Absence of impaired lymphocyte transformation to *Klebsiella* spp. in ankylosing spondylitis

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SUMMARY We have evaluated claims that impaired peripheral blood lymphocyte (PBL) transformation can occur with *Klebsiella* spp. in patients with ankylosing spondylitis (AS). PBL of four AS patients were cultured in vitro with autogenous faecal klebsiella, as were the PBL of age (± 3 years) and sex-matched pairs of 15–20 AS and normal controls cultured with heterogenous AS-derived klebsiella and control bacterial isolates. Three of four AS patients responded to their own isolates, and no significant differences were found between the matched pairs in response to heterogenous klebsiella isolates, including K21. Our studies did not show impaired PBL transformation with klebsiella in AS and therefore do not support claims of antigenic cross-reactivity between klebsiella and HLA-B27.

The aetiology and pathogenesis of ankylosing spondylitis (AS) remain unknown despite evidence which suggests an immunogenetic influence via the major histocompatibility complex (MHC) because of the strong association of AS with HLA-B27.^{1,2} In relation to this, activated humoral immunity has been suggested by elevation of serum immunoglobulin (Ig) levels, including IgA,^{3,4} as well as serum complement (C) activation, and elevated levels of circulating immune complexes.^{4–6} Perhaps surprisingly the possible participation of cell-mediated immunity (CMI) in AS has been extremely controversial,⁷ though clarification of this has recently been suggested by studies which have implicated a role for organisms of the *Klebsiella* spp.^{8,9} In view of these studies we wish to report our studies of in-vitro peripheral blood lymphocyte (PBL) transformation induced by isolates of *Klebsiella* spp. and control organisms from AS patients.

Materials and methods

STUDY GROUPS

Twenty AS patients (16 males and 4 females) were matched by sex and age (± 3 years) with 20 normal controls. All AS patients had primary disease except

two who developed inflammatory bowel disease during these studies two and five years after the onset of AS. Active disease, as defined previously by us,⁵ was present in 18 AS patients whose disease duration was 1–34 years (mean 10.7 years). Sixteen patients were withdrawn from all medication except acetaminophen at least 36 hours before study. Seventeen patients were positive (+) and three negative (–) for HLA-B27.

Control subjects were selected to include 13 health care workers, since recent reports suggest that people who frequent health care facilities are liable to have increased, asymptomatic intestinal colonisation with klebsiella.^{10,11} All controls were healthy, though two were HLA-B27 (+).

BACTERIAL ISOLATES

Twenty-one stool cultures from 17 AS patients were propagated on trypticase soy broth (Difco) and yielded 5 klebsiella serotypes. The latter were kindly determined by Dr R. P. Rennie, of McMaster University, to represent two capsular strains of K7, and one each of K21, K33, and K68.¹² Aliquots of washed isolates were propagated serially, as well as quick frozen, for use in in-vitro PBL transformation. Prior to the latter, viable isolates were fixed in 20% formalin, washed three times in Hanks's balanced salt solution (HBSS), recultured to ensure non-viability, and then resuspended in HBSS in appropriate concentrations.

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Control bacterial isolates consisted of a klebsiella pool (KP) of two urine and two stool strains, as well as a pool of *Escherichia coli* (EC) of two urine and two stool strains from non-AS donors. Serotyping of control isolates was not done.

LYMPHOCYTE CULTURES

PBL were recovered by density gradient separation (Lymphoprep-Nyegaard), washed, and resuspended in 10% human AB serum in Roswell Park Memorial Institute solution (RPMI) 1640 (Gibco). Isolated PBL were characterised as T-PBL by E rosettes and B-PBL by surface Ig staining.¹³ Mean values ($\pm$ SEM) derived from more than 150 normal control isolations were $73\pm 9\%$ T-PBL and $9\pm 2\%$ B-PBL: no significant differences were found for T and B-PBL between AS patients and their matched controls in the present studies.

In 26 preliminary experiments microcultures containing 10^5 PBL from normal subjects were established with 10^4 , 10^5 , 2×10^5 , 5×10^5 , and 10^6 bacterial isolates to determine optimal and suboptimal PBL: bacterial ratios. Culture conditions were otherwise identical to those subsequently employed (see below) and established 10^5 and 10^6 bacteria per 10^5 PBL as suboptimal and optimal ratios respectively.

Triplicate experimental cultures containing 10% autologous plasma (AP) or AB serum in RPMI-1640 (Gibco), 10^5 PBL, and 10^5 and 10^6 bacterial isolates were maintained in parallel for 132 hours in a humidified atmosphere of 5% CO₂ in air, pulsed with 0.5 μ Ci tritiated thymidine (Amersham Searle) in the final 18 hours, and then automatically harvested on filter discs (Skatron). Unstimulated controls, and mitogenic controls of phytohaemagglutinin (PHA-P, Wellcome, 0.025 and 0.05 μ g/culture) and pokeweed mitogen (PWM, Gibco, 1/10 and 1/40 stock dilutions in HBSS/culture) were established with each experiment. Radioactivity was calculated by liquid scintillation spectrometry (Beckman LS-3133T). Results are expressed as a stimulation index (SI) derived from the mean counts per minute (cpm) in stimulated (experimental) cultures divided by the mean cpm in unstimulated cultures.

Statistical calculations. Paired Wilcoxon rank sum and Student's *t* tests, as appropriate, were used to compare the results of experimental and control groups by a TRS-80 computer with custom programs. Twenty pairs of AS and control subjects were compared for the KP, K7(1), and EC isolates, and 18, 17, and 15 pairs for the K7(2), K68, and K21 isolates respectively.

Results

Transformation responses to autogenous klebsiella

isolates. The results obtained when optimal doses of four klebsiella isolates from AS patients were cultured with their own PBL are shown in Table 1. Three of the four patients showed enhanced PBL transformation (SI > 5) with their autogenous isolates in AP and/or AB serum. One patient (4) did not respond to his own K21 isolate or to the three other AS-derived isolates. All patients were HLA-B27 (+), and all had active disease of at least moderate severity subjectively and objectively.

Under identical experimental conditions except for the use of suboptimal (10^5) doses of autogenous isolates, two patients (1 and 4) showed no mitogenic response (SI < 1.0). Patients 2 and 3 gave SI of 15.0 and 8.9 with suboptimal doses of their own isolates. Responses in AB serum with suboptimal doses of isolates were not determined.

Transformation responses to heterogenous klebsiella isolates. Maximum SI for the matched groups in response to optimal doses of heterogenous KP, K7(1), K7(2), K68, and K21 in AP are shown in Fig. 1. No significant differences ($p > 0.05$) were found between the study groups for any klebsiella isolate, by Wilcoxon's rank sum test to compare the non-parametric results with KP, K7(1), K7(2) and K68, and Student's *t* test for the parametric data of K21. The distribution of results with EC was virtually identical to that with K21 and was similarly not significantly different (data not shown).

Comparison of the SI of the matched study groups with suboptimal doses of each isolate in AP, and with optimal doses of all isolates in AB serum, were not significantly different (data not shown). Although group responses in AP compared with AB were not significantly different, more than two-fold

Table 1 *Peripheral blood lymphocyte transformation by autogenous klebsiella of AS patients*

Patient	Serum Supplement	Stimulation index [†] with			
		K7(1)	K7(2)	K68	K21 [‡]
1	AP	15.3 [§]	ND	ND	ND
	AB	240.0	ND	ND	ND
2	AP	0.4	43.2	71.1	ND
	AB	1.1	1.2	1.2	ND
3	AP	16.0	3.7	0.8	ND
	AB	4.5	13.1	7.6	ND
4	AP	1.6	0.5	0.3	0.6
	AB	1.7	0.2	1.3	1.8

^{*}AP=autologous plasma and AB=human AB serum.

[†]Stimulation index=SI derived as per text.

[‡]Designates capsular serotype: K7(1) and (2) represent identical serotypes but different biotypes.

[§]SI expressed in italics are values obtained with autogenous klebsiella isolates; ND=not done.

Fig. 1 Peripheral blood lymphocyte transformation by heterogeneous klebsiella. Abbreviations correspond to those in Table 1. ●=the SI for each AS patient, ○=the SI for each matched normal control. ---=group means.

changes were occasionally seen in the SI of some subjects' PBL in parallel cultures with AP and AB serum. For example, in response to isolate K7(1) the SI of four AS were higher in AP, whereas three were higher in AB, and 13 were not significantly different.

No relationship was apparent between status for HLA-B27 and the magnitude or direction of PBL transformation to any isolate. In the AS group the same B27(−) patient hyperresponded to all isolates except K21, whereas all other AS hyperresponders were B27(+). In the normal control group, all hyperresponders were B27(−) regardless of isolate tested.

No significant differences were found between the AS and normal control groups in their unstimulated and PBL transformation responses to all doses of PHA and PWM (data not shown).

Discussion

In contrast to some recent reports^{9 14} we found no significant differences between AS and matched normal controls in their PBL transformation responses to several strains of klebsiella. Specifically, we found no impairment of AS-PBL transformation in response to AS-derived autogenous and heterogeneous *Klebsiella* spp., to a pool of *E. coli* and to the primary T and B cell mitogens, PHA and PWM, respectively. Thus we would conclude that, in AS patients, CMI as measured by in-vitro PBL transformation is normal. Further, our data do not support claims that some form of antigenic cross-reactivity exists between all⁸ and some strains^{9 14} of *Klebsiella* spp. and HLA-B27.

The proponents of the klebsiella-B27 cross-reactive association have reported impaired AS-

PBL transformation with certain strains of klebsiella, notably those which might carry the K43 and K21 capsular specificities.^{15 16} These observations, in conjunction with the results of microbiological,⁸ serological,^{17 18} and lymphocytotoxicity studies^{9 14-16} have popularised the notion that antigenic cross-reactivity between certain strains of klebsiella and HLA-B27, or a spatially related structure on the MHC, somehow induces AS.^{8 9 14-18} In view of the profound biological implications of this hypothesis our studies have attempted to confirm the basic observations from which the hypothesis was apparently deduced. Some of our other studies, particularly those concerning lymphocytotoxicity,¹⁹ have been reported in preliminary form and, as with our present report, also do not substantiate a cross-reactive association between klebsiella and HLA-B27.

We would emphasise that our present studies were deliberately restricted to AS-derived faecal klebsiella, since it was assumed that these would more likely provide 'pathogenetic' strains. Indeed, of the five isolates which were serotyped one was identified as K21, reputedly a frequent participant in the putative cross-reaction with B27.^{15 16} As with all klebsiella strains tested by us, however, AS and matched normal control PBL did not differ in their responses to these organisms, nor did three of four klebsiella donors show impaired responses to their own organisms. Thus the absence of impaired PBL transformation to AS-derived klebsiella in our studies, and in those of Enlow *et al.*,²⁰ does not support a role for cross-reactivity via these organisms and B27 in AS.

We have recently reviewed pertinent factors which might explain reported differences between those studies which support, and those which negate, a role for klebsiella in AS.⁷ Accordingly, our studies were designed to evaluate the possible contradictory influences of control subjects' prior exposure to klebsiella,⁷ the inhibitory effects of anti-inflammatory medication,²¹ the modulating effects of age and sex on CMI,²² and the effect of serum supplements on in-vitro PBL transformation.²³ We believe that we have reasonably accommodated all of the foregoing considerations in our present studies and yet failed to substantiate the affirmative reports by Seager *et al.*⁹ and Geczy *et al.*^{14 15}

In conclusion, we and others^{10 24} have not found persistent or specific intestinal colonisation by klebsiella serotypes, nor evidence of impaired in-vitro PBL transformation²⁰ to klebsiella in AS patients. Although we accept the overwhelming epidemiological and clinical evidence of an as yet ill-defined association among HLA-B27, various Gram-

negative bacteria, and spondylitic syndromes,⁷ we and others^{20 25 26} have found no consistent evidence to implicate *Klebsiella* spp. in such an association.

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