routinely detected such reactions. Effective antigen processing and presentation pathways are required to facilitate mHAg recognition [5]. It has been more recently demonstrated that there can be a high expression of mHAgs on dendritic cells "matured" *in vitro* (mDC) [6]. These cells also possess efficient antigen processing machinery to proteolytically cleave the mHAg proteins into 8–9 amino acid peptides, which have been clearly described to be presented by class I MHC molecules and also possibly into 13–18 amino acid peptides for presentation by class II MHC molecules to $CD8^+$ and $CD4^+$ T cells, respectively [7,8]. The mDC is able to present MHC-peptide complex both directly and indirectly, which makes it an elite antigen presenting cell (APC) of either donor or recipient origin in classical HLA disparate allograft immunity [6,9]. However, if a primary *in vitro* reaction between HLA*gi* pairs were to be routinely used (other than engendering mHAg specific clones), it must also take into account the presentation of non-mHAg auto-antigens by mDC of either the donor or recipient. With these impediments in mind, by using the mDC as an APC in HLA*gi* combinations, we have developed a modified Cylex assay to measure primary T cell activation via ATP levels, i.e., an early energy step requirement in a reaction involving $CD4^+$ responding cells, designated as a mixed lymphocyte dendritic cell culture (MLDC-ATP). For comparison, we also tested responding T cells in a ^{3}H-thymidine uptake assay using the same DC stimulating cells (MLDC-^{3}H). Normal HLA*gi* sibling volunteers (NLs) and HLA*gi* sibling D/R pairs pre- and postrenal transplant were tested in these assays, and compared with normal volunteer non-HLA-identical controls.

MATERIALS AND METHODS

Cell Subset Sample Preparations

Human peripheral blood was collected from HLA*gi* NLs, from pre- and postrenal transplant HLA*gi* D/R pairs, and from non-HLA-identical control volunteers after obtaining informed consent in an institutional review board approved study. In the HLA*gi* pair members, both male ↔ female and the converse occurred in the individual pairs. The specific information on these pairs is presented in the appropriate *Results* section. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient at 2,000 rpm for 20 min at 20°C, and the cells were washed with Phosphate buffered saline (PBS). Two procedures were used to generate mDC. In procedure #1, PBMC were placed into 6-well plates at $8–12 \times 10^6$ cells/well and incubated at 37°C for 30–40 min. The nonadherent cells were removed, placed in culture for seven days in 10% AB human serum RPMI-1640 (complete) medium and designated as T-cell preparations. The adherent cells were then scraped off the well bottoms with a rubber policeman, transferred into a plate at 2×10^6/well containing 15 ng/ml of GM-CSF and 5 ng/ml of IL-4 (BD PharMingen, San Diego, CA) in 10% fetal calf serum (FCS) RPMI-1640 medium, and then cultured for five days. This adherent cell population was cultured for an additional two days in the presence of a DC maturation cocktail containing concentrations of IFN-γ (10 ng/ml), TNF-β (50 ng/ml), IL-6 (100 μ/ml), and lipopolysaccharide (LPS) (20 ng/ml) (BD PharMingen, San Diego, CA). We have previously reported the quantitative characterization of these cells with respect to CD34, CD40, CD86, CD83, and Dcsign phenotypes [10]. In procedure #2, monocytes were isolated from PBMC using a $CD14^+$ Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Briefly, PBMC were incubated with CD14-selective microbeads (20 μl per 1×10^7 cells) for 15 min at 4°C. The cells were washed and separated by positive selection through columns placed in the magnetic field of a MACS separator (Miltenyi Biotec). A cell purity of more than 92% was consistently achieved as confirmed by flow cytometry. The CD14-negative cells were cryopreserved at −80°C with 90% FCS and 10% DMSO, to be used as responders in the cell proliferation experiments. The $CD14^+$ (monocytes) were then cultured in 6-well plates at a final concentration of 2×10^6 cells in 3 ml of complete media (RPMI-1640 with 10% human AB serum) supplemented with 5 ng/ml human recombinant GM-CSF and 10 ng/ml IL-4 (both from Sigma, St. Louis, MO) for 7–8 days at 37°C, in 5% CO_2 and 97% humidity. To induce mDC, complete medium supplemented with 10 ng/ml TNF-alpha, 1 μg/ml PGE_2 (both from Sigma, St. Louis, MO) and 10 μg/ml IL-1β (BD Pharmingen, San Diego, CA) was added for the final 48 hours. Fresh complete media with growth factors was added every second day during the entire culture period. Mature DCs were harvested for analysis on day 7–8 and cell viability was assessed by trypan blue.

Measurement of the T-cell Responses in Mixed Culture Using ATP Levels

The responder cells were obtained from the nonadherent cell fraction derived from the initial mDC production step above. In some experiments, these nonadherent cells were further fractionated into $CD3^+$ and $CD4^+$ populations using CD3 and CD4 isolation kits (Miltenyi Biotec) and the manufacturer's protocol (*vide supra*), before using them as responding cells in culture. The cultures contained 2×10^6 responders/well versus 0.5×10^5 mitomycin C-treated mDC-enriched autologous stimulators (negative controls), HLA*gi* sibling stimulators (experimental groups), or indifferent (non-HLA-identical)

allogeneic cells (I) (positive controls). Cultures were performed in quadruplicate for 48–72 hours (24–120 hours in preliminary experiments) in 96-well plates, with baseline values also recorded for responding and stimulating cells cultured separately. According to the manufacturer's instructions, the $CD4^+$ responder cell ATP level was obtained after the $CD4^+$ cells were isolated from the MLDC reaction using the CD4 cell isolation ImmuKnow kit (Cylex Inc.). In this procedure, 50 μl of CD4 beads were added into each well and incubated at room temperature for 30 min. The $CD4^+$ cells bound to the beads were pelleted using a magnet tray. After aspirating the medium, the pellets were washed with wash buffer once and lysed with 100 μl of lysis reagent to release ATP. Then 50 μl of cell lysate were transferred into the measurement plate, and 100 μl of luminescence-generating reagent (the ATP detection reagent) was added into each well. The plate was read using a luminometer after three to 10 minutes. An ATP value of responder to the HLA-identical stimulator partner greater than the control (responder-to-responder) plus two SD was considered as a positive reaction. To avoid complexity in nomenclature, even including normal volunteer HLA*gi* pairs, a designation of D and R was used as in the patient donor/recipient pairs, e.g., R+Dx > R+Rx (+ two SD), or D+Rx > D+Dx (+ two SD), in which R and D were designated as recipient and donor clinically as well as volunteer responder and stimulator, or vice versa, respectively. In addition "x" was designated as inactivation of the stimulating cells by either irradiation or mitomycin-C treatment (*vide infra*). The amount of ATP elevation in ng/ml over the autologous controls (+ two SD), i.e., the δATP was also recorded.

Measurement of the T-Cell Proliferative Response Using ^{3}H-Thymidine Uptake

The cryopreserved CD14-negative cells (*vide supra*) were thawed and used as responding cells cultured with irradiated (3,000 rad), stimulator mDC derived from either autologous cells, the HLA*gi* sibling, or allogeneic cell donors. The cell purity of these stimulators was equivalent to that in preparation #1. The cultures, performed in triplicate, were carried out in 96-well flat-bottom plates using 2×10^5 responders and 0.5×10^5 mDC in a total volume of 200 μl/well of complete culture medium at 37°C in a 5% CO_2 atmosphere for 5, 7, and 9 days. The wells were pulsed with 1 μCi/well of ^{3}H-thymidine for the last 18 hours before the cultures were harvested, with radioactive incorporation measured in a "WALLAC 1205 Betaplate" Liquid Scintillation Counter (LabX, Midland, ON, Canada). The same calculation of positive versus negative was performed as in the MLDC-ATP reaction,(i.e., R+Dx > R+Rx [+ two SD]). As mentioned, for simplification, the lower-case x as in Dx or Rx or Ix is used to describe either mitomycin-treated or x-irradiated inactivated stimulating cells.

HLA and mHAg Typing

HLA typing of subjects was performed on fresh peripheral blood mononuclear cells by the University of Miami Histocompatibility Laboratory, using serologic techniques confirmed by genotyping according to the standardized laboratory procedures. For mHAg typing, genomic DNA was extracted from whole blood with the QIAGEN kit (Qiagen Inc., Valencia, CA). The mHAgs were typed using the Dynal Allset (Invitrogen Corporation, Carlsbad, CA) typing kit and interpretative resolution was accomplished with agarose gel electrophoresis. This kit employs Sequence Specific priming PCR amplification for 11 mHAg alleles. Resolution delineates alleles that putatively produce peptides that are immunogenic and/or nonimmunogenic.

The identity of mHAg reactive (graft-versus-host) D versus R and (host-versus-graft) R versus D pairs was determined using the knowledge database of the Leiden University Medical Center Minor Histocompatibility Workshop (part of the 14th International HLA and Immunogenetics Workshop, Melbourne, Australia, November 29–December 3, 2005). After submitting the R/D minor and major histocompatibility typing, the program would predict the immune reactivity of R versus D and/or D versus R.

Statistics

All bars on graphs represent one standard error above and below the mean. Statistical evaluation of the data was performed with student's t tests, values of $P < 0.05$ were considered statistically significant.

RESULTS

Kinetics of the MLDC Reactions: ATP Levels and ^{3}H-Thymidine Uptake

To optimize the MLDC T lymphocyte response, the kinetics using ATP levels were studied in MHC mismatched normal volunteers and volunteer HLA*gi* sibling pairs. The MLDC-ATP assay was cultured from 24 hours to 120 hours, and ATP levels determined at 24-hour intervals. The results presented in Figure 1 show that the peak responses (ATP level) occurred at 72 hours using HLA*gi* sibling pairs ($n = 6$) and pairs of MHC-mismatched normal volunteers ($n = 3$). However, the peak ATP levels obtained at 48 hours was 90% of that obtained at 72 hours, with the maximum difference between the autologous response versus the response to the HLA*gi* siblings also occurring at 48 hours (not shown). Therefore, a 48 hour time period was used for the remainder of the studies.

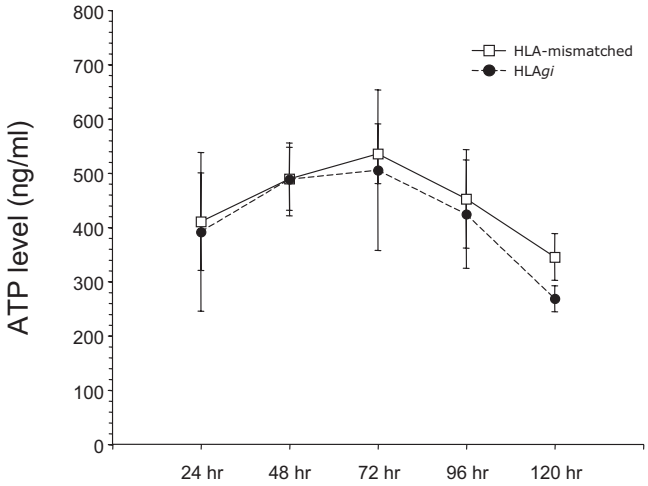

FIGURE 1 Kinetics of the MLDC-ATP assay response in normal HLA-mismatched (allogeneic) and HLA*gi* pairs. The MLDC-ATP assay was performed using HLA*gi* sibling pairs ($n = 6$) and non-HLA*gi* normal volunteers ($n = 3$) in time intervals of 24–120 hours. The data points ± one S.E. are depicted. A 48 hour incubation time was taken to be the optimum for the HLA*gi* MLDC-ATP assay. The control autologous responses fall at least two standard deviations lower than the HLA*gi* responses (see *Materials and Methods* and Table 1).

In contrast to the MLDC-ATP assay, the MLDC-^{3}H assay peaked (and differed the most from autologous controls, i.e., in a stimulation index) at 7 days ($n = 4$) (Figure 2), which is similar to the kinetics of the allogeneic MLR [11].

Parallel Measurements of MLDC Using ATP Levels Versus ^{3}H-Thymidine Uptake Assays

When we compared the MLDC-ATP versus the MLDC-^{3}H assays using peak kinetics in five HLA*gi* responder/stimulator (R versus D and D versus R) nonimmunosuppressed sibling pairs, the MLDC-^{3}H and the MLDC-ATP assays appeared to mirror each other in four of the five pairs tested. However, in one HLA D/R combination (pair #5, Table 1), MLDC-^{3}H was considered negative, whereas the MLDC-ATP assay was positive. The MLDC-ATP assay as used here is a measurement of only the $CD4^+$ responding cells. In a few experiments, when $CD3^+$ isolated cells were used with a $CD3^+$ isolation kit, there did not appear to be any more marked ΔATP changes than those already depicted using the $CD4^+$ kit (not shown). Therefore, δATP from the $CD4^+$; cells only were measured (see *Materials and Methods*) for all subsequent assays. As additional controls in all experiments, the responding cells (D and R) were cultured separately and the stimulating (R and D) mDCx backgrounds were always less than the autologous D+Dx or R+Rx reactions in both the MLDC-ATP and MLDC-^{3}H assays (not shown).

After analyzing the kinetics of the two assays (MLDC-^{3}H versus MLDC-ATP) (Figs. 1 & 2), and this preliminary comparison of the frequency of positive responses (Table 1), we concluded that it would be more expedient to utilize the MLDC-ATP assay in the subsequent studies to be described. Moreover, the MLDC-ATP assay is a modification of the Cylex ImmuKnow assay that is being used to provide a global assessment in monitoring the cellular immune status of immunosuppressed organ transplant patients [12, 13, and might be compared in the future with monitoring MLDC assays postoperatively in HLA*gi* D/R pairs.

The MLDC-ATP Assay Detects NonMHC Antigen Differences in Normal HLA*gi* Sibling Pairs

We analyzed, using the MLDC-ATP assay, five normal volunteer HLA*gi*, sibling pairs, and one family with three HLA*gi* siblings for a total of 16 possible responder/stimulation combinations. Each member of a pair was designated as R or D (see nomenclature in *Materials and Methods*). In Figure 3A, three pairs (pairs #1, 3, and 5) tested were reciprocally positive (R+D and D+R). Pairs #2, 4, and 7 showed either a single nonreciprocal positive R-to-D or D-to-R response, and pair #8 had no response in either direction. However, the reproducible variations of the response (when the same pairs were tested at two different time intervals) appeared to demonstrate a dif-

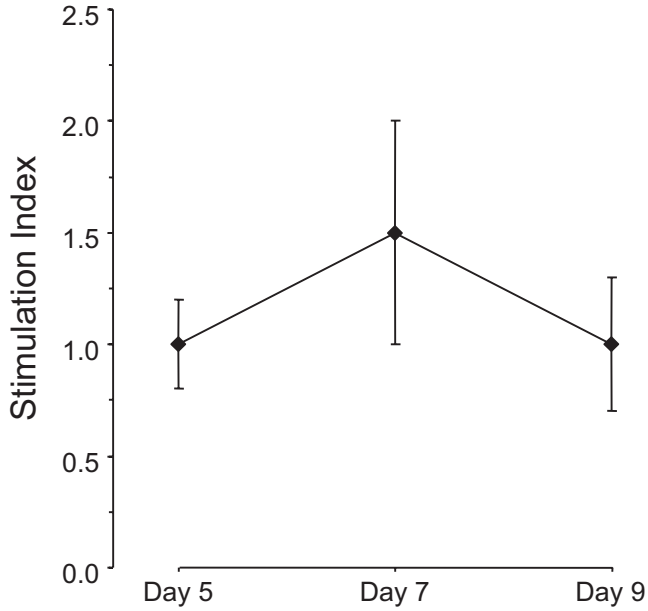

FIGURE 2 Kinetics of the MLDC-^{3}H response. The MLDC-^{3}H assay was performed in intervals of 5, 7, and 9 days ($n = 4$ in data points ± one SE.). The autologous reactions were also more than two standard deviations different than the HLA*gi* pairs, but the differences were more variable (see Table 1).

TABLE 1 MLDC-ATP and MLDC-^{3}H-TdR reactions in HLA*gi* sibling pairs

Volunteer sibling or patient D/R pairs[a] and HLA typing	^{3}H-TdR (cpm) 7 day assay (mean ± SD)	ATP (ng/ml) 48-hour assay (mean ± SD)	MLDC response[b] Δcpm	Interpretive result	ΔATP	Interpretive result
1. R (*m*): A-,2;B35,-;DR3,8						
D (*f*): A-,2;B35,-;DR3,8						
R+Rx	1,589 ± 229	619 ± 34				
R+Dx	870 ± 548	376 ± 33	—	neg	—	neg
D+Dx	585 ± 91	426 ± 29				
D+Rx	1,860 ± 179	571 ± 82	1,093	pos	87	pos
2. R (*f*): A24,33;B44,52;DR11,15						
D (*f*): A24,33;B44,52;DR11,15						
R+Rx	316 ± 32	479 ± 38				
R+Dx	320 ± 56	376 ± 16	—	neg	—	neg
D+Dx	304 ± 110	188 ± 6				
D+Rx	571 ± 67	404 ± 50	47	pos	204	pos
3. R (*f*): A24,34;B14,53;DR1,8						
D (*f*): A24,34;B14,53;DR1,8						
R+Rx	1,328 ± 753	331 ± 44				
R+Dx	1,671 ± 1,490	334 ± 58	—	neg	—	neg
D+Dx	3,641 ± 2,391	747.82 ± 59.32[c]				
D+Rx	2,987 ± 2,231	747.84 ± 59.35	—	neg	—	neg
4. R (*f*): A1,29;B35,44;DR4,7						
D (*m*): A1,29;B35,44;DR4,7						
D+Dx	1,399 ± 386	133 ± 19				
D+Rx	1,159 ± 431	136 ± 46	—	neg	—	neg
R+Rx	1,812 ± 642	201 ± 44				
R+Dx	2,180 ± 1,015	217 ± 17	—	neg	—	neg
5. R (*f*): A24,31;B44,35;DR9,17						
D (*m*): A24,31;B44,35;DR9,17						
D+Dx	16,479 ± 6,624	618 ± 62				
D+Rx	15,331 ± 7,358	585 ± 28	—	neg	—	neg
R+Rx	7930 ± 2101	170 ± 8				
R+Dx	11,470 ± 2,984	371 ± 33	—	neg	185	pos

[a] Pairs #1, 2, and 3 are laboratory volunteer HLA*gi* sibling pairs. Pairs #4 and 5 are renal pre-transplant HLA*gi* D/R pairs (see *Methods* for R/D, D/R nomenclature). **The (*m*) or (*f*) indicates male or female**.

[b] Δcpm (^{3}H-TdR incorporation assay) and ΔATP values of responder to the HLA-identical partner greater than the control (responder to responder) plus two SD is considered a positive reaction, (e.g., R+Dx > R+Rx plus two SD). The same calculation was applied to D+Rx vs. D+Dx. A negative value is not interpreted in this study and, therefore, not recorded other than by a negative sign.

[c] Coincidental equivalence. Differences only demonstrated by extension to two decimal points.

ferential ability of HLA*gi* siblings to stimulate and/or respond to each other. The frequency of a positive response in all combinations was 63% for normal HLA*gi* siblings, with the ATP value of responder to the HLA-identical partner (R+Dx or D+Rx) greater than the control (R+Rx or D+Dx) plus two SD considered as a positive reaction (see *Materials and Methods*). Figure 3B summarizes the 10 positive responder/stimulator combinations. There was a significant difference between the ATP levels of the autologous responses as contrasted with the positive sib versus sib responses ($p = 0.0003$). These findings were consistent with the notion that, in HLA*gi* siblings, the MLDC assay could reproducibly amplify the detection of non-MHC antigens recognized by $CD4^+$ responding cells.

Mature DC Induce Variable But Reproducible Primary $CD4^+$ T-Cell Responses to NonMHC Antigens in MLDC ATP Assays in a Set of Three HLA*gi* Siblings

Three HLA*gi* volunteer siblings (two males and one female) were analyzed by the MLDC ATP assay. Male #1 and Male #2 responded strongly to the Female (Fig. 4). The Male #1 response to Male #2 and to the Female was five- and sixfold, respectively, when compared to self. In contrast, Male #2 did not respond to Male #1 and the response was only 1.5-fold versus the Female when compared to self, i.e., did not differ by two SD. The Female response to Male #1 was two-fold that to Male #2 (and significant by greater than two SD). Therefore, the pattern of response in the MLDC-ATP assay ($CD4^+$ cells)

A

MLDC ATP levels

Pairs	R+Dx partner D+Rx partner	R+Rx self D+Dx self	2 S.D.	ΔATP	Interpretive Result
1. R	418	308	98	12	pos
D	527	346	60	121	pos
2. R	611	472	24	115	pos
D	529	513	90	-	neg
3. R	467	119	42	306	pos
D	628	119	42	467	pos
4. R	133	226	42	-	neg
D	343	226	42	75	pos
5. R	196	103	34	59	pos
D	354	103	34	217	pos
6. R	376	619	68	-	neg
D	571	426	58	87	pos
7. R	376	479	76	-	neg
D	404	188	12	204	pos
8. R	334	331	88	-	neg
D	748	748	108	-	neg

B

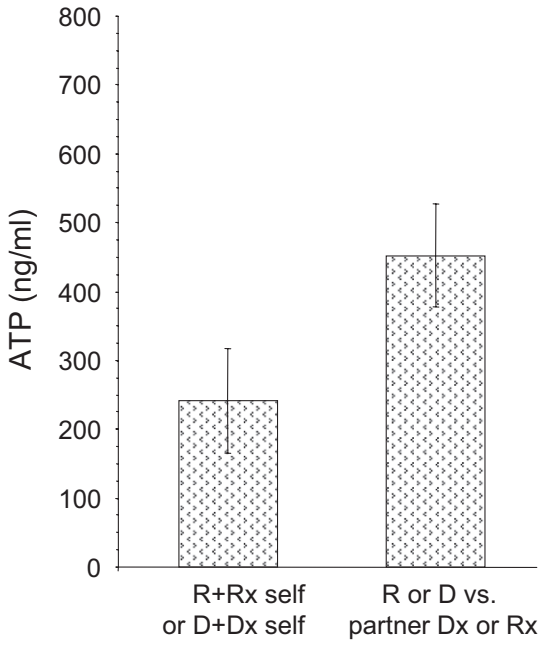

FIGURE 3 The MLDC-ATP assay detects a primary immune response in HLA*gi* normal siblings. (A) Eight normal HLA*gi* sibling pairs were tested using the MLDC-ATP assay (five pairs of HLA*gi* sibling NLs and one family of three HLA*gi* sibling NLs). In (B), when we summarized the 10 positive responder pairs, a δATP mean value for positive responders was found of 116 ± 75 SE ($n = 10$). When the average autologous response versus the average (positive) response to the HLA*gi* siblings were compared, a p value of 0.0003 was obtained.

was different for each of the HLA-identical siblings, suggesting that the stimulatory determinants (mHAg) exhibited polymorphism. Additionally, there was no direct gender-associated response. However, when taking into account the HLA A, B, and DR typing, it is also consistent with the notion that this $CD4^+$ cell-dependent MLDC-ATP assay was not detecting classical mHAgs previously shown to be presented by class I MHC molecules in a $CD8^+$ CTL response (2) (*vide infra*, Table 3).

The MLDC-ATP Assay Detects a Primary $CD4^+$ T-Cell Response Between HLA*gi* D/R Pairs Both Pre- and Post-transplant

Ten HLA*gi* kidney transplant D/R pairs were analyzed with the MLDC-ATP assay pre-transplant. In the D+R and R+D combinations, two pairs of 10 were reciprocally positive in the ATP assay (Figure 5A, pairs #1 and 4). Moreover, four of the 10 recipients responded to their (eventual) donors, whereas six of the donors responded to their recipients (Figure 5A). Therefore, the overall frequency of a positive MLDC-ATP response was 50% for the D/R pairs (D+Rx or R+Dx) pre-transplant (compared with the 63% for normal volunteer HLA*gi* sibling pairs) (Figure 3A).

Eighteen HLA*gi* D/R pairs postrenal transplant were then assayed by MLDC-ATP, whose recipients were on stable immunosuppressive regimens and were between 4 and 180 months after transplantation (Table 2). The responding cells had been precultured for seven days prior to preparing the assay (as per the *Materials and Methods* section description re: nonadherent cells), which may have reduced an immunosuppressive drug effect. Nine of the D+Rx reactions (50%) and seven of the R+Dx reactions (39%) were positive. Four of the pairs (#1, 2, 3, and 4) were reciprocally positive (see p values in footnote to Table 2).

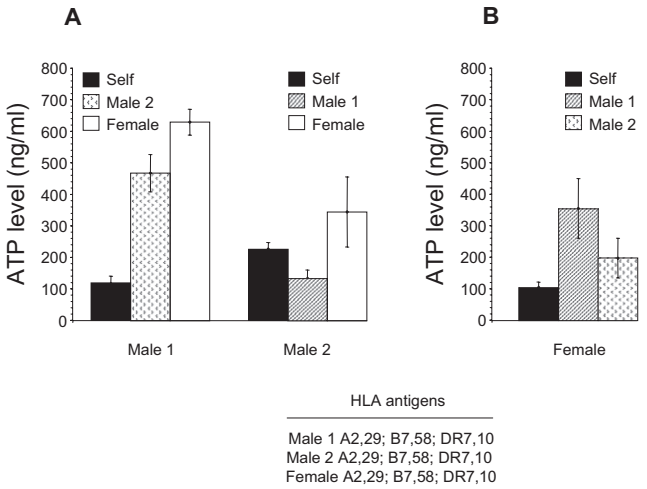

FIGURE 4 The MLDC-ATP assay exhibited a differential response in three normal HLA*gi* siblings. The MLDC-ATP assay was performed on three HLA*gi* siblings (two males and one female). In (A) is shown the response of Male #1 and Male #2 versus each other and the female. The response of Male #1 was stronger than Male #2. Also shown in (A) was the observation that Male #2 did not respond to Male #1. In (B) is shown the response of the female to both Male #1 and Male #2. Vertical bars on the graphs represent one standard error.

The Effect of mHAg Polymorphism on the MLDC-ATP Assay Measurement in HLA-Identical Donor/Recipient Pairs Post-transplant

As stated above, the mHAgs have been reported to be presented mainly to $CD8^+$ cells in the context of MHC class I molecules [2, 5, 14]. However, the MLDC-ATP assay measures the primary immune reactivity of $CD4^+$ cells. It is in this context that we compared the mHAg DNA typing of nine HLA-identical D/R pairs post-transplant with their MLDC-ATP responses. The nine D/R pairs represented recipients transplanted from 6–168 months previously. The theoretical R→D and D→R responses were determined based upon the mHAg mismatches of the D/R immunogenic peptides that should induce a positive response (in $CD8^+$ cells) by the presentation of the mHAg peptides by class I MHC molecules to T cells [15]. In Table 3, the predicted immune responses of the nine D/R pairs by mHAg genotyping that should have been induced in the R→D and D→R reactions were compared with the observed responses as measured by the MLDC-ATP assay. The MHC class I molecules by histocompatibility typing of the HLA*gi* pairs and the disparate mHAg peptides, along with the HLA restriction molecules for these mHAg peptides [15], are shown. The theoretical negative reactions of pair #4 correspond with the MLDC-ATP assay with respect to D and R responses, in that nothing should have been reactive, and that nothing was. However, the other pairs do not exactly correspond. Moreover, in pairs #4, 5, 6, and 9, mHAg typing would predict that there should be no response in these pairs. However, in pairs #5 and 6, there was a positive D→R MLDC response (29 and 56 ng δATP/ml), whereas in pair #9 there was a positive R→D MLDC (86 ng δATP/ml). Moreover, when examining the D/R pairs with respect to MHC class I antigens that could serve as restriction molecules (i.e., could present the D/R pair disparate peptide), only three of the nine pairs were found. Pair #1 (DD/LD) HLA-A1 was disparate at HA-3, such that the recipient DD (nonimmunogenic M peptide) could respond to the donor LD (T immunogenic peptide) using HLA-A1 restriction molecule. In pair #2, using A2/HA-1, and pair #8, using B44/HB-1 combinations, reactivity might also have occurred. However, there was no R→D or D→R MLDC-ATP response in pair #1, and no R→D in pair #8. In the other pairs that were disparate in mHAgs to produce R→D and D→R responses (pairs #3 and 7), the appropriate HLA-restriction molecule to present the D/R disparate peptide was not present, i.e., there was no correlation of these observations with the presence of a positive MLDC-ATP assay response. If one assumes that seven days in culture of the responding (nonadherent) cells might have removed the immunosuppressive effect, then the *ex vivo* positive MLDC-ATP assays would have reflected the immune status of the recipients to donor immune stimulator determinants that activated $CD4^+$ cells in these HLA*gi* patients. This is consistent with the notion that the stimulatory molecules for the MLDC-ATP assay response may differ from the classical mHAgs, in that class II MHC molecules and associated peptides could be involved in the MLDC-ATP assay.

DISCUSSION

A long-term goal in transplantation is to induce permanent immunologic tolerance. Even today, however, when HLA*gi* D/R kidney transplant pairs are followed, graft loss occurs because of alloimmune mechanisms, although using partial myeloablation and donor bone marrow infusions, some success in tolerance reduction appears to have recently been attained [16]. Therefore, there has been a renewed focus on non-MHC molecules, including mHAgs, and their involvement in organ transplantation [17]. We feel that matching mHAgs and newly-defined polymorphic alleles involved in activation and/or regulation in HLA*gi* donor/recipient pairs may promote a tolerant state. In contrast to the concept that mHAg matching may be helpful, the opposite has been reported. In this study, the mismatching of HA-1 mHAgs induced $CD8^+$ T regulatory cells that specifically suppressed HA-1 specific effectors [14]. However, there is currently no routine method for monitoring the responses to

TABLE 2 MLDC-ATP assay in HLA*gi* D/R pairs post-renal transplant

Pair #	HLA*gi* D/R pairs[a]	ΔATP D→R[b]	Interpretive result	ΔATP R→D[b]	Interpretive result
1	PW (*f*)/GW (*m*)	57	pos	111	pos
2	GJ (*f*)/AJ (*f*)	320	pos	69	pos
3	EP (*m*)/RP (*m*)	50	pos	16	pos
4	DL (*f*)/SC (*m*)	6	pos	45	pos
5	SE (*f*)/DE (*m*)	64	pos	—	neg
6	IA (*m*)/EA (*m*)	109	pos	—	neg
7	MG (*f*)/GM (*f*)	56	pos	—	neg
8	CD (*f*)/AD (*f*)	29	pos	—	neg
9	AW (*m*)/MW (*f*)	222	pos	—	neg
10	CN (*f*)/TO (*f*)	—	neg	86	pos
11	C,CAR (*m*)/C,CAM (*f*)	—	neg	62	pos
12	NI (*f*)/AI (*f*)	—	neg	160	pos
13	AL (*f*)/BL (*f*)	—	neg	—	neg
14	RF (*m*)/JS (*f*)	—	neg	—	neg
15	DC (*m*)/RC (*m*)	—	neg	—	neg
16	LD (*f*)/DD (*f*)	—	neg	—	neg
17	EG (*m*)/MH (*f*)	—	neg	—	neg
18	MC (*f*)/LC (*m*)	—	neg	—	neg

The (*m*) or (*f*) indicates male or female.

[a] ATP value of responder to the HLA-identical partner greater than the control (responder-to-responder) plus two SD is considered a positive reaction, i.e., R+Dx greater than R+Rx plus two SD. The ΔATP is that calculated value in ng/ml. The same calculation was applied to D+Dx and R+Dx. When all positive combinations were averaged, the *p* value for D+Rx vs. D+Dx = 0.00048 and R+Rx vs. R+Dx = 0.0016. A negative value (as in Table 1) is not interpreted in this study and, therefore, not recorded other than by a minus sign.

mHAgs or other non-MHC D/R responses in transplant recipients other than the generation of specific T-cell clones *in vitro*. A few isolated reports have demonstrated the feasibility of applying a modified MLR reaction that uses mDC as stimulators [18, 19]; the *trans vivo* DTH [20], and CTL [21] have also been used. In this study, we have utilized the unique capacity of mDC to primarily stimulate lymphocytes in an energy consumption (and lymphoproliferation) assay in HLA*gi* sibling volunteers and in HLA*gi* sibling D/R pairs pre- and post-transplant. We measured this stimulation of T cells (mainly $CD4^+$) by intracellular ATP levels and compared it with ^{3}H-thymidine uptake in reactions termed MLDC-ATP and MLDC-^{3}H. Although the reactions were somewhat similar, the peak kinetics of the reactions in HLA*gi* pairs presented in Figures 1 and 2 and in Table 1, revealed that the MLDC-ATP assay could be more reproducibly measured at 48 hours versus a seven-day peak reaction that appeared not to be as sensitive in the more classical MLR reaction of the MLDC-^{3}H assay. In normal HLA*gi* sibling pairs, 63% of the siblings tested gave a positive MLDC-ATP response, as defined by greater than two SD of δATP from the autologous control (the latter presenting autoantigens as well in an auto-MLR) 22]. Although only 37.5% of the sibling pairs showed a reciprocal response, in some the magnitude of the response (δATP) was as high as 5:1 versus the autologous controls, suggesting that pair members could reproducibly differ in varying degrees in their stimulatory and/or responding elements. We further examined three HLA*gi* siblings of both genders and found that the nongender-related pattern of responses (putatively to nonclass I-associated mHAgs) of Male #1 versus Male #2 and of the female versus the males was unique for each individual sibling, suggesting polymorphism in stimulatory determinants (Fig. 4) or perhaps additional reactivity associated with other amplifying nonhistocompatibility immune response genes, not yet as well characterized [23–25].

We next examined ten HLA*gi* D/R pairs pre-transplant using the MLDC-ATP assay, (i.e., recipients [R] with end-stage renal disease) before transplantation from their HLA*gi* sibling donors (D) (Figure 5). The results were similar with somewhat less frequently positive MLDC reactions than those seen using normal HLA*gi* siblings (Figures 3 and 4). Additionally, we tested 18 HLA*gi* renal D/R pairs post-transplant (culturing the responding cells for seven days prior to the MLDC-ATP assay, which possibly removed immunosuppressive effects), with even somewhat fewer reactions than in patients with end stage renal disease (ESRD) pre-transplant. The descending order of the frequency of positive reactive pairs in the three groups, after further confirmation by adding more HLA*gi* paired samples, might be a reflection of the uremic disease state pre-Tx and immunosuppressive state post-Tx.

TABLE 3 The effect of mHAg polymorphism on the *ex vivo* MLDC-ATP measurement in HLA-identical donor/recipient pairs at 6 to 168 months post-Tx

Pair #	(Recipient initials/ donor initials)*	Class 1 HLA antigens and MHC restriction[a]	mHAgs[d]/HLA restriction											Predicted immune response[e]	Observed MLDC-ATP		Month post-Tx
			HA-1 A2,B60	HA-2 A2	HA-3 A1	HA-8 A2	HB-1 B44	ACC-1 A24	ACC-2 B44	HwA-9 **A3**	HwA-10 **A3**	UGT2B17 A29	HY B8		ΔATP	ng/ml[f]	
1	DD (*f*)	A1 11 B35,18[a]	R	[i]**V**/M	M	R/P	H	C	G	**R**/G	R/stop	EXt1A	NEG	R→D	R+D	Neg	(6)
	LD (*m*)	(+)[b]	R	**V**	[i]**T**/M	R/P	H	C	G	R/G	R/stop	EXt1A	NEG	D↛R	D+R	Neg	
2	BL (*f*)	A2 11 B40,46	[i]**H**/R	**V**	**T**/M	P	[i]**H**	Y/C	G	R	STOP	NEG	NEG	R↛D	R+D	Neg	(167)
	AL (*f*)	(+)	R	**V**	**T**/M	P	**H**/Y	Y/C	G	R	STOP	NEG	NEG	D→R	D+R	Neg	
3	EA (*m*)	A2 B7.35	**H**	**V**/M	**T**	R/P	**H**	C	G	G	R/stop	EXt1A	POS	R→D	R+D	Neg	(44)
	IA (*m*)	(−)[c]	**H**/R	**V**/M	**T**/M	R/P	**H**	C	G	[i]**R**/G	R/stop	EXt1A	POS	D↛R	D+R	109	
4	JS (*f*)	A28,30 B44,18	R	**V**	**T**/M	P	**H**	[i]**Y**/C	[i]**D**/G	**R**/G	[i]**R**	EXt1 A	POS	Neg	R+D	Neg	(168)
	RF (*m*)	(−)	R	**V**	**T**	P	**H**	**Y**	D	**R**	**R**/stop	EXt1A	POS	Neg	D+R	Neg	
5	AD (*f*)	A2,30 BW42 BW53	R	**V**	**T**	P	**H**	C	G	**R**/G	**R**	EXt1A	NEG	Neg	R+D	Neg	(21)
	CD (*f*)	(−)	R	**V**	**T**	P	**H**	C	G	**R**/G	**R**	EXt1A	NEG	Neg	D+R	29	
6	MG (*f*)	A2,3 B35,53	**H**/R	**V**/M	**T**/M	R	**H**	**Y**/C	**D**/G	**R**	**R**	EXt1A	NEG	Neg	R+D	Neg	(66)
	GM (*f*)	(−)	**H**/R	**V**/M	**T**	R	**H**	**Y**/C	**D**/G	**R**	**R**	EXt1A	NEG	Neg	D+R	56	
7	MW (*f*)	A26,33 B35,35	**H**/R	**V**	M	[i]**R**/P	**H**/Y	**Y**/C	**D**/G	**R**	**R**/stop	EXt1A	POS	R→D	R+D	Neg	(66)
	AW (*m*)		**H**/R	**V**	**T**/M	**R**	**H**/Y	**Y**/C	G	**R**	**R**	NEG	POS	D→R	D+R	222	
8	LC (*m*)	A29,31 B44,60	**H**	M	**T**/M	P	Y	C	G	G	**R**/stop	NEG	NEG	R→D	R+D	Neg	(10)
	MC (*f*)	(+)	**H**	**V**/M	**T**/M	**R**/P	**H**/Y	**Y**/C	**D**	G	**R**/stop	NEG	NEG	D↛R	D+R	Neg	
9	OT (*f*)	A2,3 B35,44	R	**V**	M	P	**H**	C	G	**R**	**R**	EXt1A	NEG	Neg	R+D	86	(162)
	NC (*f*)	(−) (−)	R	**V**	M	P	**H**	C	G	**R**	**R**	EXt1A	NEG	Neg	D+R	Neg	

The (*m*) or (*f*) indicates male or female.

[a] Underline indicates HLA restriction molecule(s) are present in the donor and recipient HLA antigens for presentations of mHAgs, i.e., in pair #1, donor LD is A1 that presents HA-3 peptide T to recipient DD, therefore R→D, see footnote e. Class 1 HLA antigens that are not restriction molecules are not underlined. As such, there is no minus (−) or plus (+) sign accompanying pair #7, because no known restriction molecule is present in the antigens of this pair.

[b] The (+) indicates that the D/R pair can utilize the restriction molecule for mHAg peptide presentation.

[c] The (−) indicates that the disparate mHAg peptide(s) in the D/R pair is not presented by the HLA restriction molecules (i.e., in pair #3, A2 does not present H or V since they are present in both donor and recipient).

[d] The box indicates D and R disparities in mHAg peptides. [i] = immunogenic peptide (in bold); the other letters represent the nonimmunogenic peptides.

[e] The predicted reaction R→D, D→R is based upon the mHAg mismatches that theoretically should induce a response in $CD8^+$ cells (14,15) by the presentation of mHAg peptides by MHC class 1 molecules. By mHAg genotyping, R→D = The recipient (R) response is predicted to respond to the donor (D); D→R, the donor (D) is predicted to respond to the recipient (R). By mHAg genotyping, R↛D = The recipient (R) is predicted not to respond to the donor (D); D↛R = the donor (D) is predicted not to respond to the recipient (R). In this Predicted Immune Response column, negative (Neg) = no response would be predicted in either direction.

[f] For the calculation of ΔATP, see Methods i.e., a positive reaction is the ΔATP in ng/ml greater than two SD from the autologous controls.

A

Donor	D+Rx	D+Dx	2 S.D.	ΔATP	Interpretative Result
1	666	86	82	498	pos
2	763	501	20	242	pos
3	915	613	86	216	pos
4	553	330	14	209	pos
5	431	381	40	10	pos
6	423	353	112	-	neg
7	359	341	44	-	neg
8	136	133	38	-	neg
9	585	618	124	-	neg
10	330	303	26	1	pos

Recipient	R+Dx	R+Rx	2 S.D.	ΔATP	Interpretative Result
1	692	336	120	236	pos
2	113	126	52	-	neg
3	727	777	98	-	neg
4	461	323	136	2	pos
5	703	622	200	-	neg
6	314	241	80	-	neg
7	741	572	66	103	pos
8	217	201	88	-	neg
9	371	170	16	185	pos
10	279	259	46	-	neg

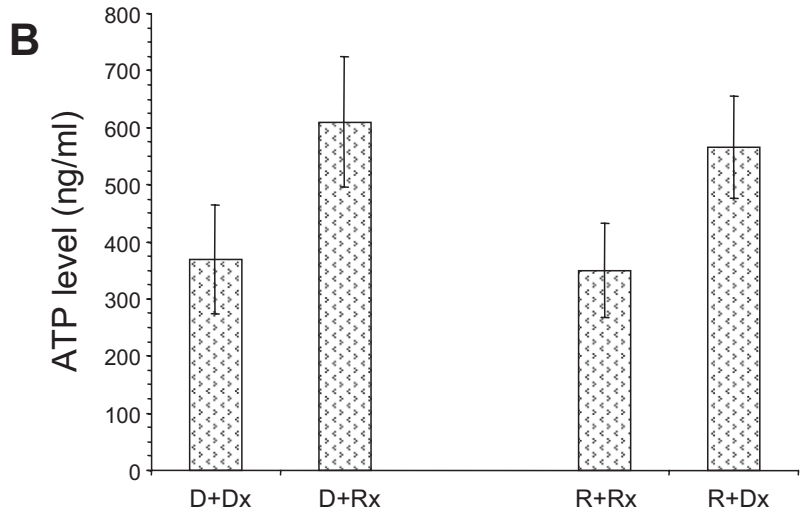

FIGURE 5 The MLDC-ATP assay detects a primary $CD4^+$ cell response in HLA*gi* pre-transplant D/R pairs. Ten HLA*gi* pre-transplant D/R pairs were assayed. In (A) is shown the data for 10 pairs. The frequency of response for the D+Rx and the R+Dx are 60% and 40%, respectively. In (B), the data for the six D+Rx positive response pairs and the four R+Dx positive response pairs is summarized graphically. The overall δATP mean value for positive responders D+Rx was 196 (±96 SE) ($n = 6$), whereas the overall δATP mean value for positive responders R+Dx was 132 (±51 SE) ($n = 4$). The p value of 0.016 ($n = 6$) was obtained for D+Dx versus D+Rx, and 0.010 ($n = 4$) for R+Rx versus R+Dx.

We finally compared the mHAg type by SSP analysis of the known mHAgs characterized thus far, with the MLDC-ATP assay response in nine HLA*gi* D/R pairs postrenal transplant (6–168 months). It was seen (Table 3) that one of the nine pairs in MLDC-ATP assays (negative) corresponded to the predicted (negative) immune reactivity and eight pairs did not. Several pairs (Table 3), which were theoretically negative using the mHAg genotyping calculation, were positive with respect to either the D+Rx or R+Dx MLDC-ATP assays. Reactivity or nonreactivity in the MLDC-ATP assay to date are more consistent with the concept that the $CD4^+$ cells are activated by nonclassical mHAgs. These findings are similar to those reported previously [19] that demonstrated in MLDC-^{3}H reactions that both the proliferative and cytotoxic ($CD8^+$ clone) induced T cells were not mHAg antigen-specific. We therefore hypothesize that MHC class II-associated peptides possibly amplified by other non-MHC (more recently defined) immune response gene polymorphism components [23–25] might be involved. Such MLDC reactions may help to facilitate the characterization of these genes and the role they play in transplantation immunity, as the MLR helped clarify the HLA system a generation ago.

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