Brief Definitive Report

THREE HLA-D REGION ANTIGENS DEFINED BY PRIMED LD TYPING*

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Definition of cell-surface antigens determined by the genes of the major histocompatibility complex in man, HLA, has been performed for purposes of matching donor and recipient for transplantation, for studying associations between HLA antigens and disease, and for genetic studies (1). Four HLA loci, HLA-A, HLA-B, HLA-C, and HLA-D have been identified.

Until recently, HLA-D disparity could only be assayed in mixed leukocyte culture (MLC) tests (2, 3). In 1972, Bradley et al. (4) introduced the homozygous typing cell (HTC) test in which HLA-D antigens were defined by stimulating cells in MLC that were homozygous for the locus (loci) determining the MLC stimulatory, or LD, antigens. If the responding cell in MLC carried the same HLA-D antigen(s) as the HTC, no response occurred. The HTC test has been used in man to define eight "clusters" of HLA-D antigens called HLA-DW1 through HLA-DW6, LD 107, and LD 108 (5).

We have developed a new test, called primed LD typing or PLT, to define HLA-D antigens (6–9). The reagents ("PLT cells") are prepared in vitro, in an MLC in which the responder and stimulator differ by only one HLA haplotype. The test is based on the observation that lymphocytes "primed" in MLC by stimulating with allogeneic cells will give a rapid, secondary-type proliferative response when restimulated 10 days later with cells of the original sensitizing cell donor (10–14). We were able to show, in addition, that this secondary proliferation can be used to "type" for the HLA-D antigens on cells from unrelated donors (6, 7).

A PLT cell that is maximally useful for antigen definition is one that is highly discriminatory, i.e. a PLT cell that will respond strongly to some restimulating cells and much more weakly to others, allowing a clear separation between "positive" and "negative" restimulation. We have studied 13 such discriminatory PLT cells. These 13 cells were chosen from 24 PLT cells, prepared in six different families, that were first screened on a panel of nine unrelated individuals to find the discriminatory reagents. The results suggest that seven of the

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PLT cells, prepared against seven independent haplotypes, can be used to define three antigens (or antigenic clusters) which we have called PL1, PL2, and PL3; two more PLT cells may define antigen(s) PL4.

The PLT test has recently been described in detail (8). In brief, PLT cells are prepared in MLCs using cells of family members known to share one HLA haplotype. The cells are incubated for 10 days, after which the remaining cells are frozen in aliquots for storage. For testing, the PLT cells are thawed and cultured in triplicate in microtiter plates (15) with restimulating cells from: the individual who was the donor of responding cells for the primary MLC ("control" cells), the donor of the sensitizing or stimulating cells for the primary MLC ("reference" cells), or other individuals ("test" cells). All restimulating cells also had been frozen. These secondary cultures were labeled with tritiated thymidine from hours 40–48 and mean counts per minute incorporated were determined.

All PLT cells used in the studies described here were prepared in MLCs between cells of parent-child pairs. Each family was given a number, and the father was always called A, the mother B, and the child C or D. PLT cells were designated according to family and to cell donors used in the sensitizing MLC. For example, 11 BC_x is a PLT cell from family 11, made with responding cells from the mother (B) and X-irradiated stimulating cells from a child (C). Positive and negative reactions were determined subjectively (before any attempts to group different PLT cells) on the basis of apparent separation of high and low stimulating groups.

We have tested reproducibility of the PLT assay using highly discriminatory PLT cells such as those shown in Fig. 1. A given PLT cell was prepared and frozen in multiple vials; cells in one vial were tested against a panel of nine unrelated individuals, and at a different time cells in another vial were tested against the same panel. Such repeat experiments using the same PLT cell and panel of restimulating cells gave 95% concordant results, i.e., in 95% of all cases, a given stimulating cell gave either both positive or both negative reactions in the two repeat tests.

The results of testing a panel of 47 unrelated cells against 9 of the 13 PLT cells, that appear to define PL antigens, are given in Fig. 2. A significant positive association between the results obtained with two different PLT cells suggests that the cells may be recognizing at least one antigen in common. Pairwise associations were tested by analyzing data in a two by two contingency table by the χ^2 statistic and Fisher exact test. It appears that three groups of highly associated PLT cells can be used to "define" three different antigens. For instance, PLT cells 7 BC_x and 11 BC_x show a pattern of identical reactions as well as some that are not identical; their reaction patterns show a highly significant association ($\chi^2 = 19.47$; $P = 0.00009$). Similarly, PLT cells 9 CB_x and 5 CB_x can be grouped ($\chi^2 = 29.07$; $P = 0.000008$), as can the cells 11 DA_x, 11 DB_x, and 9 AC_x (χ^2 values for pairwise combinations among the three cells range between 15.35 and 25.2; P values between 0.00001 and 0.0005). Although much less clear, the PLT cells 7 CB_x and 5 BC_x can also be grouped ($\chi^2 = 10.41$; $P = 0.013$). This last association could well have occurred by chance alone, however, since there are 78 possible pairwise comparisons for 13 PLT cells. Using the PLT cells to define these antigens, individuals 3 and 8 carry antigens PL1 and PL3 and individual 6 carries antigens PL1 and PL4. (Since compiling these data, we have found one more PLT cell defining PL4 which shows a highly significant χ^2 with 7 CB_x ($\chi^2 = 25.0$), and two more pairs of PLT cells defining antigens PL5 and PL6.)

All of the data presented to date suggest that the antigens that cause restimu-

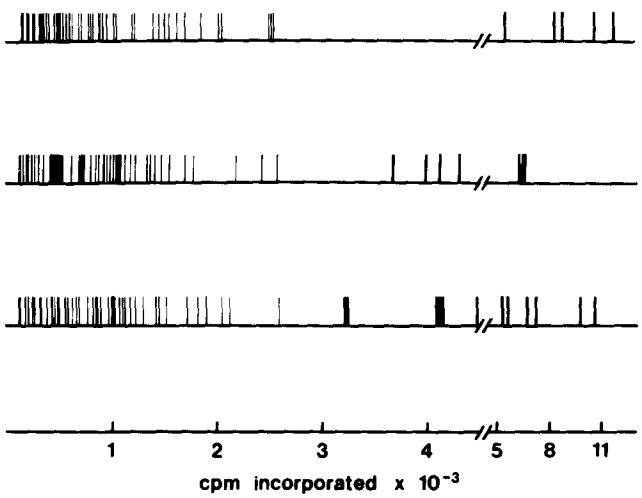

FIG. 1. The data obtained by testing each of three discriminatory PLT cells (plotted on the top three horizontal lines) against a panel of 47 test cells from unrelated individuals are shown. On the bottom horizontal line are counts per minute (cpm) representing tritiated thymidine incorporated into individual cultures where a PLT cell is stimulated with a given test cell. Each vertical bar on one of the top three horizontal lines represents the results (in cpm) of restimulation with one test cell. For these three PLT cells, those test restimulating cells that led to incorporation of 4,000 cpm or greater were called positive, as was the one restimulating cell for the middle PLT cell that caused incorporation of approximately 3,650 cpm. The two test cells leading to incorporation of approximately 3,200 cpm in the third PLT cell were considered negative.

lation in PLT are determined by genes in the HLA-D region (6, 7, 16–18), in all likelihood the HLA-D antigens themselves. We are referring to the antigens as PL antigens so as not to prejudice this point.

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 (9, 19). The theoretical basis of the PLT test would suggest that PLT cells can be prepared against individual determinants of the HLA-D region. Such specific cells would result 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 priming sequentially with cross-reactive cells (20).

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 cells in the PLT test. We would anticipate that as more PLT cells are developed or other techniques are used to obtain more specific PLT cells, the antigens PL1, 2, 3, and 4 will be "split" into "shorter" antigens, i.e., antigens detected by PLT cells which react with fewer members of the unrelated panel.

Summary

We have recently described a new method, primed LD typing or PLT, for specific identification of HLA-D antigens. Highly discriminatory PLT cells have

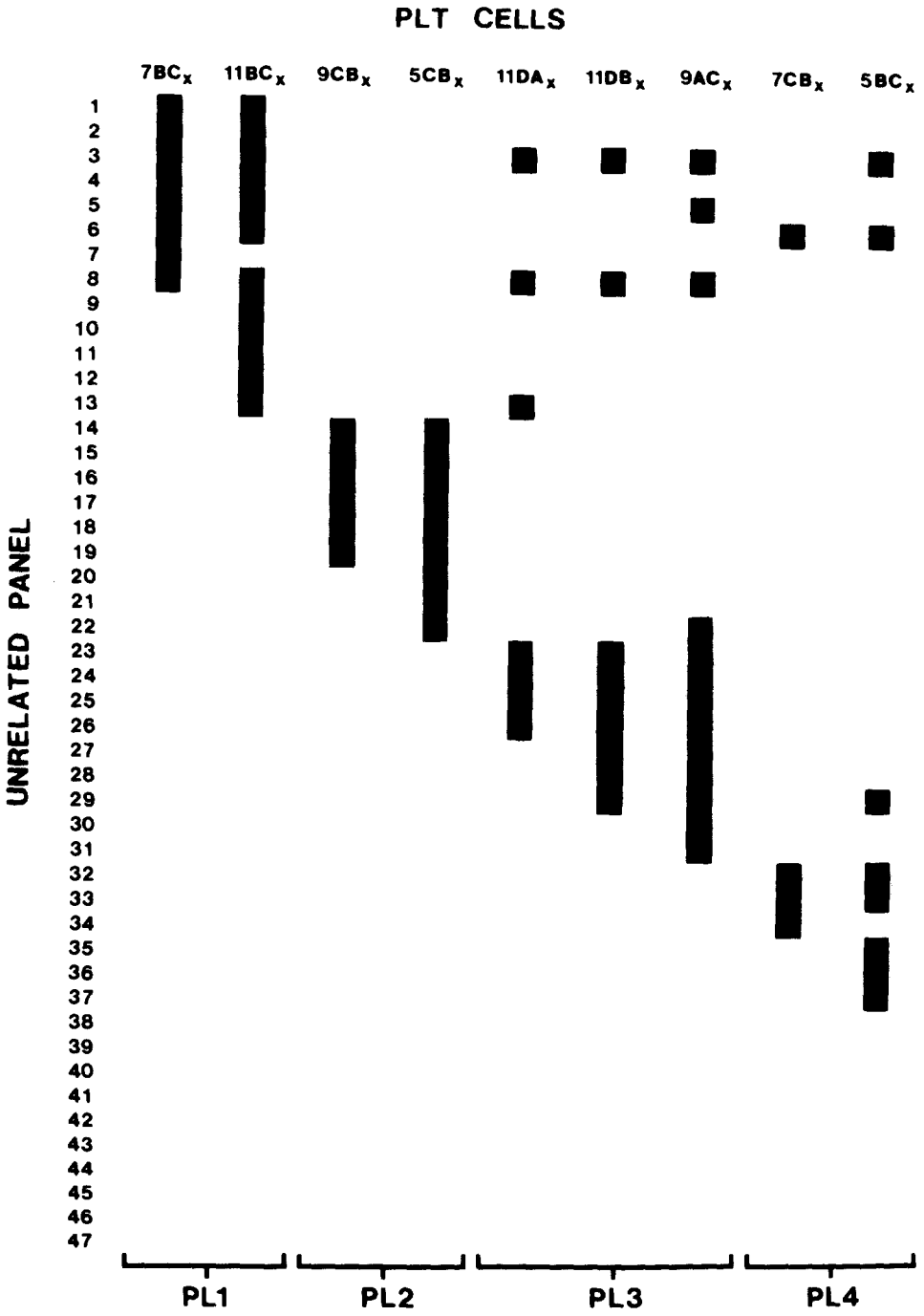

FIG. 2. Nine different PLT cells listed across the top of the figure were tested against a panel of 47 unrelated test cells. The vertical black bars indicate those combinations of test restimulating cells and PLT cells which gave positive restimulation as discussed in the text and shown in Fig. 1.

been developed which clearly differentiate between cells of individuals that restimulate strongly and those that restimulate weakly. Seven such discriminatory PLT cells have been used to define three antigens called PL1, PL2, and PL3; two more PLT cells may define antigen(s) PL4.

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