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Identification of HLA-B27M1 and -M2 cross-reactive antigens in *Klebsiella, Shigella* and *Yersinia*

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The human leucocyte antigen B27, which is subdivided into $M1^+M2^+$ and $M1^+M2^-$ variants, is closely linked to several forms of reactive arthritis (a sterile synovitis associated with an infection elsewhere in the body) and related diseases, notably ankylosing spondylitis and Reiter's syndrome (Brewerton *et al.*, 1973; Woodrow, 1973). Their aetiologies are based on circumstantial evidence incriminating certain bacteria (Keat, 1982). Molecular similarity between HLA-B27 and certain bacterial antigens is thought to result in selective immune tolerance and thus in low titres, which only gradually rise (Ebringer, 1983; Young, Ebringer & Archer, 1978). Recently, observations supporting this concept have been noted (van Bohemen *et al.*, in preparation). The identification of these cross-reactive antigens, would support the above theory and also pave the way for further study of their role in the pathogenesis of ReA. This paper identifies these antigens in *Shigella flexneri* (mol. wt. 20,000) and *Klebsiella pneumoniae* and *Yersinia enterocolitica* (mol. wt. 16,000) with the aid of monoclonal antibodies.

The identification of HLA-B27 cross-reactive antigens in several suspected bacteria was achieved, as shown in Fig. 1, by separating their cell envelope antigens on polyacrylamide gels and subsequently incubating thin longitudinal slices of these gels with monoclonal anti-HLA-B27 antibodies, which discriminate for the M1 and M2 B27 epitopes (Grumet, Fendley & Engleman, 1981; Grumet *et al.*, 1982; Kaneoka, Engleman & Grumet, 1983).

The anti-M2 antibody only reacted with a *S. flexneri* antigen of mol. wt. 20,000. The anti-M1 variant reacted with an antigen of mol. wt. 16,000 of *Y. enterocolitica* type 9 and *K. pneumoniae* type K21 and K43. All reactivities were excellently reproducible for the strains investigated in this study. Non-specific monoclonal antibodies of the appropriate (sub-) class (namely IgM and IgG2a) that had been raised against cell envelope antigens of non-related bacteria (*Haemophilus, Mycobacterium* and *Neisseria*), did not display any reactivity; neither did ascites fluid of unprimed mice. This excluded a possible aspecific adherence of the investigated immunoglobulins to the identified antigens. The anti-HLA-B27 monoclonal antibodies used in this study have been characterized previously (Grumet *et al.*, 1982). A control monoclonal antibody to HLA-A11,24 did not display reactivity. However, the existence of other HLA cross-reacting cell envelope antigens on intestinal bacteria is probable, especially as removal of the intestinal microflora is known to reduce clearly the graft-versus-host reaction (van Bekkum & Knaan, 1977). The HLA-B27 cross-reactive antigens identified in this paper are not the so-called major outer membrane proteins (mol. wt. approximately 35,000), which due to their cross-reacti-

Abbreviation: ReA, reactive arthritis.

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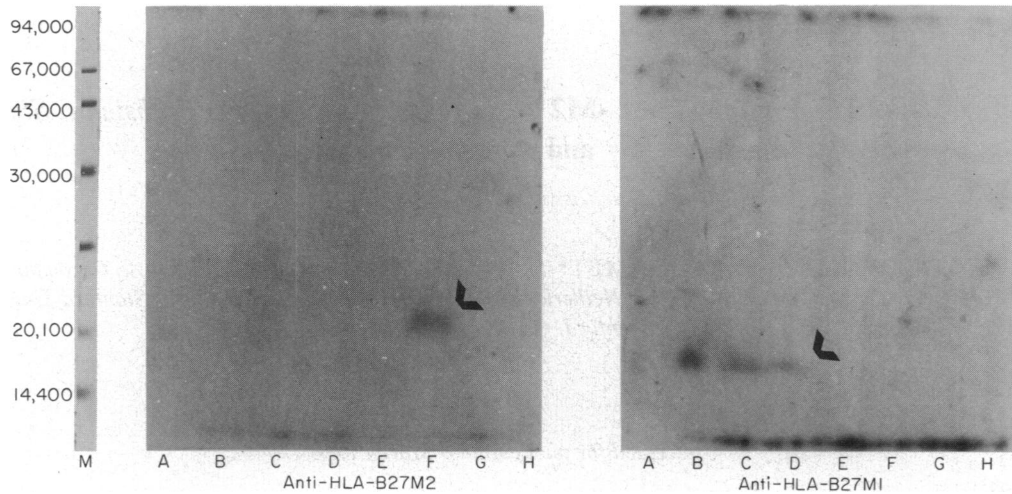

Figure 1. Identification of HLA-B27M1 and M2 cross-reactive antigens in *Enterobacteriaceae* cell envelopes. A = *Yersinia enterocolitica* type 3; B = type 9; C = *Klebsiella pneumoniae* K21; D = K43; E = *Enterobacter aerogenes*; F = *Shigella flexneri* type 2a; G = *Salmonella typhimurium* phage type 505; H = unstained molecular weight markers; M = Coomassie Brilliant Blue stained markers.

The above bacteria were subcultured as described elsewhere (Cowan & Steel, 1979). Cell envelopes were obtained by ultrasonic desintegration (Branson, Danbury, CT) followed by differential centrifugation (Sorvall, Newtown, CT), after which the total protein concentration of the sonicates was determined (Lugtenberg *et al.*, 1975; Markwell *et al.*, 1978). Samples were diluted to 1 mg protein/ml in Tris-HCl buffer (0·625 M, pH = 6·8) containing sodium dodecyl sulphate (2%), glycerol (10%), mercaptoethanol (5%) and bromphenol blue (0·01%), after which they were boiled for 5 min. Routinely 20 μg of protein was applied to an 11% polyacrylamide gel (5 × 4·5 × 0·3 cm) and electrophoresis was carried out at room temperature, applying a current of 20 mA to stack the components and 30 mA for the actual separation (Laemmli, 1970). Electrophoresis was terminated when the tracking dye (bromphenol blue) had proceeded to about 1 cm from the bottom of the gel. To visualize proteins, gels were stained overnight with Coomassie brilliant blue (0·1%, dissolved in acetic acid : methanol : water, 10 : 50 : 40). Destaining was carried out in the same solvent mixture. Molecular weights were estimated (Weber & Osborn, 1969) using a ready-made calibration kit (Pharmacia, Uppsala, Sweden). Unstained gels were frozen on metal holders in liquid nitrogen and sliced at −30° into 40 μm longitudinal sections with a model 5030 microtome-cryostat (Bright, Huntington, U.K.) (Poolman & Zanen, 1980). The slices were stored at −20° in ethanol : acetic acid (6 : 1 vol/vol) for 1–14 days, after which they were thawed, put into 1 ml plastic reaction vials (Sarstedt, Nümbrecht, FRG) and princubated in phosphate-buffered saline (PBS: NaCl, 140 mM; Na_2HPO_4, 9 mM; NaH_2PO_4, 2 mM; pH = 7·4) containing 5% bovine serum albumin (BSA: essentially globulin free, Sigma, St. Louis, MO) and 0·05% Tween-80 for 1 hr at room temperature. The slices were subsequently rinsed (20 min) three times in the same solution but without BSA and incubated in 150 μl monoclonal anti-HLA-B27M1 or M2 (Grumet, 1983), diluted (1:10 vol/vol) in PBS, for 1 hr at 37° followed by 16–18 hr at 0°; controls received non-specific monoclonal antibodies belonging to the same immunoglobulin (sub-) class (IgG2a and IgM respectively) or ascites fluid. They were then rinsed (20 min) three times again in 50 ml PBS-Tween and incubated overnight at room temperature in 150 μl (10^6 d.p.m.) chloramine-T iodinated (^{125}I, Amersham, U.K.) protein A (Hunter & Geenwood, 1962), after which the slices were rinsed at regular intervals in 50 ml PBS-Tween during 5 days under continuous rotating at 4° (Thomas, Philadelphia, PA). All investigated monoclonal antibodies interacted with protein A. Finally, the gel slices were spread onto glass slides (17·5 × 7·5 cm), dried and placed in a cassette (Ilford, Basildon, U.K.) and exposed for autoradiography to Kodak SO-282 X-ray film (Eastman-Kodak, Rochester, NY) under an intensifying screen (Ilford) at −80° for 2–5 days.

vity among many *Enterobacteriaceae* and their noted immunogenicity in ReA patients, are also of interest (van Bohemen *et al.*, 1983). Nor are they the antigens of the *K. pneumoniae* cell envelope (mol. wt. 40,000–52,000), which based on indirect experiments, were thought to cross-react with HLA-B27 (Druery *et al.*, 1980).

Cell envelope antigens of *Y. enterocolitica* type 3 did not react with the anti-HLA-B27 preparations, indicating that the possession of an HLA-B27 cross-reacting antigen is a serotype selective trait. This is supported by the lack of reactivity with *Enterobacter aerogenes* and *Salmonella typhimurium*. The first is phylogenetically related to *K. pneumoniae* and also suspected of disease involvement (Ebinger *et al.*, 1976) and the latter has been closely associated with

HLA-B27 and ReA (Aho *et al.*, 1975). Although the existence of additional B27 epitope-like antigens is likely (Grumet, 1983), it is also possible that the aetiology of ReA is restricted to certain serotypes of the suspected causative organisms. This aspect has hitherto not been fully appreciated.

K. pneumoniae has been incriminated as a causative agent in ankylosing spondylitis (Ebringer *et al.*, 1978), however, this is contested by some reports (Warren & Brewerton, 1980; Eastmond *et al.*, 1980). The above identification of HLA-B27 cross-reactive antigens in *K. pneumoniae* supports its potential involvement in this disease. Noteworthy, however, is the absence of reports indicting *K. pneumoniae* in relation to ReA. Perhaps those cases diagnosed as ReA, but in which the causative infectant has not been isolated, harbor *K. pneumoniae* infections.

Despite the lack of one-to-one correlations with disease, *Enterobacteriaceae* involvement in some forms of non-rheumatoid arthritis is steadily acquiring greater validity as a relevant concept. The above identification of HLA-B27M1 and -M2 cross-reactive antigens in the cell envelopes of some of these bacteria should facilitate further studies regarding the exact role of these pathogens in ReA and related diseases.

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REFERENCES

Aho K., Ahvonen P., Alkio P., Lassus A., Sairanen E., Sievers K. & Tillikainen A. (1975) HLA-B27 in reactive arthritis following infection. *Ann. rheum. Dis.* **34** (Suppl.), 29.

Brewerton D.A., Caffrey M., Hart F.D., James D.C.O., Nichols A. & Sturrock R.D. (1973) Ankylosing spondylitis and HL-A27. *Lancet*, **i**, 904.

Cowan S.T. & Steel K.T. (1979) *Manual for the identification of medical bacteria*, 2nd edn. Cambridge University Press, Cambridge.

Druery C., Bashir H., Geczy A.F., Alexander K. & Edmonds J. (1980) Search for *Klebsiella* cell wall components cross-reactive with lymphocytes of $B27^+AS^+$ individuals. *Human Immunol.* **1**, 151.

Eastmond C.J., Willshaw H.E., Burgess S.E.P., Shinebaum R., Cooke E.M. & Wright V. (1980) Frequency of faecal *Klebsiella aerogenes* in patients with ankylosing spondylitis and controls with respect to individual features of the disease. *Ann. rheum. Dis.* **39**, 118.

Ebringer A. (1983) The crosstolerance hypothesis and B27 disease. *Br. J. Rheum.* **22** (Suppl. 2), 53.

Ebringer R.W., Cadwell D.R., Cowling P. & Ebringer A. (1978) Sequential studies in ankylosing spondylitis. Association of *Klebsiella pneumoniae* with active disease. *Ann. rheum. Dis.* **37**, 146.

Ebringer A., Cowling P., Ngwa Suh N., James D.C.O. & Ebringer R.W. (1976) Cross-reactivity between *Klebsiella aerogenes* species and B27 lymphocytes antigens as an aetiological factor in ankylosing spondylitis. In: *HLA and Disease* (eds J. Dausset and A. Svejgaard), p. 28. Inserm, Paris.

Grumet F.C. (1983) Monoclonal antibodies to HLA-B27. *Br. J. Rheum.* **22** (Suppl. 2), 110.

Grumet F.C., Fendley B.M. & Engleman E.G. (1981) Monoclonal anti-HLA-B-27 antibody (B27M1): production and lack of detectable typing differences between patients with ankylosing spondylitis, Reiter's syndrome and normal controls. *Lancet*, **ii**, 174.

Grumet F.C., Fendley B.M., Fish L., Foung S. & Engleman E.G. (1982) Monoclonal antibody (B27M2) subdividing HLA-B27. *Human Immunol.* **5**, 61.

Hunter W.M. & Greenwood F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)*, **194**, 495.

Kaneoka H., Enleman E.G. & Grumet F.C. (1983) Immunochemical variants of HLA-B27. *J. Immunol.* **130**, 1288.

Keat A.C. (1982) HLA-linked disease susceptability and reactive arthritis. *J. Infect.* **5**, 227.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**, 680.

Lugtenberg B., Meijers J., van der Hoek P., Peters R. & van Alphen L. (1975) Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. *FEBS Lett.* **58**, 254.

Markwell M.A.K., Haas S.M., Bieber L.L. & Tolbert N.E. (1978) The modification of the Lowry procedure to simplify protein determinations in membrane and lipoprotein samples. *Analyt. Biochem.* **87**, 206.

Poolman J.T. & Zanen H.C. (1980) Detection of antibody activity in human sera against meningococcal cell wall antigens using a gel-immuno-radio-assay (GIRA). *FEMS Microbiol. Lett.* **7**, 293.

van Bekkum D.W. & Knaan S. (1977) Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. *J. natn. Cancer Inst.* **58**, 787.

van Bohemen Ch.G., van Alphen A.J.W., Zanen-Lim O.G., Dekkers-Saeys A.J. & Zanen H.C. (1983) Antibodies against cell envelope antigens of *Yersinia enterocolitica* in reactive arthritis and ankylosing spondylitis. *Br. J. Rheum.* **22**, (Suppl. 2), 83.

WARREN R.E. & BREWERTON D.A. (1980) Faecal carriage of *Klebsiella* by patients with ankylosing spondylitis and rheumatoid arthritis. *Ann. rheum. Dis.* **39**, 37.

WEBER K. & OSBORN M. (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. biol. Chem.* **244**, 4406.

WOODROW J.C. (1973) HL-A-27 and Reiter's syndrome. *Lancet*, **ii**, 671.

YOUNG C.R., EBRINGER A. & ARCHER J.R. (1978) Immune response inversion after hyperimmunisation: possible mechanism in the pathogenesis of HLA linked disease. *Ann. rheum. Dis.* **37**. 152.