Rapid Publications

Specific HLA-DR4-associated Histocompatibility Molecules Characterize Patients with Seropositive Juvenile Rheumatoid Arthritis

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Abstract. The structural and functional heterogeneity of HLA-DR4-associated specificities was investigated in patients with seropositive juvenile rheumatoid arthritis, a DR4-associated disease. Using a combination of HLA-D analysis by mixed lymphocyte culture and electrophoretic analysis of immunoprecipitated Ia molecules by two-dimensional polyacrylamide gels, we observed a surprisingly homogeneous pattern of HLA-D antigen expression. All patients expressed common structural products of the DR and DS loci, and 7/12 homozygous DR4 patients expressed a rare and subtle HLA-D heterozygous phenotype.

Introduction

The presence of particular genetic markers has been linked to disease susceptibility in patients with some autoimmune or immune-mediated diseases (1–9). To test the hypothesis that specific class II molecules associate with a particular HLA-linked disease, and thus identify putative disease-susceptibility Ir genes, we have analyzed the HLA-DR4-associated specificities in patients with seropositive juvenile rheumatoid arthritis (JRA).[1] HLA-DR4 is a serologically defined specificity present in diverse populations, but in mixed lymphocyte response assays T cells recognize at least six distinct HLA-DR4-associated specificities, which are defined as HLA-D antigens (10–12). These HLA-DR4-associated haplotypes also differ from each other in recognizable electrophoretic variants of products of the DR locus and to a lesser extent of the DS locus (13, 14). We initiated this study to look for a class II molecule(s) common to JRA patients that would be a candidate for a DR4-associated disease-susceptibility product contributing an Ir gene-regulated function.

Methods

Patient selection and typing. Patients attending the Arthritis Clinic at Children's Orthopedic Hospital and Medical Center, Seattle, WA with a diagnosis of JRA were used for this study. 27 out of 400 patients were diagnosed as having seropositive JRA by using previously described criteria (15). Most of these patients have a late childhood onset of symmetrical, erosive, and usually severe and unremitting polyarthritis with high titer rheumatoid factor; they have frequent subcutaneous nodules; and they are female. The 22 Caucasian patients in this group, and their families, were selected for study.

HLA-DR typing was performed on nylon-wool-purified peripheral blood B cells or B lymphoblastoid cell lines as previously described, by using a panel of antisera that recognize HLA-DR1 through HLA-DRw10 (16). HLA-D typing was performed on peripheral blood lymphocytes by using a standard mixed lymphocyte culture as previously described (17).

Four homozygous typing cells were used to define Dw4 (JAH, LGY, GW1, and JOL). Four homozygous typing cells were used to define LD40 (2075, THO, BIN40, and LS40). Dw4 and LD40 are two of the six recognized HLA-D clusters associated with HLA-DR4 (10, 12). Homozygous cells that type as LD40 respond in mixed lymphocyte culture against homozygous Dw4 cells, even though they both express the DR4 specificity (10). The double normalized value method of Ryder et al. (18) was used to adjust data from each HLA-D typing experiment.

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1. *Abbreviations used in this paper:* JRA, juvenile rheumatoid arthritis; RR, relative risk.

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Double normalized values of <45 were considered to be "typing responses", and assignments were made according to the Ninth Histocompatibility Workshop method (17).

Antibodies and cells. Antibody P4.1 is a murine monoclonal antibody specific for a determinant on HLA-DR molecules (12). Monoclonal antibody SG465 recognizes a determinant present on DS molecules, which are molecules that are homologous by partial sequence analysis to murine I-A antigens (19). Cell lines were established from peripheral blood B lymphocytes of patients by Epstein-Barr virus transformation (20).

Immunoprecipitation and gel analysis. Radiolabeling and neuraminidase treatment of cell lysates and subsequent immunoprecipitation were performed as previously described (12, 13). Gel analysis of the immunoprecipitates used nonequilibrium electrophoresis in the first dimension, followed by discontinuous 10% sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis in the second dimension. Gels were dried under vacuum and autoradiographed directly onto Kodak X-Omat film. HLA-DR α-chains did not display significant polymorphism or electrophoretic variation between haplotypes, so only β-chain results are shown.

Results

2-Dimensional gel analysis of DR and DS molecules from homozygous cell lines derived from patients with seropositive JRA. B lymphoblastoid cell lines derived from eight of the patients with seropositive JRA who were homozygous for HLA-DR4 were radiolabeled and lysed for analysis by immunoprecipitation and gel electrophoresis, which is shown in Fig. 1. Ia β-chains of the DR and DS products have characteristic electrophoretic patterns that vary within the DR4 specificity, in a manner that corresponds with the HLA-D-defined haplotype (13). The JRA DR β-chain immunoprecipitates (left column), however, show a remarkably homogeneous electrophoretic pattern that has little variation between individuals. Three major spots are seen in each case, with similar molecular weight and charge profiles. The patterns are indistinguishable between cell lines, except in the case of cell line 728 where a faint fourth spot is also present, migrating more acidic than the others. This extra spot may represent an additional DR4-associated product whose specificity has not been defined by homozygous typing cells.

To analyze Ia β-chains of the non-DR locus called DS, the cell lysates were precleared of DR molecules with antibody P4.1 before immunoprecipitation with anti-DS antibody SG465. The observed patterns (middle column) are distinct from the DR β-chain patterns but again show a remarkable degree of homogeneity within the individuals tested; each cell line shows a consistent gel pattern with two dominant spots, although subtle degrees of charge heterogeneity exist. Again the exception is cell line 728 where a faint basic spot is present.

After neuraminidase treatment and electrophoresis, most of the DR β-chain charge heterogeneity disappears (right column). In six cases, a single dark spot is seen, with a faint acidic shadow. In two of the cases, 728 and 813, the intensity of these same two spots differs, which may reflect different susceptibilities to desialylation, or may indicate a second β-chain polypeptide. In all cases the dominant spots co-migrate. Confirmation of this consistent electrophoretic migration was achieved both by overlapping alignment of the gels, and by mixing experiments, where immunoprecipitates from two separate cell lines were mixed before the first-dimensional electrophoresis. Thus, subtle differences in charge or size would show up as separate spots when run simultaneously. In the lower right of Fig. 1 two representative mixing experiments are shown, which reveal that in fact the major spots from different neuraminidase-treated cell lines (162 + 469, 125 + 372) are indistinguishable.

HLA-D and DR typing of patients with seropositive JRA. Complete HLA haplotypes of the patients studied by biochemical analysis are shown in Table I. Surprisingly, although all eight patients were homozygous for the serologically defined DR4 specificity, seven were actually HLA-D heterozygotes. In the one exception, the patient appeared to be homozygous for the uncommon D-locus specificity LD"40", which is the same specificity expressed by six of the seven heterozygotes. These data are summarized along with HLA typing of the entire seropositive JRA patient group in Table II.

DR4 was present in 17 of 22 patients (77%) compared with 32% of 219 controls for a relative risk (RR) of 7.2. 12 of the 22 patients (54%) were homozygous for DR4. HLA-Dw4 was present in 16 patients (73%) and in 17% of controls, for a RR of 12.9. Eight of the DR4-positive patients exhibited the relatively uncommon D-locus specificity LD"40". All eight patients with HLA LD"40" were homozygous for DR4 and seven of them were heterozygous for Dw4 and LD"40". The expected frequency of LD"40", Dw4 heterozygotes was 0.1%, thus the RR for these two antigens together was 116. None of the control population typed as LD"40", Dw4 heterozygotes. None of 25 seronegative JRA patients, including five DR4-positive females, expressed this heterozygous specificity.

Discussion

The expression of the HLA-DR4 specificity in patients with seropositive JRA is one of many such associations that correlate a single HLA specificity with a particular disease syndrome. These observations of association have generally been interpreted to represent linkage of a gene within the HLA region to a disease-susceptibility locus; in the case of HLA-D region associations, this is often attributed to an Ir gene-regulated type of event. If this model is valid, it predicts that the specific products of Ir genes within the HLA-D region, namely the Ia antigens, may be directly involved in disease susceptibility.

We present our observations using structural and functional analysis of D region products that tested this hypothesis. We found a remarkably consistent expression of particular class II molecules in our homozygous JRA patients by two-dimensional gel analysis of DR and DS β-polypeptide chains. In contrast to similar studies of nonselected HLA-DR4 homozygous cells, which revealed at least five distinct DR β-chain and three DS β-chain electrophoretic patterns, all patients cluster into very

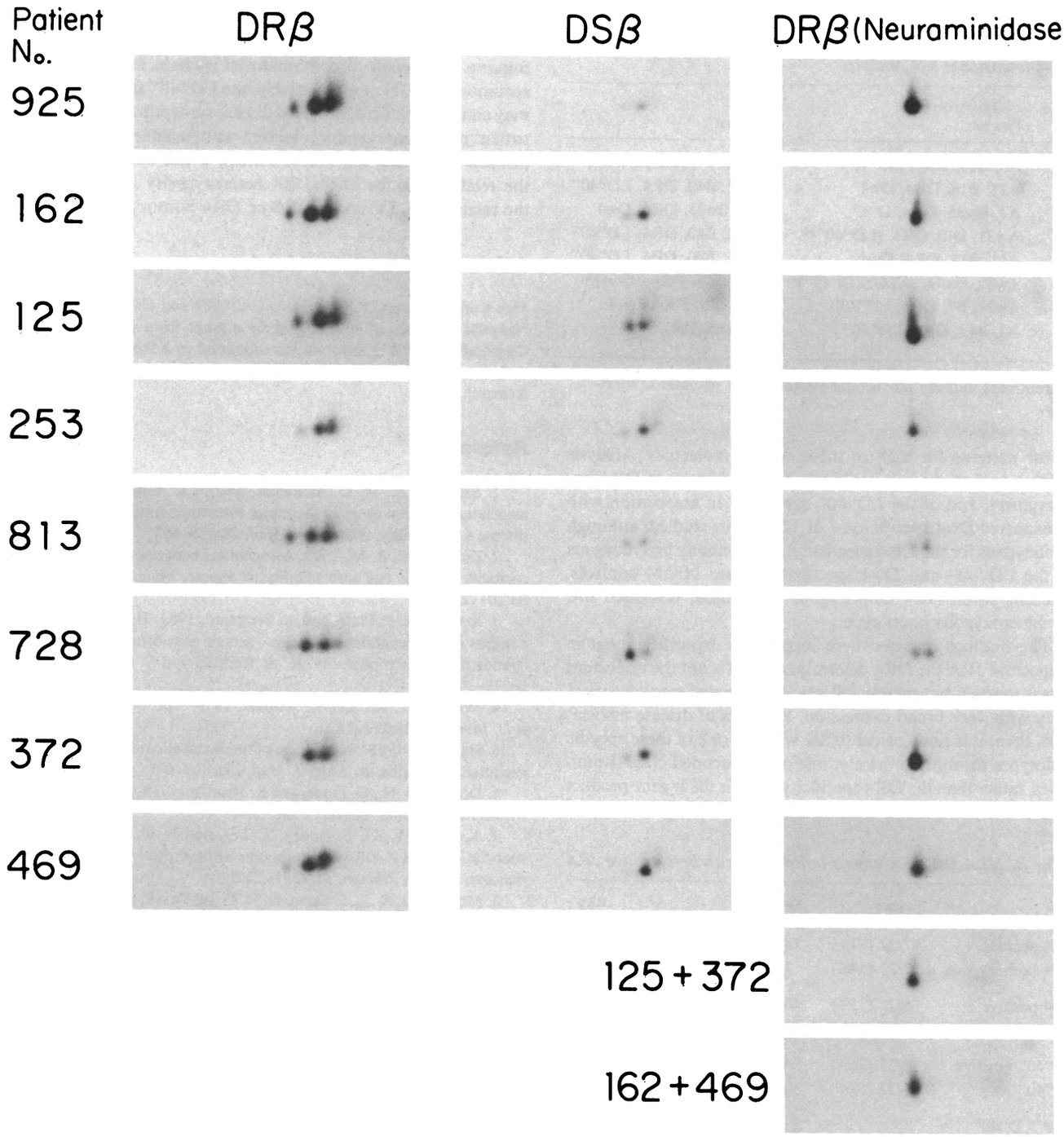

Figure 1. Two-dimensional gel analysis of Ia molecules from DR4 homozygous patients with JRA. Electrophoretic patterns of β-chains radioiodinated with lactoperoxidase and ^{125}I-NaI from immunoprecipitates of DR and DS molecules are shown, obtained from each of eight HLA-DR4 homozygous cell lines derived from patients with JRA. The DR β-gels (left and right columns) were obtained using antibody P4.1; the DS β-gels (middle column) were obtained using antibody SG465. Additive gels formed by mixing immunoprecipitates are shown (bottom right) for combinations of two different neuraminidase-treated DR β-chains. Equivalent portions of each autoradiogram are presented; the basic end of the gel (pH 8.5) is at the left and the acidic end (pH 5.0) at the right.

Table I. The HLA Haplotypes of HLA-DR4 Homozygous Seropositive JRA Patients Investigated by 2-Dimensional Gel Analysis

Patient no.	Paternal	Maternal
813	A3, B40, DR4, LD"40"	A3, Bw44, DR4, Dw4
469	A25, B18, DR4, Dw4	Aw32, B40, DR4, LD"40"
728	A3, Bw35, DR4, D"x"	A29, Bw35, DR4, Dw4
253	Aw31, B40, DR4, (LD"40")*	Aw32, B40, DR4, LD"40"
925	A11, B18, DR4, Dw4	Aw31, B40, DR4, LD"40"
372	Aw32, Bw44, DR4, (LD"40")*	Aw24, B8, DR4, (Dw4)*
125	Aw24, B7, DR4, LD"40"	A26, B15, DR4, Dw4
162	A2, B40, DR4, LD"40"	A2, B15, DR4, Dw4

* Parentheses indicate provisional assignment of parental D specificity.

similar patterns for each of these class II molecules. Analysis by HLA typing shows a strikingly high incidence of DR4 homozygosity, and of the LD"40" specificity in association with the expected Dw4 specificity; 7 of 12 patients studied, although homozygous for the DR4 specificity, were actually heterozygous for the LD"40" and Dw4 specificities. Zero of 234 controls, including seven DR4 homozygous individuals, possessed this rare heterozygous phenotype.

The findings reported here support the hypothesis that in seropositive JRA the DR4 determinant itself is not the important disease marker, but merely reflects an associated serologic specificity with very broad expression. In terms of disease associations, then, it is now logical to ask whether one of these specific haplotypes or, more precisely, one of the encoded class II molecules, rather than the DR4 specificity itself, is the Ir gene product most directly associated with disease susceptibility.

Table II. HLA-DR, D Antigens in Patients with Seropositive JRA

HLA	Patients	Controls*	χ^2	RR‡
DR4 positive	17/22 (77%)	70/219 (32%)	17.8	7.2
DR4 homozygous	12/22 (54%)	7/219 (3%)§	72.6	36.3
Dw4 positive	16/22 (73%)	40/234 (17%)	36.2	12.9
Dw4 only	4/22 (18%)‖	2/234 (0.8%)§	17.2	25.8
LD"40" positive	8/22 (36%)	4/83 (5%)	17.1	11.3
LD"40" only	1/22 (5%)‖	0/83 (0.06%)§	3.8	47
Dw4, LD"40" positive	7/22 (32%)	0/83 (0.1%)§	27.3	116

* Random healthy Caucasian donors.
‡ Relative risk.
§ Expected frequency of homozygotes, calculated from gene frequency.
‖ Provisional assignment (family study not available).

The preponderance of JRA Dw4/LD"40" heterozygotes is intriguing. Heterozygous patients may possess unique Ia determinants as a result of combinatorial α-chain and β-chain associations (21, 22) or alternatively, the LD"40" specificity alone may confer significant increased disease susceptibility. In another patient population, namely children with juvenile onset diabetes (insulin-dependent diabetes mellitus), it has been shown that the relative risk for DR3/DR4 heterozygosity is greater than the relative risk for either DR3 or DR4 homozygotes (23).

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References

1. Sasazuki, T., H. O. McDevitt, and F. C. Grumet. 1977. The association between genes in the major histocompatibility complex and disease susceptibility. *Annu. Rev. Med.* 28:425–452.

2. Zinkernagel, R. M. 1979. Associations between major histocompatibility antigens and susceptibility to disease. *Annu. Rev. Microbiol.* 33:201–213.

3. Ryer, L. P., P. Platz, and A. Svejgaard. 1981. Histocompatibility antigens and susceptibility to disease—genetic considerations. *In* Current Trends in Histocompatibility. R. A. Reisfeld and S. Ferrone, editors. II:279–301.

4. Winchester, R., and H. Kunkel. 1979. The human Ia system. *Adv. Immunol.* 28:222–282.

5. Stastny, P. 1978. Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *N. Engl. J. Med.* 298:869–871.

6. Dobloug, J. H., O. Førre, and E. Thorsby. 1979. HLA-DRw4 and rheumatoid arthritis. *Lancet.* I:548–549.

7. Karr, R. W., G. E. Rodey, T. Lee, and B. D. Schwartz. 1980. Association of HLA-DRw4 with rheumatoid arthritis in black and white patients. *Arthritis Rheum.* 23:1241–1245.

8. McMichael, A. J., T. Sasazuki, H. O. McDevitt, and R. O. Payne. 1977. Increased frequency of HLA-Cw3 and HLA-Dw4 in rheumatoid arthritis. *Arthritis Rheum.* 20:1037–1042.

9. Jaraquemada, D., C. Pachoula-Papasteriadis, H. Festenstein, J. A. Sachs, I. M. Roitt, M. Corbett, and B. Ansell. 1979. HLA-D and DR Determinants in Rheumatoid Arthritis. *Transplant. Proc.* XI:1306.

10. Reinsmoen, N. L., and F. H. Bach. 1982. Five HLA-D clusters associated with HLA-DR4. *Hum. Immunol.* 4:249–258.

11. Nose, Y., K. Sato, N. Nakagawa, K. Kondoh, H. Inouye, and K. Tsuji. 1982. HLA-D clusters associated with DR4 in the Japanese population. *Hum. Immunol.* 5:199–203.

12. Nepom, B. S., G. T. Nepom, E. Mickelson, P. Antonelli, and J. A. Hansen. 1983. Electrophoretic analysis of human HLA-DR antigens from HLA-DR4 homozygous cell lines. Correlation between β chain diversity and HLA-D. *Proc. Natl. Acad. Sci. USA.* 80:6962–6966.

13. Nepom, G. T., B. S. Nepom, P. Antonelli, E. Mickelson, J. Silver, S. M. Goyert, and J. A. Hansen. 1984. The HLA-DR4 family of haplotypes consist of a series of distinct DR and DS molecules. *J. Exp. Med.* 159:394–404.

14. Groner, J., A. Watson, and F. Bach. 1983. Dw/LD-related molecular polymorphism of DR4 B-chains. *J. Exp. Med.* 157:1687–1691.

15. Schaller, J. G. 1980. Juvenile rheumatoid arthritis. *Pediatrics Rev.* 2:163–174.

16. Danilovs, J. A., G. Ayoub, and P. I. Terasaki. 1980. B lymphocyte isolation by thrombin-nylon wool. *In* Histocompatibility Testing 1980. P. I. Terasaki, editor. UCLA Press, Los Angeles. 287–288.

17. Dupont, B., D. W. Braun, E. J. Yunis, and C. B. Carpenter. 1980. HLA-D by cellular typing. *In* Histocompatibility Testing 1980. P. I. Terasaki, editor. UCLA Press, Los Angeles, 229–267.

18. Ryder, L. P., M. Thomsen, P. Platz, and A. Svejgaard. 1975. Data reduction in LD typing. *In* Histocompatibility Testing 1975. F. Kissmeyer Nielsen, editor. Munksgaard, Copenhagen. 557–562.

19. Goyert, S., and J. Silver. 1983. Further characterization of HLA-DS molecules. Implications for studies assessing the role of human Ia molecules in cell interactions and disease susceptibility. *Proc. Natl. Acad. Sci. USA.* 80:5719–5723.

20. Hansen, J. A., S. M. Fu, P. Antonelli, M. Kamoun, J. N. Hurley, R. J. Winchester, B. Dupont, and H. G. Kunkel. 1979. B-lymphoid cell lines derived from HLA-D homozygous donors. *Immunogenetics.* 8:51–64.

21. Fathman, C. G., M. Kimoto, R. Melvold, and C. David. 1981. Reconstitution of Ir genes, Ia antigens, and mixed lymphocyte reaction determinants by gene complementation. *Proc. Natl. Acad. Sci. USA.* 78:1853–1857.

22. Lafuse, W., J. McCormick, P. Corser, and C. David. 1980. Gene complementations to generate Ia antigens (Ia.23) on hybrid molecules. *Transplantation.* 30:341–346.

23. Svejgaard, A., and L. P. Ryder. 1981. HLA genotype distribution and genetic models of insulin-dependent diabetes mellitus. *Ann. Hum. Genet.* 45:293–298.