Assay of HLA-D Disparity

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Until recently, the only method of assaying disparity for the HLA-D system has been the mixed leukocyte culture (MLC) test. While the MLC test is useful for finding two individuals who are HLA-D identical (no stimulation in MLC) and can be used to quantitate the amount of HLA-D disparity in what appears to be a meaningful way, it does not give information about the individual HLA-D antigens carried by the two donors of cells for the MLC.

The introduction by Bradley et al. of the homozygous typing cell (HTC) test represented a major step forward in this regard. For the first time, one could use the cellular techniques that have been related to the HLA-D system for “defining” the antigens carried by the cells of any one individual. The HTC test was based on the concept that if the stimulating cells used in MLC are homozygous for the LD (L determinant or lymphocyte-defined) antigens responsible for MLC stimulation, then responding cells either heterozygous or homozygous for these antigens would not react to that HTC.

In practical usage, HTC’s have been very valuable for picking out individuals who either have no response or a relatively very weak response and, with at least certain HTC’s, separate the weak response of these individuals to the HTC from the strong response of many other individuals who apparently do not carry the determinants of that HTC.

Several explanations have been offered for the relatively low-level response seen to an HTC when cells of individuals are used as responding cell donors, which purportedly carry the HLA-D haplotype of the stimulating cell. One factor that in all likelihood can play at least some role in this low level, but significant, response is “back-stimulation.” Back-stimulation occurs when the stimulating cell (treated with mitomycin C or X-irradiation) responds to foreign antigens of the responding cell and puts out factors that then cause the responding cell to proliferate. A second proposed explanation is that, in fact, the HLA-D system is complex in that a single haplotype code for more than one determinant. This concept, if correct, raises several problems regarding HTC testing. The low-level response of some cells, which might be thought to carry the haplotype of the HTC, could be explained by the fact that those cells carry only some of the determinants of that haplotype but not others. An HTC from an individual who is not genotypically homozygous secondary to inbreeding may not be truly homozygous; such an HTC might carry two HLA-D haplotypes that code for some determinants in common but that also differ to some degree.

Even given the above stated reservation, HTC testing has been useful in defining six “clusters” of the HLA-D system (HLA-Dw1 through Dw6). Two more have been defined in a more tentative manner (LD 107 and LD 108). We use the term “cluster” in this regard to refer to the possibility that a single HLA-D haplotype codes for a number of different determinants that are fre-
quent associated in terms of their presence in
the population. 6, 7

We have developed a new test for assaying
HLA-D disparity. 8-13 This test, which we
have called primed LD typing or PLT, is
in part based on the initial observation of
Andersson and Häyry 14 that lymphocytes
stimulated in vitro in an MLC will give a
secondary-type, rapid proliferative response
when restimulated with cells of the original
sensitizing cell donor several days past the
peak of the primary proliferative response.
This observation, initially made in mouse, 14, 15
has been extended to man. 16-18

Our work 8-13 demonstrated, in addition to
the finding that a secondary-type prolifera-
tive response could be elicited in man and
that this response was a method of detecting
the HLA-D determinants, 19 that it could be
used in the unrelated population for HLA-D
typing.

The test has several advantages. First, re-
sults can be obtained within 24 hr, which
makes this procedure potentially applicable
to matching for cadaver kidney transplanta-
tion. Secondly, since the lymphocytes that
are eventually used as the PLT cells are
primed to only a single haplotype, the hope
exists that PLT cells can be prepared against
individual HLA-D determinants and, to the
extent that a single HLA-D haplotype codes
for more than one determinant, individual
reagents would be available to measure
these determinants. Thirdly, and perhaps of
greater importance, is the ability to develop
reagents against essentially any HLA-D de-
determinant by preparing PLT cells within a
family in which the gene for that deter-
minant is segregating. We have recently
reviewed the evidence that suggests that it
is only the HLA-D system that is respon-
sible for restimulation in the PLT test; 20
more work on this is strongly needed, includ-
ing family segregation studies.

Several laboratories have adopted the
PLT procedure as described; the subse-
cquent data obtained provide evidence that the
antigens measured with HTC's are, in all
probability, the same as those that restimu-
late in the PLT test. 8, 21 An example of an
experiment demonstrating this is given in
Table 1 from collaborative work with Dr.
Mogens Thomsen and Dr. Arne Sveigaard. 8

In that study, individuals negative for HLA-
Dw2 were stimulated with Dw2 HTC's to
prepare PLT cells against the determinants
of the Dw2 haplotype. The PLT was then
restimulated with individuals negative for
Dw2, those heterozygous for Dw2, and
those homozygous for Dw2; the results
showed an HTC-PLT correlation.

The PLT test procedure that we have
used throughout these studies has recently
been described in detail. 13 In brief, an MLC
is set up between family members differing
by one haplotype. The cells are incubated
for 10 days, at which time they are frozen
in 10% dimethylsulfoxide (DMSO). The
cells are thawed and cultured in microtiter
plates with restimulating cells from either
the individual used as the donor of the re-
sponding cells in the primary MLC (the
control restimulator), cells from the in-
dividual used as the sensitizing restimulating
cell in MLC (the reference restimulator), or
cells from other individuals (test restimula-
tor cells). The PLT cultures are labeled with
3H-TdR from 40 to 48 hr (although
earlier labeling is possible), and counts
incorporated in the individual wells are as-
sayed by use of an automated harvester
(Otto Hiller, Madison, Wisc.) and liquid
scintillation counting.

<table>
<thead>
<tr>
<th>Table 1. PLT-Homozygous Typing Cell Correlation</th>
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<tbody>
<tr>
<td>Responding PLT</td>
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<td>Restimulating Cells</td>
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<tr>
<td>A (Dw2-negative)</td>
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<tr>
<td>BB (Dw2-negative)</td>
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<tr>
<td>BKJ (Dw2-heterozygous)</td>
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<td>MT (Dw2-heterozygous)</td>
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<td>RR (Dw2-homozygous)</td>
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A PLT cell was established in which an individual, A, who
is Dw2-negative, was sensitized with a homozygous typing
cell for Dw2. The PLT was restimulated with cells of the six
individuals shown.
A major goal of developing PLT reagents is to have cells that provide a discriminatory pattern when tested with restimulating cells from any unrelated individuals, i.e., the cells of some unrelated individuals would stimulate very strongly, as much as the "reference" cell, while others would restimulate relatively little. We find that in approximately 30%-40% of the cases, such discriminatory PLT cells result when the initial sensitizing mixture is between individuals who differ by only one HLA haplotype, the routine genetic relationship that we use for generating PLT cells.\(^9,13\) We believe that this priming with a single HLA haplotype accounts for much of the ability to type for HLA-D in unrelated individuals.

In addition to finding highly discriminatory cells by chance alone in these combinations, it is possible to use restimulation in the sensitization of "tertiary" PLT cells to develop more discriminatory and specific cells.\(^12\)

We have used highly discriminatory cells to attempt to define antigens using PLT cells. The approach has been essentially similar to that used by serologists in that a search was made for PLT cells that appear to measure, at least in part, the same HLA-D determinants.

The results of testing a panel of 47 unrelated cells against 13 PLT cells are given in Fig. 1.\(^20\) A significant positive association between the results obtained with two dif-
ifferent PLT cells suggests that the cells may be recognizing at least one antigen in common. Associations are frequently tested by analyzing data in a two-by-two contingency table by the \( \chi^2 \) statistic and the Fisher Exact Test. On this basis, it appears that three groups of PLT cells can be used to "define" three different antigens. For instance, PLT cells 7 BC, and 11 BC, show a pattern of identical reactions as well as some that are not identical (\( \chi^2 = 19.47; p = 0.00009 \)). Similarly, PLT cells 9 CB, and 5 CB, can be grouped (\( \chi^2 = 29.07; p = 0.000008 \)) as well as the cells 11 DA, 11 DB, and 9 AC (\( \chi^2 \) for pair-wise combinations between the three cells range between 15.35 and 25.2, \( p \) between 0.00001 and 0.0005, for all comparisons). Although less clear, the PLT cells 7 BC, and 5 BC, can also be grouped (\( \chi^2 = 10.41; p = 0.013 \)). Using the PLT cells to define these antigens, individuals 3 and 8 carry antigens PL1 and PL3 and individual 6 carries antigen PL1 and PL4.

All of the data presented to date suggest that the antigens that cause restimulation in PLT are determined by genes in the HLA-D region. In all likelihood the HLA-D antigens themselves, although this has not been proven. We are referring to the antigens as PL antigens so as not to prejudice this point. We anticipate that these PL antigens will be the same as those detected in primary MLC or with homozygous typing cells.

It has been suggested, on the basis of results obtained with homozygous typing cells and with the PLT method, that there are several antigenic determinants associated with a single HLA-D haplotype. The theoretical basis of the PLT test might suggest that PLT cells can be prepared against individual determinants of the HLA-D region either on the basis of the antigenic relationship of responder and stimulator used in the primary MLC for the preparation of the PLT cell or on the basis of restimulation.

In the study reported in this paper, we have shown that PLT cells prepared against independent haplotypes can be used to detect the same antigen. We use the word "antigen" in this case to refer to the antigen or antigens detected by the clones of reactive lymphocytes in the PLT cell. We would anticipate that as more PLT cells are developed or other techniques are used to obtain more specific PLT cells, that the antigens PL1, 2, 3, and 4 will be "split" into antigens that, in analogy with the terminology used in studies with antisera, could be referred to as "shorter" antigens, i.e., antigens detected by PLT cells that react with fewer members of the unrelated panel.

It is encouraging that even in the first 13 cells tested on a panel of size 47, that more than one PLT cell was found to detect each of the four antigens described and that nine PLT cells could be grouped to detect four antigens. In terms of further definition of the system controlling the PL antigens, the problem is greatly simplified, since one can generate reagents in vitro for essentially any haplotype. We would stress again that it is of the utmost importance to determine whether all restimulation is caused by HLA-D determinants and to keep in mind that the correlations to date leave open the possibility, which we regard as remote, that antigens of the HLA-D region, which are different from the HLA-D antigens as detected in primary MLC, are responsible for causing restimulation in PLT tests.

ACKNOWLEDGMENT

The authors wish to dedicate this paper to Dr. Felix Milgrom of the State University of New York at Buffalo on the occasion of the 30th anniversary of his research activities.

REFERENCES