Klebsiella 'modifying factor': binding studies with HLA-B27⁺ and B27⁻ lymphocytes

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SUMMARY On the basis that extracts of some klebsiella organisms bind selectively to the lymphocytes of HLA-B27⁺ individuals and induce the appearance of new antigens, attempts were made to detect the binding of klebsiella products to HLA-B27⁺ and B27⁻ lymphocytes by a number of different techniques. Firstly, blocking of the binding of two different HLA-B27 specific monoclonal antibodies to HLA-B27⁺ lymphocytes has been examined following exposure of the lymphocytes to a cell-free culture filtrate from *K. pneumoniae* K21 and K43. There was no reduction in the cytotoxicity of either antibody, suggesting that neither of the epitopes detected by the anti-HLA-B27 monoclonal antibodies is a binding site for klebsiella products. Secondly, we have studied the binding of partially purified, radiolabelled klebsiella products to healthy HLA-B27⁺ and B27⁻ lymphocytes. There was no significant difference either in terms of numerical counts bound or by comparing, by SDS-PAGE analysis, the molecules bound to each cell type. At the level of sensitivities of these techniques we can detect no difference in binding of klebsiella products to the lymphocytes of healthy HLA-B27⁺ and B27⁻ individuals.

Despite the demonstration of the strong association between HLA-B27 and ankylosing spondylitis (AS)^{1, 2} the mechanism which links them is obscure. The lack of disease concordance among B27⁺ identical twins³ and the observation that only a subpopulation of B27⁺ individuals acquires AS argue strongly for an environmental factor which triggers the disease in susceptible individuals. The demonstration that individuals with AS shed *Klebsiella pneumoniae* in their faeces more frequently than normal B27⁺ individuals⁴ suggests that infection with this organism may predispose certain individuals to spondylarthropathy. In support of this, an increase in the frequency of faecal isolation of klebsiella species during disease activity (as compared with quiescent disease) has been demonstrated.⁵ Geczy and his coworkers first showed, using a ⁵¹Cr release assay, that sera raised in rabbits against certain subtypes of *K. pneumoniae* will specifically lyse AS⁺B27⁺ peripheral blood lymphocytes (PBLs), but not B27⁺ PBLs from healthy individuals.⁶ Furthermore, the cells from healthy AS⁻B27⁺ individuals became susceptible to

lysis by the same antisera after incubation with a cell-free culture filtrate derived from such strains of klebsiella.⁷ Thus the cell surface of healthy B27⁺ cells becomes 'modified' after contact with klebsiella products. The ability of B27⁺ PBLs from normal subjects (but not B27⁺ PBLs from subjects with AS) to absorb this 'modifying activity' from klebsiella supernatants strongly suggests the presence of a soluble factor released from klebsiella which is able to adhere to a receptor on B27⁺ cells and initiate cellular changes which are somehow involved in the induction of AS. This is an attractive hypothesis and provides a direct link between the disease, the organism, and the HLA-B27 antigen. Initial characterisation of this soluble factor by means of Biogel P100 chromatography suggested it to be a glycoprotein of 26-30Kd,⁸ and more recently it has been shown that the ability to produce this modifying factor is transmissible to other organisms by a plasmid, in that *Escherichia coli* which take up this plasmid subsequently become able to modify B27⁺ PBLs from normal subjects.⁹

It should be noted that all of this testing involved the use of the ⁵¹Cr release assay and its inhibition, and recently there has been some difficulty in

reproducing the ^{51}Cr assay.¹⁰ Other workers have unsuccessfully attempted to demonstrate specific binding of anti-klebsiella antibodies to AS⁺B27⁺ lymphocytes by a variety of other techniques, including enzyme immunoassay, dye-release cytotoxicity, and cytofluorographic analysis (P R Russell, R W Ebringer, D Wakefield, personal communications.)

In view of the crucial importance of these findings in explaining the pathogenesis of an HLA-associated disease we have attempted to demonstrate the binding of klebsiella products to B27⁺ PBLs by means other than the ^{51}Cr release assay (i.e., by direct binding). Using a variety of techniques we are unable to demonstrate specific binding of klebsiella products to healthy B27⁺ PBLs.

Materials and methods

Subjects studied. All HLA-B27⁺ individuals denied symptoms of inflammatory arthritis, had no past history of uveitis, psoriasis, or inflammatory bowel disease and no family history of spondylarthropathy. Clinical examination revealed none of the stigmata of AS.

Monoclonal antibodies. B27m2 (a kind gift of Professor F Carl Grumet) and HLA-ABC-m3, both murine monoclonal antibodies which specifically detect HLA-B27, have been described elsewhere.^{11 12}

Bacterial cultures. *K. pneumoniae* K21, K43 (subtypes to which rabbit antisera have been raised and cross-react with AS⁺B27⁺ PBLs) and F77 (a non-cross-reacting strain) were kind gifts of Dr Pamela Russell (Kolling Institute, Royal Prince Alfred Hospital, Sydney, Australia). Antibiotic sensitivities were tested before and after each experiment by the Royal Melbourne Hospital Microbiology Department. Cell-free culture filtrates were obtained by harvesting supernatant from 24-hour cultures of the organisms in TSB (tryptone soya broth, Oxoid Ltd, Basingstoke, England) and passage through a 0.22 μm filter (Gelman Sciences, Australia). Filtrates were stored at -20°C .

Blocking studies. PBLs were prepared sterilely by passage of heparinised peripheral blood through an Isopaque-Ficoll gradient, washing, then resuspending for 2–8 h at 37°C in bacterial culture supernatant (K21, K43, F77, or TSB) diluted in 1:2 with either phosphate buffered saline or TSB. Cells were washed once with Leibovitz medium (Flow Laboratories, Irvine, Scotland) with 0.5% (w/v) bovine serum albumin, adjusted to $1.5 \times 10^6/\text{ml}$, and tested in the standard National Institutes of Health (NIH) microcytotoxicity assay.¹³

Partial purification of factors from supernatants of klebsiella cultures. Klebsiella modifying factor was partially purified by a modification of the method of Sullivan *et al.*¹⁴ Log phase culture supernatant, 400 ml, of K21 and F77 in TSB was collected, concentrated 100-fold across a PM-10 membrane (Amicon, Australia), and applied to a Fractogel TSK HW 55(S) column (E. Merch, Darmstadt, Federal Republic of Germany). Molecules of Mr ranges 200K–50K, 50K–18K, and smaller than 18K were separately collected, reconcentrated, and radio-labelled.

Radiolabelling. Equal amounts of purified K21 and F77 klebsiella supernatants were radiolabelled with ^{125}I by the chloramine T method and specific radioactivity calculated at approximately 1.0 Ci/ μg (>80% trichloroacetic acid precipitable counts). Iodinated products were used immediately. Supernatant proteins were biosynthetically labelled by adding 2.5 mCi ^3H -leucine to klebsiella cultures overnight. Filtered, biosynthetically labelled material was purified as described above.

Radioactive binding assays. PBLs (2.5×10^6) were incubated with radioiodinated or biosynthetically labelled material (5×10^5 cpm) at 37°C for 0–20 hours. Cells were harvested, washed with phosphate buffered saline containing 0.5% (w/v) bovine serum albumin, and the bound radioactivity was detected in a gamma or scintillation counter. All assays were performed in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Following osmotic lysis of cells and removal of nuclear material, radiolabelled species bound to B27⁺ and B27[−] cells were resuspended in buffer containing 20 mM dithiothreitol and analysed in 12.5% one-dimensional Laemmli slab gels.¹⁵

Results

The aim of this study was three-fold: firstly, to demonstrate that the klebsiella organisms used and their products were identical to those used in previous studies; secondly, to determine whether klebsiella products bind to HLA-B27⁺ cells via any of the epitopes defined by anti-HLA-B27 monoclonal antibodies, and, thirdly, to demonstrate either in a quantitative and/or a qualitative fashion a difference in binding of K21 or K43 klebsiella products to HLA-B27⁺ and B27[−] PBLs.

1. Characterisation of klebsiella isolates. The isolates of klebsiella K21, K43, and F77 were subcultured directly from organisms previously extensively characterised.⁹

(a) Antibiotic sensitivity of klebsiella isolate.—To

ensure that the klebsiella organisms used had not altered or lost plasmids during culture, antibiotic sensitivities were determined before and after each experiment and were found to be identical, in each case, to those previously published⁹ (Table 1).

(b) Absorbance profiles of F77 and K21 *K. pneumoniae* supernatants following Fractogel chromatography.—The absorbance profiles (280 nm) for K21 and F77 coincided with each other and with the previously published absorbance data for these klebsiellae¹⁵ (Fig. 1). Molecules of 50 Kd and 18 Kd were collected at elution volumes of 106 and 118 ml respectively.

2. Blocking of anti-HLA-B27 monoclonal antibody binding. Although klebsiella factor has been shown to be bound only by normal B27⁺ (but not

B27⁺ AS⁺ or B27⁻ AS⁻) lymphocytes, the binding site for this factor has not been demonstrated. We therefore investigated whether several of the known epitopes on the B27 molecule are binding sites using two anti-HLA-B27 monoclonal antibodies. Preincubation of B27⁺ PBLs from three healthy individuals with culture supernatants from K21 (diluted

Fig. 1 Absorbance profiles (280 nm) of eluates derived from Fractogel TSK HW-55(S) chromatography of klebsiella K21 (●—●) and F77 (*—*). Molecules of 50 Kd and 18 Kd eluted at 106 and 118 ml respectively.

Fig. 2 Reactivity of two lymphocytotoxic anti-HLA-B27 monoclonal antibodies, HLA-ABC-m3 (a) and B27m2 (b) with B27⁺ PBLs preincubated with K21 supernatant (●—●) and untreated (○—○).

Table 1 Antibiotic sensitivity profile of klebsiella K21

	Smx	Cm	Tm	Fus	Rf	Sm	Sp	Tc	Tp	Ap	Cp	Km	Gm
Previously reported ⁹	R	R	R	R	R	R	R	R	R	R	R	R	R
This study	R	R	R	R	R	R	NT*	R	R	R	R	R	R

*Not tested

Smx: sulphamethoxazole. Cm: chloramphenicol. Tm: tobramycin. Fus: fusidic acid. Rf: rifampicin. Sm: streptomycin. Sp: spectinomycin. Tp: Trimethoprin. Tc: tetracycline. Ap: ampicillin. Cp: cephalothin. Km: kanamycin. Gm: gentamicin.

either with PBS or TSB) for two or eight hours failed to reduce the cytotoxicity titre of either HLA-ABC-m3 or B27m2 monoclonal antibodies, which detect separate antigenic determinants on the B27 molecule¹¹ (Fig. 2a and 2b). Identical results were obtained following incubation with K43 and F77 supernatants (not shown). This suggests that neither of the polymorphic determinants detected by B27m2 or HLA-ABC-m3 antibodies is a binding site for klebsiella products. It should be noted that the blocking conditions were identical to those which inhibit ⁵¹Cr release from labelled B27⁺ lymphocytes, the only differences being the use of

Fig. 3 Radioactivity bound to PBLs from healthy HLA-B27⁺ (○—○) and B27⁻ (●—●) individuals following incubation for 0, 2, 6, 12, or 20 hours with ¹²⁵I-labelled supernatants from klebsiella K21 (a) and F77 (b). Counts bound are expressed as the mean of triplicate binding assays ± standard error.

monoclonal antibodies in this study and the measurement of cytotoxicity by dye rather than ⁵¹Cr release.

3. *Binding of radiolabelled klebsiella products to B27⁺ and B27⁻ PBLs.* Previous absorption studies have demonstrated the removal of the modifying factor from K21 supernatants by B27⁺ PBLs within two hours¹⁶; furthermore, B27⁺ PBLs which absorb this material become susceptible to lysis by anti-klebsiella sera within eight hours.¹⁶ The fact that modifying factor is absorbed *specifically* by healthy B27⁺ cells should permit identification of the factor by using these cells to bind any factor present in klebsiella culture supernatant. We have thus monitored the radiolabelled K21 and F77 supernatant material (18–50 Kd) which binds to the PBLs of three HLA-B27⁺ and three HLA-B27⁻ individuals over 0–20 hours' incubation. The radioactivity bound increased to a maximum of approximately 1.5% of counts added by 12 hours. However, there was no quantitative difference in the binding of K21 or F77 products to B27⁺ and B27⁻ PBLs for any of the incubation periods studied (0, 2, 6, 12, and 20 hours; Fig. 3 a, b). Similarly, no difference was detected after incubation with either the 50–200 Kd or the 18 Kd fraction (not shown).

In order to exclude the possibility that klebsiella

Fig. 4 Radioactivity bound to PBLs from healthy HLA-B27⁺ and B27⁻ individuals following 16 hours' incubation with K21 culture supernatant biosynthetically labelled with ³H-leucine. Counts bound are expressed as the mean of triplicate binding assays ± standard error.

Fig. 5 SDS-PAGE analysis of radioactive material bound to HLA-B27⁺ and B27⁻ PBLs following incubation for 16 h with culture supernatants from klebsiella K21 (left panel) and F77 (right panel) biosynthetically labelled with ³H-leucine.

factor is not a suitable substrate for iodination, or that iodination inactivates the molecule(s), the binding studies described were repeated with biosynthetically labelled material. For these studies ³H-leucine was used for incorporation into klebsiella proteins (in preference to ³⁵S-methionine in case the protein(s) in question lack sufficient methionine residues). Again, no difference in binding to HLA-B27⁺ and B27⁻ cells was detected over 16 hours (Fig. 4). Thus, we are unable to demonstrate by quantitative means, the *specific* binding of a factor derived from klebsiella supernatant to healthy HLA-B27⁺ PBLs.

We also attempted to demonstrate a qualitative difference in the 18–50 Kd products bound to B27⁺ and B27⁻ PBLs by analysing bound radiolabelled molecules with one-dimensional SDS-PAGE (Fig. 5). As expected following Fractogel chromatography, the principal molecules bound from both K21 and F77 klebsiellae fall within a confined range of apparent molecular weights (approx 25–60 Kd) and

there is a similarity of the molecular weights of molecules bound from the K21 and F77 isolates. We were unable to identify molecules which either specifically bind to or are absent from B27⁺ cells. In particular, no molecule of apparent molecular weight of approximately 46 Kd, the previously reported apparent molecular weight of klebsiella factor on polyacrylamide gels,¹⁴ was found to be exclusively present in the tracks of labelled protein from B27⁺ cells.

Discussion

The finding that certain klebsiella subtypes produce a factor which can bind to B27⁺ cells from AS⁻ individuals, but not to B27⁺AS⁺ cells has generated great interest, as this could provide a link between the bacterium and the disease. Furthermore the observation could be of great importance in directly linking HLA molecules and disease states, since HLA-B27 could be a receptor for the bacterial

product. Unfortunately the studies have not been easily reproducible, and we have attempted, by sensitive radiolabelling techniques, to define a factor which binds to B27⁺ cells and does not bind to B27⁻ cells. We were unable to find such a factor.

We explored three different means of demonstrating the selective binding of K21 and K43 klebsiella products to the surface of normal HLA-B27⁺ PBLs. Firstly, blocking of the binding of anti-HLA-B27 monoclonal antibodies to B27⁺ cells following exposure to klebsiella supernatants was attempted. Secondly, quantitative differences in the binding of radiolabelled klebsiella products to B27⁺ and B27⁻ cells were sought. Finally, attempts were made to define, by SDS-PAGE analysis, a klebsiella molecule binding specifically to cells bearing the HLA-B27 specificity. In each case no differences in binding to B27⁺ and B27⁻ lymphocytes could be demonstrated.

The study is the first to use specific monoclonal antibodies to determine whether some of the epitopes on the B27 molecule act as binding sites for klebsiella products. Indeed previous studies have produced no strong evidence that the B27 molecule (as opposed to some other molecule, possibly the product of a gene linked to HLA-B27), is the putative receptor. Since at least four HLA-B27 related epitopes have been defined by murine monoclonal antibodies (and others may exist), our study does not exclude these other determinants as binding sites. However, our study has utilised the only two monoclonal antibodies which define epitopes known not to occur on a variety of HLA-B27⁻ cells.¹² Thus, if the determinants defined by B27m1¹⁷ and ME1¹⁸ (the two anti-HLA-B27 monoclonals not used in this study) were sites of attachment for klebsiella products, a substantial number of B27⁻ cells should absorb klebsiella modifying factor. This has been shown *not* to be the case.¹⁶

The inability of B27⁺ PBLs from AS patients to absorb modifying factor would suggest a polymorphism in the structure of the HLA-B27 molecule if B27 itself were the binding site. However, such a polymorphism has not been detected in two studies using two-dimensional PAGE and tryptic and chymotryptic peptide mapping.^{19,20} In fact no difference in structure of B27 was noted between subjects with AS and normal subjects. The polymorphism in B27 structure defined by B27m2 (12% of individuals typed as B27⁺ by conventional means lack the determinant detected by this monoclonal antibody) does not correlate with susceptibility to AS.¹¹ Taken together all of these studies argue strongly against klebsiella products binding directly to the HLA-B27 molecule.

At their face value our findings do not support

those of Geczy *et al.*, but using different techniques in a different laboratory leads us to question our findings and to discuss the shortcomings of our investigations. An inability to demonstrate either a quantitative or qualitative difference in binding of radiolabelled klebsiella products to HLA-B27⁺ and B27⁻ PBLs could be compatible with the findings of Geczy *et al.* for any of several reasons. Firstly, having bound to HLA-B27⁺ cells, the modifying factor could be rapidly internalised and degraded *in vitro* and therefore not detected. This could be the case, although we note that B27⁺ cells taken from AS⁻ individuals (who therefore bind klebsiella factor) and which are then transformed with Epstein-Barr virus into cell lines, still have the phenotypic trait for up to two days, suggesting that, once bound, the factor is stable.²¹ Secondly, the interaction between receptor and ligand may be of low affinity, so that modifying factor might be shed during washing prior to estimation of radioactivity bound. This would suggest an affinity well below that seen for well characterised interactions such as those between antigen and antibody or hormones and their receptors. Thirdly, modifying factor may be present in amounts small enough to evade detection by the techniques we have used. This would suggest a great biological potency for the small number of klebsiella molecules involved. Finally, since only 80–90% of healthy HLA-B27⁺ individuals have been shown to absorb modifying factor,¹⁶ our results may indicate a random selection of individuals who lack this ability. However, since each of our studies utilised the cells of at least three B27⁺ healthy individuals, the chance of a false negative result for this reason is small (<0.8% for each study). We are thus unable to explain why we cannot find the factor discussed by Geczy *et al.*

In view of the difficulty in reproducibility of the ⁵¹Cr release assay and the inability to demonstrate the specificity of binding of klebsiella products to B27⁺ cells by a variety of techniques in various laboratories, it is also possible that the *in-vitro* interaction observed between klebsiella and B27⁺ PBLs may be fortuitous and of questionable significance *in vivo*. Certainly it would be greatly reassuring if the association between HLA-B27⁺ PBLs and the products of certain strains of klebsiella could be defined precisely at a molecular level by some technique other than the ⁵¹Cr release cytotoxicity assay.

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