EFFECT OF KLEBSIELLA CAPSULAR ANTISERA ON LYMPHOCYTES FROM PATIENTS WITH ANKYLOSING SPONDYLITIS

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SUMMARY. The cytotoxicity of klebsiella capsular antisera for lymphocytes from 10 HLA B27-positive patients with ankylosing spondylitis and eight B27-negative normal control subjects was examined. None of the antisera was cytotoxic for lymphocytes from nine patients but several antisera were cytotoxic for the lymphocytes from one patient. There were no differences between the results obtained with lymphocytes from the nine patients and those with lymphocytes from the normal controls. If klebsiella carriage is important in the pathogenesis of ankylosing spondylitis, the results of this investigation suggest that the capsular antigen is probably not involved.

INTRODUCTION

Seronegative arthritis may develop after infection by intestinal bacteria particularly, though not exclusively, in individuals who have the HLA B27 tissue antigen. Reiter's syndrome has followed infections with *Shigella*, where four out of five patients examined in one series were B27-positive (Calin and Fries, 1975), *Salmonella* (Vartiainen and Hurri, 1964) and *Yersinia* (Ahvonen, Sievers and Aho, 1969), but there are no reports of such infections giving rise to ankylosing spondylitis (AS), the disease most closely linked to the possession of the HLA B27 antigen.

Ebringer *et al.* (1976) demonstrated cross reactivity between B27-positive lymphocytes and *Klebsiella aerogenes*, *Enterobacter aerogenes* and *Yersinia enterocolitica* and Ebringer (1978) suggested that molecular mimicry may be the pathogenic mechanism. Ebringer *et al.* (1977) found that faecal carriage of klebsiellas in patients with AS was associated with active disease, but other workers could not confirm this observation (Eastmond, Cooke and Wright, 1978; Warren and Brewerton, 1979a). However, Ebringer, Cawdell and Ebringer (1979) and Eastmond *et al.* (1980) showed an association between klebsiella carriage and anterior uveitis but this was not supported by the findings of Warren and Brewerton (1979b), and sequential studies of klebsiella

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carriage have also produced conflicting results (Ebringer *et al.*, 1978; Warren and Brewerton, 1980; Eastmond *et al.*, 1981).

Geczy and co-workers (Geczy and Yap, 1979; Geczy *et al.*, 1980*a* and *b*); showed that the lymphocytes from patients with AS who were B27 positive reacted with antisera prepared against klebsiellas of capsular types 21 and 43 but lymphocytes from B27-positive or B27-negative control subjects without AS did not. They concluded that there is cross reactivity between some klebsiella antigen(s) and either a cell-membrane structure closely associated with the B27 antigen or a genetically modified B27 antigen itself.

In an attempt to identify the cross-reacting antigens, we have investigated the lymphocytotoxicity of antisera prepared against 52 of the standard klebsiella capsular serotypes.

MATERIALS AND METHODS

The methods used to determine lymphocytotoxicity were based on those of Seager *et al.* (1979).

Sources of lymphocytes and antisera. Lymphocytes were obtained from 10 patients with AS and eight normal subjects. All the patients were B27 positive, two were female and all were outpatients. Two had acute anterior uveitis at the time of sampling and two had a previous history of this condition. All the controls were B27 negative, one was female and none had any history or familial association with AS or acute anterior uveitis.

Antisera against the standard klebsiella capsular serotypes were available in the laboratory. They had been prepared as described by Edmondson and Cooke (1979) for capsular typing of klebsiella by the quellung method. Antisera against 52 capsular types (K1-49, 52, 53 and 62) were used; antisera against the serotypes that we have found to be most common were used first and then the others in numerical order. The antisera had titres in the range 2-64, but neat antisera were used in all cases. Lymphocytes from patients from whom we had isolated klebsiellas in previous studies were always tested against antisera to the serotypes isolated. Human anti-B27 serum was tested against all the lymphocyte preparations.

Lymphocyte separation and labelling. Up to 60 ml of blood was collected by venepuncture, preservative-free heparin 10 units/ml were added and the mixture was diluted with an equal volume of Hanks's bile salts solution (HBSS). This was layered on to a Ficoll-Trisil density gradient (sp. gr. 1.077) and centrifuged at 650 *g* for 20 min. The lymphocyte layer was removed and washed three times in HBSS with heparin 1 unit/ml and resuspended at a final concentration of 10⁷ lymphocytes/ml in RPMI 1640 (Flow Laboratories, Irvine, Scotland) containing 10% human AB serum. ⁵¹Cr (Radiochemical Centre, Amersham, Bucks) 100 µCi/ml was added to the lymphocyte suspension and incubated at 37°C for 60 min. The cells were then washed three times in RPMI and resuspended in RPMI with 10% human AB serum at a concentration of 3 × 10⁶ cells/ml.

Lymphocytotoxicity test. Antiserum (100 µl) was added to 10 µl of labelled cells and incubated at 24°C for 30 min; 100 µl of rabbit complement were then added and incubation was continued for a further 60 min. The tubes were centrifuged at 1500 *g* for 15 min and 100 µl of the supernate were removed for counting in a Packard Auto-gamma Spectrometer (model no. 3320, Packard Instruments, Caversham, Berks).

Minimal release was determined from a control preparation with cells, RPMI and complement. Maximal release was measured by adding 200 µl of 2% Tween 80 to 100 µl of cell suspension.

The results were expressed as a percentage of maximal ⁵¹Cr release, calculated by the formula:

$$^{51}\text{Cr release (\%)} = 100 \times \frac{R_1 - R_2}{R_3 - R_2}$$

where R_1 = radioactivity released by antiserum, R_2 = radioactivity released in absence of antiserum and R_3 = radioactivity released by cells disrupted by Tween 80.

Each subject's lymphocytes were tested against anti-B27 serum and as many anti-klebsiella sera as the number of cells would allow. All tests were done in duplicate. In no case were there enough lymphocytes to test against all 78 klebsiella capsular antisera.

Statistical analysis. The results were analysed in two ways. Initially 50% ^{51}Cr release was selected arbitrarily as the dividing line between a positive and negative result for cytotoxicity. The proportion of patients giving a positive or equivocal result with any of the antisera was compared with the proportion of controls giving a positive or equivocal result, by Fisher's exact test (Armitage, 1971). In the second analysis, the distribution of percentage ^{51}Cr release in the patients and controls was compared for each antiserum in turn, by the Mann-Whitney U test (Armitage, 1971). This test is equivalent to the Wilcoxon two-sample test. It avoids the assumptions of the t test.

RESULTS

Two hundred and eighty-five tests with lymphocytes from patients and 117 tests with lymphocytes from controls were performed with klebsiella capsular antisera, and each lymphocyte suspension was also tested against anti-B27 serum. The results are shown in the table.

With 50% ^{51}Cr release taken as a positive result, tests with anti-B27 serum gave one false negative (34% and 41% release) and one equivocal result (84% and 49%). Most of the negative results obtained with klebsiella antisera were

TABLE

Cytotoxicity of anti-B27 and anti-klebsiella sera for lymphocytes from patients with AS and controls

Subject no. and status	Result with anti-B27 serum	Number of klebsiella antisera tested	Number of results in category*		
			negative	positive	equivocal
1. AS	—	20	19	0	1
2. AS	+	15	15	0	0
3. AS	+	22	19	0	3
4. AS	+	13	13	0	0
5. AS	+	43	42	1	0
6. AS	+	49	31	11	7
7. U(NA)	+	51	48	0	3
8. U(NA)	+	25	25	0	0
9. U(A)	+/-	26	18	0	8
10. U(A)	+	21	21	0	0
11. Control	—	33	32	0	1
12. Control	—	27	27	0	0
13. Control	—	14	13	0	1
14. Control	—	3	3	0	0
15. Control	—	6	6	0	0
16. Control	—	7	7	0	0
17. Control	—	8	7	1	0
18. Control	—	19	18	0	1

AS = patient with ankylosing spondylitis but without history of uveitis; U(NA) = patient with ankylosing spondylitis and with a history of uveitis; U(A) = patient with ankylosing spondylitis and active acute anterior uveitis. + = Positive; — = negative; +/- = equivocal.

* A positive result = ^{51}Cr release $\geq 50\%$.

in the range 0–25%. Of the 402 tests done with klebsiella antisera, 25 gave equivocal results and 15 of these were with lymphocytes from two patients. Fisher's exact test showed no significant difference between the proportions of patients and controls that gave a positive result.

The results obtained with lymphocytes from patient no. 6 (see table) differed from those with lymphocytes from other patients and from the controls. The lymphocytes from patient no. 6 were tested against 49 antisera (K1–44, 46, 47, 49, 52 and 62). Eleven were cytotoxic (K1, 29, 30, 31, 34, 36, 37, 39, 40, 41 and 42) and seven gave equivocal results (K5, 20, 22, 24, 25, 32 and 35); the negative results in five of these were in the range 40–45%.

To assess the effects of the individual antisera, the Mann-Whitney test was applied to the results obtained with the 13 antisera for which there were sufficient data (K1, 2, 3, 6, 7, 9, 10, 12, 13, 16, 21, 27 and 35). A statistically significant difference between patients and controls was found for one capsular antiserum (K13) at the 5% level ($0.05 > p > 0.01$). Amongst 13 tests, one significant result at this level might easily occur when true differences are not present.

Lymphocytes from none of the four patients from whom klebsiellas had been isolated in previous surveys gave a positive or equivocal result with the antisera against the capsular types previously isolated.

DISCUSSION

In this study we have generally failed to demonstrate lymphocytotoxicity of klebsiella capsular antisera for lymphocytes from B27-positive patients with AS, although the lymphocytes of one patient were lysed by several antisera. Capsular antisera to types K21 and K43 were found to be cytotoxic for lymphocytes from patients with AS by Geczy *et al.* (1980a) but these antisera were not cytotoxic for lymphocytes from this patient. If there is any association between klebsiellas and AS, it is probably complex. Moreover, several mechanisms for the association of the HLA B27 antigen and AS have been proposed. These include the two-gene or linkage-disequilibrium theory which proposes that a disease-susceptibility gene is closely associated with the major histocompatibility complex (McDevitt and Bodmer, 1974). Other theories propose an interaction between the B27 antigen and an external agent. The cross-tolerance or molecular-mimicry theory is based on the possession by micro-organisms of antigens similar to human antigens (Jenkin, 1963). A more recent development has been the demonstration by Geczy *et al.* (1980b) that B27-positive lymphocytes from control subjects without AS can be modified by a filtrate of klebsiella serotype K43 so that they become as susceptible to lysis by klebsiella antisera as are B27-positive lymphocytes from patients with AS.

One difficulty in assessing this type of study is that the antigenic determinant that may be important has not been defined and may not have been exposed in the preparation of our klebsiella antisera. In the present study, as in that of Geczy and co-workers (Seager *et al.*, 1979), formalinised

cultures were used to raise antisera but the immunisation schedules were different and we did not use Freund's adjuvant, which may increase the immunological response and, possibly, lower its specificity.

Klebsiella antisera were cytotoxic for lymphocytes from only one patient in the present study and the lymphocytes of this patient reacted with antisera to many klebsiella capsular types. The antisera that were cytotoxic for this patient's lymphocytes did not show cross reactions in the quelling reaction and a common klebsiella antigen was probably involved.

With the exception of this evidence of toxicity of klebsiella antisera for lymphocytes of one patient, the results reported here suggest that the results obtained by other workers are probably not due to the klebsiella capsular antigen but may be caused by some other bacterial structure that was antigenic in their immunisation schedules but was not antigenic in our schedule for the production of capsular antisera.

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