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Electrophoretic analysis of human HLA-DR antigens from HLA-DR4 homozygous cell lines: Correlation between β-chain diversity and HLA-D

(HLA-DR β chains/*DR4* haplotype/HLA-D and mixed lymphocyte culture reaction/two-dimensional gel electrophoresis)

BARBARA S. NEPOM*, GERALD T. NEPOM†‡§, ERIC MICKELSON†, PAOLO ANTONELLI†, AND JOHN A. HANSEN†¶‖

†Fred Hutchinson Cancer Research Center, ¶Puget Sound Blood Center, and the Departments of *Pediatrics, ‡Pathology, and ‖Medicine, University of Washington School of Medicine, Seattle, WA 98104

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ABSTRACT **Two-dimensional gel electrophoresis of immunoprecipitated human HLA-DR antigens from cells expressing the *HLA-DR4* haplotype shows distinct clustering of β-chain patterns. Six unique electrophoretic variants were observed among 17 HLA-DR4 homozygous cell lines (HCL) analyzed. These patterns correlate precisely with the HLA-D phenotype of the HCL donor as determined by reactivity in mixed lymphocyte culture. All DR4 HCL that belong to one of the well-defined HLA-D antigen groups (Dw4, Dw10, LD "40", LD "DYT", LD "KT2", or LD "TAS") have identical DR β-chain patterns; DR4 HCL belonging to different HLA-D antigen groups do not. The concordance of the functional expression in mixed lymphocyte culture of a specific D phenotype with a distinct DR β-chain pattern on gel analysis provides a direct structural basis for understanding the genetic control of HLA-D polymorphisms; HLA-D specificities as revealed by T-cell recognition in mixed lymphocyte culture thus might be accounted for by DR β-chain polymorphisms. The extent of this β-chain diversity within a single *DR* haplotype may aid in understanding variations in Ia-regulated functions, such as *Ir* gene control and certain disease susceptibilities.**

The relationship between HLA-D and HLA-DR has remained a puzzling and unresolved issue in human immunology. This has been due in part to the differing methods used to identify and characterize D and DR antigens. Whereas DR antigens are defined by the reactivity of selected antisera for "Ia-like" molecules, D antigens are defined by the patterns of reactivity elicited in mixed lymphocyte culture (MLC) when cells from *HLA-D* homozygous donors are used as "typing cells" (HTC). The composite array of Ia-like antigens expressed on the cell surface is likely to account for HLA-D typing results; on the other hand, identification of a particular DR antigen on the same cell may require only the expression of a single *DR*-encoded epitope. Consequently, some cells sharing the same *DR* haplotype express different D antigens.

This distinction is particularly apparent for the *HLA-DR4* haplotype. In the Eighth International Histocompatibility Workshop, the gene frequency of *HLA-DR4* in Caucasians was greater than 10%, whereas the observed gene frequency of *HLA-Dw4* was only 7%. Thus, less than 70% of *DR4* haplotypes in Caucasians are Dw4 positive (1). The relationship between *DR* and *D* can change from one population to another. For example, among Japanese, less than 10% of *DR4* haplotypes type as Dw4 (2), and among Yakima Indians of the Pacific Northwest, only 11% of *DR4* haplotypes type as Dw4 (3). In these populations, a different HLA-D antigen(s) is associated with *DR4*. Comprehensive populations studies with *DR4*-associated HTC have led to the description of several distinct *DR4*-associated HLA-D antigens, including Dw4, Dw10, LD "40", LD "DYT", and LD "KT2" (4–8). There are several possible explanations for these apparent *DR4* "splits." First, HLA-Dw4 and DR4 may be the products of different loci, each coding for distinct "D region"-associated antigens. A second possibility is that Dw4 and DR4 are products of a single gene, the primary product of which is recognized by DR4 alloantisera, but through post-translational modification sufficient phenotype variation occurs to cause the differences detected in MLC by T cells. In a third model, the haplotypes expressing Dw4, Dw10, LD 40, LD DYT, and LD KT2 may be encoded by different alleles but share a common crossreactive or "supertypic" determinant recognized by DR4 alloantisera.

To answer such questions, we have begun a detailed analysis of the two-dimensional gel electrophoresis patterns of DR antigens expressed by B-lymphoblastoid cell lines (HCL) derived from *HLA-DR4* homozygous donors. Since most of the polymorphism attributed to DR molecules has been correlated with structural heterogeneity of the DR β-chain (9–13), we have prepared cell lines from donors of *HLA-D* homozygous typing cells representing each of the distinct HLA-D antigens associated with *DR4* and have studied the relationships among isolated DR4 β-chain polypeptides. Our results demonstrate a striking correlation between the functionally distinct HLA-D phenotypes of individual HCL and the two-dimensional gel patterns of the DR β chains isolated from these HCL.

MATERIALS AND METHODS

HCL. Seventeen B-lymphoblastoid cell lines homozygous for an HLA-DR4-associated *D* locus were used in this study (Table 1). The specificities Dw4, Dw10, LD 40, LD DYT, LD KT2, and DB3 have been defined by the Eighth Workshop (5), by Reinsmoen and Bach (4), and by Nose *et al.* (2). In addition, we have used HCL derived from a unique HTC, "TAS," described by Amar *et al.* (20), that is mutually reactive in MLC with cells from each of the other clusters and that may represent yet another apparent split of the *DR4* haplotype.

Established B-lymphoblastoid cell lines were available for some of the above HTC, otherwise new B-lymphoblastoid lines were prepared from cryopreserved HTC by cocultivation with Epstein–Barr virus (EBV) as described (21). Briefly, 1–2 × 10^6

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Abbreviations: HCL, homozygous B-lymphoblastoid cell lines; MLC, mixed lymphocyte culture; HTC, homozygous typing cells; EBV, Epstein–Barr virus; SaCl, *Staphylococcus aureus* (Cowan 1 strain).

§To whom reprint requests should be addressed at: Fred Hutchinson Cancer Research Center, 1124 Columbia, Seattle, WA 98104.

Table 1. *HLA-DR4*-associated *HLA-D* HCL

HCL	Workshop No.	*HLA-D*	Source of HTC	Refs.
WALK*	8W132	Dw4	Local	5, 14
ER*	8W128; 6W3003		B. Dupont (Sloan–Kettering Institute)	2, 14–16
NIN†			F. Bach (University of Minnesota)	
HA*	8W131; 7W547; 6W3004		Local	2, 5, 14–17
PRIESS‡	7W507; 6W12006		M. Thomsen (University of Copenhagen)	17
FS*	8W212; 7W548	Dw10	B. Dupont (Sloan–Kettering Institute)	5, 14, 17, 18
EM*	8W210; 7W543		E. Yunis (Sydney Farber Institute)	5, 14, 16, 17, 19
THO†		LD 40	Local	4
LS40†			F. Bach (University of Minnesota)	2, 4
BIN40†			F. Bach (University of Minnesota)	
KT3†		LD DYT	N. Kashiwagi (Kitasato University)	8
HAS-15†			F. Bach (University of Minnesota)	2, 4
JHa†		DB3	H. Festenstein and J. Sachs (London Hospital)	2, 4, 5
SST†			S. Hsu (Johns Hopkins University)	4
KT2†	8W407	LD KT2	N. Kashiwagi (Kitasato University)	2, 5, 8
KT13†			N. Kashiwagi (Kitasato University)	
TAS†		LD TAS	C. Brautbar (Hadassah University)	20

* Established by EBV transformation of cryopreserved lymphocytes from *HLA-D/DR* homozygous donors as described (21).
† Established for this study.
‡ The donor of this cell line was originally described by Thomsen *et al.* (22) and the cell line was supplied to us by W. F. Bodmer.

B cells/ml were mixed with an equal volume of culture supernatant from the EBV-secreting line B95-8 and plated into multiple wells of tissue culture micro plates. Cultures with growth were expanded and established within 15–60 days.

Definition of *HLA-DR*. *HLA-DR* was originally defined as the "*HLA-D* region-related" gene(s) controlling the expression of human Ia-like alloantigens on B cells and monocytes (23). HLA-DR typing was performed on nylon wool purified peripheral blood B cells or B-lymphoblastoid cell lines as described (24, 25) using a panel of antisera recognizing HLA-DR1 through DRw10.

Definition of *HLA-D*. The *HLA-D* locus (MLR-S) was originally defined as the genetic region causing stimulation in MLC (26), based on an association analysis of MLC typing responses (5). Cells from individuals known to be homozygous for *HLA-D* are used as typing reagents (HTC) that can be "clustered" according to the reactivity patterns elicited from responder cells tested in HLA-D typing experiments. The HLA-Dw4 antigen was first defined in the Sixth International Histocompatibility Workshop (27). The criteria for the assignment of HLA-D antigens by this method have been verified by extensive segregation analysis within families (5).

Monoclonal Anti-Ia Antibodies. Antibody P4.1 is a murine monoclonal antibody specific for all HLA-DR molecules ("monomorphic"). P4.1 was characterized as recognizing an Ia-like antigen by virtue of its reactivity with a p29,34 complex from human B cells, lack of reactivity with unstimulated T cells, and failure to react with an *HLA-DR* deletion mutant cell line, 6.1.6 (28). P4.1 is similar to antibody L203 described by Lampson and Levy (29) and characterized as anti-DR (mature form) (30). Antibody P4.1 also binds the molecule recognized by the monoclonal anti-DR antibody SG157 but not SG465 (anti-DS) (31) in sequential immunoprecipitation experiments. Antibodies S1.19 (32) and 7.2 (33) have been described.

Radiolabeling and Immunoprecipitation. Cells were labeled with ^{125}I by incubation of 4.5×10^7 cells in 0.5 ml of phosphate-buffered saline/5 mM glucose containing 50 μg of glucose oxidase, 0.7 unit of lactoperoxidase (Calbiochem), and 2 mCi of $Na^{125}I$ (1 Ci = 37 GBq; Amersham) for 20 min at room temperature. After washing, cells were lysed with 0.5 ml of lysis buffer (34) containing 1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride for 1 hr at 0°C. Lysates recovered after centrifugation at $1,500 \times g$ were cleared with 300 μl of a washed 10% solution of formalin-fixed heat-killed *Staphylococcus aureus* (Cowan 1 strain; SaCl; Bethesda Research Laboratories) followed by centrifugation at $15,000 \times g$.

^{125}I-Labeled lysate ($2–10 \times 10^7$ cpm) was incubated overnight at 0°C with approximately 50 ng of hybridoma antibody. One milligram of SaCl was then added for 20 min, and this was followed by repetitive washing in SaCl wash buffer (34).

Two-Dimensional Gel Electrophoresis. Samples were suspended in 8.5 M urea/2% Nonidet P-40 with 5% 2-mercaptoethanol for loading onto nonequilibrium pH gradient polyacrylamide tube gels (70×1.5 mm) using pH 3.5–10 Ampholines as described by O'Farrell and co-workers (35, 36). Gels were electrophoresed for 1,500 V·hr, then equilibrated with $NaDodSO_4$-containing sample buffer and directly applied to 10% $NaDodSO_4$/polyacrylamide slab gels run in a discontinuous buffer system (37). Gels were dried and autoradiographed for 4 hr to 10 days at −70°C with Kodak XR film and a Rarex intensifying screen. Direct comparisons between separate samples were made only on gels that were simultaneously prepared and electrophoresed side-by-side on the same apparatus. Representative autoradiographs were aligned for comparison by overlapping the cathodic origin and any high molecular weight invariant spots that appeared.

RESULTS

Radiolabeled DR4 Immunoprecipitates Show Several Different Two-Dimensional Gel Patterns. Table 1 lists the HCL used in this study, each derived by EBV transformation of B cells from *HLA-D* homozygous typing cell donors representing seven distinct *DR4*-associated HLA-D specificities. Each of these cell lines was radiolabeled by using ^{125}I rather than a metabolic label because we were primarily interested in external determinants that would be likely to be available as recognition antigens in MLC.

The immunoprecipitated Ia antigens from each of the cell lines in Table 1 were analyzed by two-dimensional gel electrophoresis. Autoradiographs of the β-chain profiles from seven of the *DR4* HCL are shown in Fig. 1. In the immunoprecipitate from cell line WALK (line A), a member of the Dw4 cluster, there are five distinct spots in the β-chain region that have similar molecular weights but different charges. The relative positions and overall pattern were not only reproducible from day to day using WALK cells but also were identical to the β-chain pattern of PRIESS, HA, NIN, and ER cells, other HCL from the Dw4 group. This gel pattern is similar to that reported by others for DR4 antigens (38).

Fig. 1 (lines *B–F*) illustrates a striking finding. Several distinctly different β-chain gel patterns were obtained using other *DR4* non-Dw4 cell lines, differing not only from the WALK cell pattern (line A) but also from each other. Line B, for example, is the β-chain gel pattern of cell line EM, and the spots are at a much more acidic position than those of any other cell line. Each pattern is distinct, except for lines E and F, which appear to have identical β-chain patterns of three major spots each. Of the 17 cell lines analyzed, only these six patterns were seen. All of the other cell lines from Table 1 gave β-chain gel patterns identical to one of the above examples (see below). Several of these experiments were repeated using monoclonal anti-p29,34 antibodies S1.19 and 7.2. In each case, the patterns seen were identical to those seen with antibody P4.1 (data not shown).

Consistent β-Chain Patterns Among Cell Lines Correlate with HLA-D Specificity. The relationships among the two-dimensional gel patterns observed with each of the *DR4* HCL were analyzed by mixing experiments, in which immunoprecipitates from two cell lines were mixed together just prior to two-dimensional gel analysis. Thus, slight variations in polypeptides might show up as multiple spots whereas identical polypeptides should comigrate and appear as a single spot. Very small differences in amino acid or carbohydrate composition that do not significantly alter either charge or size, however, might not be distinguished by this method.

First, immunoprecipitates of β chains from HLA-D identical cell lines were mixed and analyzed on two-dimensional gels.

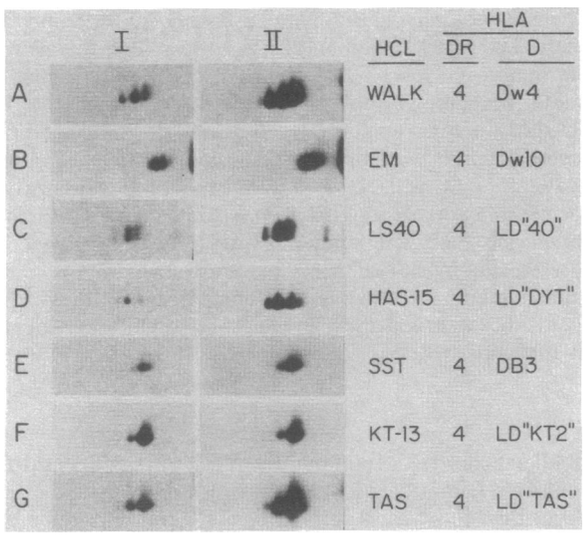

FIG. 1. Two-dimensional gel patterns of immunoprecipitated β chains from *DR4* HCL. Two exposures of each gel are shown: I, brief exposure (5–18 hr); II, long exposure (24–72 hr). Each anode is aligned on the right. In some cases, a small amount of radiolabeled material is seen near the anode, representing material that did not enter the first-dimension gel.

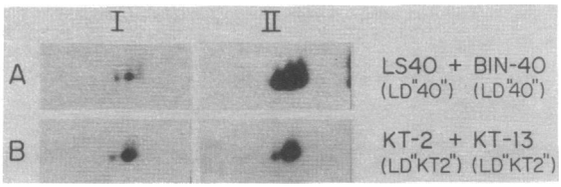

FIG. 2. Comparisons of two-dimensional gel patterns from different *DR4* HCL within the same HLA-D cluster. Each line shows the composite gel pattern of β-chain immunoprecipitates from two different cell lines, whose Ia antigens were immunoprecipitated independently and then mixed before electrophoresis. I and II are short and long exposures, respectively, of the same gels.

Two examples are shown in Fig. 2. As shown in line A, the combined β chains from two cell lines, each representing the LD 40 cluster, gave three distinct spots, a pattern indistinguishable from the pattern seen when β chains from either cell line alone or from other cells in the LD 40 cluster were electrophoresed (compare Fig. 1, line C).

Similarly, line B shows the results of mixing immunoprecipitates from two cell lines representative of the KT2 cluster. Only three spots were obtained, each identical to the β-chain pattern of either cell alone. Similar direct comparisons of combined immunoprecipitates from all cell lines of the same HLA-D cluster were carried out for all of the cell lines in Table 1. Every cell line in an HLA-D cluster was compared. As shown in Fig. 2 for the LD 40 and LD KT2 groups, a striking correlation was found between the gel patterns and the HLA-D cluster. In only one combination did we fail to observe a unique β-chain pattern for each of the two distinct D antigens: Cell lines JHa and SST gave patterns overlapping with cell lines KT2 and KT13, indicating that this method did not distinguish between the DB3 and LD KT2 HLA-D clusters.

Different β-Chain Patterns Share Some Characteristic Features. We next analyzed the relationships among different β-chain patterns when immunoprecipitates from cells of different D clusters were combined. Fig. 3 shows three examples. In line A, precipitates from cell lines EM and WALK, representing clusters Dw10 and Dw4, respectively, were mixed. The β-chain pattern has eight spots. By comparing this with each β-chain pattern run individually on the same day, we can identify the two central spots in Fig. 3 (line A) as present in both patterns, identical in charge and size. The three more basic spots are contributed solely by cell line WALK and the three more acidic spots are contributed by cell line EM.

Similarly, line B shows mixing of BIN40 (LD 40) and WALK (Dw4) immunoprecipitates. In this case, only five spots were seen, identical to cell line WALK alone. By overlapping gels of the individual cell lines run simultaneously, we can assign the

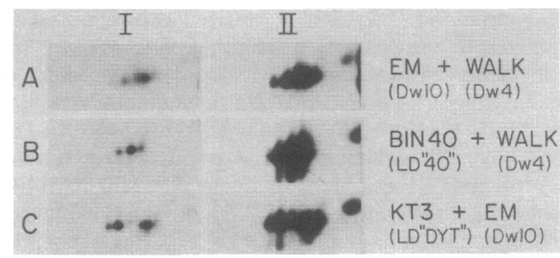

FIG. 3. Two-dimensional gel patterns of immunoprecipitates from two *DR4* HCL from different HLA-D clusters. Ia-like antigens from each cell line were immunoprecipitated individually and mixed before electrophoresis. Spots on the far right represent α chains and some material near the anode that did not enter the first-dimension gel. I and II are short and long exposures, respectively, of the same gels.

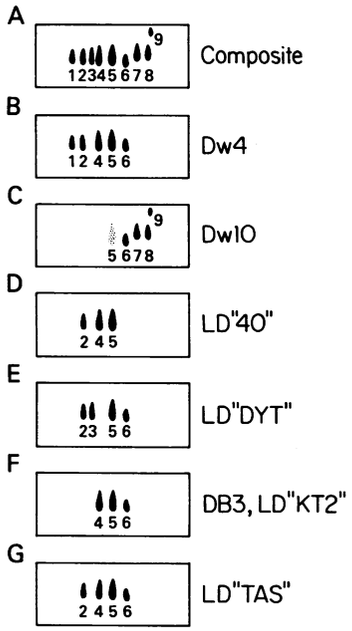

FIG. 4. Diagrammatic representation of β-chain two-dimensional gel patterns found among *DR4* HCL. (*A*) Composite drawing on which spots are arbitrarily numbered from most basic (spot 1) to most acidic (spot 9). (*B–G*) Consistent electrophoretic variants that correlate with HLA-D cluster analysis. Spot 5 in *C* is stippled to indicate that this spot is occasionally absent.

three central spots as the pattern identical in charge and size shared by both cell lines, with the most acidic and the most basic spots being contributed by cell line WALK alone.

A final example is shown in line C. Here, immunoprecipitates from cell lines KT3 (LD DYT) and EM (Dw10) were mixed, resulting in seven spots. The two most basic spots are part of the KT3 profile, the three most acidic spots are part of the EM pattern, and the remaining two spots are found in both cell lines.

Similar combinations of immunoprecipitates from the different cell lines have allowed us to define the relationships of distinct β-chain spots representative of each of the subgroups with respect to each other; these are summarized diagrammatically in Fig. 4. Fig. 4*A* presents a composite of all the individual β-chain spots detected. Each spot is numbered arbitrarily, from most basic to most acidic. Fig. 4 *B–G* illustrates the unique β-chain patterns of each of the *HLA-DR4*-associated D clusters studied.

DISCUSSION

Heterogeneity within individual *DR* haplotypes has been reported for *DR2* (6, 39–41), *DRw6* (42, 43), *DR7* (44–48), and *DRw8* (49), as well as for *DR4* (2, 4, 50). Each has been split on the basis of HLA-D reactivity. Of these, HLA-DR4 is the most complex antigen. Although well defined as an apparent allele of the *DR* system, several different patterns of reactivity have been seen for putative *DR4* HTC (2–6, 17, 50). Thus, there clearly exist antigenic differences between cells grouped within the *DR4* haplotype.

We report here a clear diversity of DR antigens isolated from *DR4* HTC-derived lymphoblastoid cell lines. Our results provide an electrophoretic map of a family of DR4 β chains. We found consistent and distinctive β-chain variations that correlated in a striking way with the *HLA-D* polymorphism defined by HTC typing. Among the seven *HLA-DR4*-associated *D*-locus-defined subgroups analyzed, we detected unique electrophoretic DR β-chain patterns for six.

The gel patterns were consistent between experiments using the same cell lines and strikingly consistent between different cell lines that are *HLA-D* identical. Reinsmoen and Bach (4) have summarized data (5, 6, 17) describing five *HLA-D* clusters associated with the *DR4* haplotype, each defined by a distinctive antigenic profile recognizable in MLC. Interestingly, the two patterns that were indistinguishable in our study, DB3 and LD KT2, were the only two HLA-D clusters that could not be clearly separated by the HTC experiments (4).

Similar two-dimensional gel analyses of immunoprecipitated DR antigens have been used to describe differences among homozygous cell lines of different *DR* haplotypes. Charron and McDevitt (38) compared two-dimensional gels of different HCL and found that *DR* haplotypes 1, 2, 3, 4, 5, w6, 7, and w8 gave distinct patterns. Recently, Goyert *et al.* (51) studied patterns of human Ia-like antigens precipitated from a similar set of HCL by using an antibody against a second set of HLA-D region-linked Ia-like antigens called DS. They concluded by two-dimensional gel analysis that each HLA haplotype expresses both a *DR*- and a *DS*-encoded β chain. The present study analyzes the patterns within a particular *DR* haplotype, DR4, and reports that there is apparently as much diversity within the HLA-DR4 family as among cells of different *DR* haplotypes.

The antibodies used for this study, P4.1, S1.19, and 7.2, are all anti-DR, and all gave the same results. This makes it unlikely that products of other loci are also precipitated and contribute to the gel patterns observed. Because consistent and identical patterns were observed among several different *D*-identical HCL, it is unlikely that the variations seen can be attributed to heterozygosity of the lines. In other words, we would expect a heterozygous profile to look like one of our mixing gels, with both of two haplotypes represented.

The remarkable correlation between the structural polymorphism of DR β chains as shown by unique gel patterns and the polymorphism defined by reactivity in the MLC assay suggests that DR β chains may be a major contributor directly responsible for causing stimulation in MLC. However, recent analysis of the complexity of the *D* region, using both monoclonal antibodies and genomic probes (reviewed in ref. 52), suggests that multiple products of loci within the *D* region are likely to be expressed as potential MLC-stimulating determinants. It seems probable, therefore, that the actual molecular basis of D reactivity represents a composite of multiple antigens.

An extension of the approach described here should assist in more detailed descriptions and identification of the specific D-region determinants responsible for MLC reactivity and, indeed, for Ia-regulated immune recognition phenomena. Further molecular studies using endoglycosidase treatment, metabolic labeling and peptide analysis, or restriction mapping of the *D*-region genome should further clarify the basis for the structural polymorphism we describe. The ability to define in molecular terms the basis for such structural variables will contribute to understanding the mechanism of DR4 restriction phenomena and associated disease susceptibilities.

Finally, we note that Groner *et al.* (53) have reported electrophoretic analysis of *DR4* HCL in experiments published after completion of this manuscript. Using six HCL analyzed by isoelectric focusing followed by $NaDodSO_4$/polyacrylamide gel electrophoresis, they observed three different patterns that parallel the LD 40, Dw10, and LD DYT data described here. The study reported here using 17 *DR4* HCL and a nonequilibrium electrofocusing technique resolves an additional three clusters and distinguishes between Dw4 and LD 40 HCL.

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