Ankylosing spondylitis, HLA-B27, and klebsiella: a study of lymphocyte reactivity of anti-klebsiella sera*

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SUMMARY Twenty three anti-klebsiella antisera were tested for their cytotoxic activity and four for their binding capacity for peripheral blood lymphocytes (PBL) from patients with HLA-B27 positive ankylosing spondylitis (AS⁺B27⁺) and from B27 positive (AS⁻B27⁺) and B27 negative (AS⁻B27⁻) healthy individuals. None of the antisera showed specific activity against PBL from any particular group. The antisera tested included two anti-klebsiella K43 sera provided by an Australian group, who have reported them to be specifically cytotoxic for AS⁺B27⁺ PBL, four antisera raised against a klebsiella K43 strain provided by this group, and an antiserum from another group, who have reported it as having increased binding capacity for AS⁺B27⁺ and AS⁻B27⁺ PBL compared with AS⁻B27⁻ PBL. The results of other workers who have attempted to reproduce the results of either group are reviewed and the possible reasons for the repeated failure to confirm the reported findings are discussed.

Of the numerous associations between diseases and particular HLA antigens, perhaps the most striking is that between ankylosing spondylitis (AS) and HLA-B27.¹ The increased frequency of B27 in other seronegative arthropathies such as Reiter's disease,² and reactive arthritis after gastrointestinal infection with shigella,^{3,4} salmonella,^{5,6} or yersinia⁵ is also well established. The association of B27 with these diseases could be either because B27 is directly involved in disease susceptibility or because there is linkage disequilibrium between the B27 gene and a disease susceptibility gene.⁷

It has been suggested by two different groups of workers that *Klebsiella pneumoniae* may have a role in the pathogenesis of AS in B27 positive individuals. Ebringer and his colleagues have based this suggestion partly on their finding that anti-klebsiella serum binds more strongly to PBL from AS⁺B27⁺ and AS⁻B27⁺ subjects compared with AS⁻B27⁻ PBL in enzyme linked immunosorbent assays (ELISA).⁸ This, together with other evidence,⁹⁻¹¹ has been interpreted by Ebringer⁸ as providing evidence for the 'cross tolerance' hypothesis for the pathogenesis of AS, klebsiella infection giving rise

to antibodies, which are then capable of cross reacting with the B27 antigen on certain tissues.

On the other hand, Geczy and his coworkers in Australia have used a complement dependent, chromium release cytotoxic assay and found that certain anti-klebsiella sera are cytotoxic for PBL from the majority of AS⁺B27⁺ individuals, but not for cells from AS⁻B27⁺ individuals, even when they are first degree relatives of AS patients.¹²⁻¹⁴ They have interpreted this work, together with subsequent studies,^{15,16} as suggesting that there is present on the surface of nucleated cells of AS⁺B27⁺ individuals a component of the cell wall of certain klebsiella strains which is in close association with the B27 antigen.

It is apparent that there is a fundamental divergence between the experimental findings of these two groups of workers. This study is concerned with trying to confirm the results of either group with anti-klebsiella sera and by three techniques: chromium release cytotoxicity assays, dye exclusion microcytotoxicity assays, and ELISA.

Patients and methods

PATIENTS AND CONTROLS

All patients were diagnosed as having AS according to the New York criteria.¹⁷ The healthy controls were members of staff and students with no present or previous history of joint disease. All patients and

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controls were tissue typed by a two stage micro-lymphocytotoxicity assay.¹⁸

BACTERIAL CULTURES

Bacteria were grown in tryptone soya broth (TSB; Oxoid) and killed by adding 0.25 ml of 40% formaldehyde to a 20 ml suspension of the organisms (10^9 – 10^{10} /ml) in TSB. The formalin killed bacteria were washed twice with sterile 0.9% NaCl and stored at -20°C .

ANTISERA

Antisera were raised in New Zealand white rabbits and BALB/c mice against 13 local klebsiella isolates and a klebsiella K43 isolate from Dr A F Geczy of the New South Wales Red Cross Blood Transfusion Service, Sydney, Australia.

Rabbits (3–5 kg) were immunised with formalin killed bacterial suspensions according to the method of Seager *et al.*¹² Briefly, the animals were each injected intravenously with approximately 10^9 bacteria in 2 ml 0.9% NaCl and two weeks later intramuscularly with approximately 10^{10} bacteria in 2 ml Freund's complete adjuvant (Difco). Ten days later the rabbits were boosted intravenously with 1 ml of bacterial suspension (10^8 – 10^9 organisms) in 0.9% NaCl and two weeks after this the rabbits were bled and the serum stored at -40°C .

Mice in groups of 10–15 were each injected intraperitoneally with 0.1 ml of bacterial inoculum (10^9 – 10^{10} organisms/ml 0.9% NaCl) and were then boosted intraperitoneally on two occasions at weekly intervals with 0.1 ml of bacterial inoculum. Two weeks after the last injection the mice were bled and the serum was pooled and stored at -20°C .

Each antiserum was tested against concentrated bacterial culture supernatants of various strains of klebsiella by the double diffusion technique.¹⁹ Precipitin lines were observed for all antisera against homologous culture supernatants and in some cases against heterologous culture supernatants.

Antisera were also obtained from Dr A Ebringer of the Department of Rheumatology, Middlesex Hospital, London and from Dr A F Geczy.

CHROMIUM RELEASE LYMPHOCYTOTOXICITY ASSAY

Peripheral blood lymphocytes were obtained from defibrinated blood by centrifugation in Ficoll-Paque (Pharmacia Ltd). They were washed three times in Hanks's balanced salt solution (HBSS; Wellcome) containing 0.035% sodium bicarbonate. 10×10^6 PBL were resuspended in 1 ml of HBSS supplemented with 10% decomplemented newborn calf serum (NBCS; Flow Laboratories Ltd) and were incubated with $100 \mu\text{Ci}$ ^{51}Cr (Amersham) for 60 min

at 37°C . After washing three times with complement fixation test diluent (CFT; Flow) containing 2% NBCS (CFT/NBCS) the cells were suspended at a concentration of 1×10^6 PBL/ml. Ten serial doubling dilutions of each test serum were prepared with CFT/NBCS and $100 \mu\text{l}$ of the ^{51}Cr labelled PBL suspension was added to $100 \mu\text{l}$ of each serum dilution. This was followed by the addition of $100 \mu\text{l}$ of rabbit complement (Buxted Rabbit Co, Sussex) which had been diluted 1:5 with CFT/NBCS. After one hour at 37°C 1.5 ml of cold 0.9% NaCl was added to each tube and after centrifugation the supernatants were removed for radioactivity measurements in a gammacounter (LKB Instruments).

The amount of radioactivity in the samples was compared with the radioactivity present in the supernatants from tubes containing PBL in medium plus complement only, and with that present in tubes containing cells which had been lysed by the addition of $200 \mu\text{l}$ of 0.1% Triton-X 100. The results are expressed as the percentage of maximum ^{51}Cr released at any serum dilution:

$$\text{Percentage } ^{51}\text{Cr release} = \frac{\begin{array}{c} \text{Radioactivity} \\ \text{released by} \\ \text{antiserum} \end{array} - \begin{array}{c} \text{Radioactivity} \\ \text{released in} \\ \text{absence of} \\ \text{antiserum} \end{array}}{\begin{array}{c} \text{Radioactivity} \\ \text{released by} \\ \text{Triton-X} \end{array} - \begin{array}{c} \text{Radioactivity} \\ \text{released in} \\ \text{absence of} \\ \text{antiserum} \end{array}} \times 100$$

Rabbit antihuman lymphocyte antiserum (ALS) was used as a positive control in all the lymphocytotoxicity assays, and maximum levels of cytotoxicity ranged from 75 to 95% chromium release.

DYE EXCLUSION

MICROLYMPHOCYTOTOXICITY ASSAY

Five serial doubling dilutions of serum were prepared in CFT and $0.5 \mu\text{l}$ pipetted into wells under liquid paraffin. $0.5 \mu\text{l}$ of PBL at a concentration of 10^7 cells/ml was incubated with each serum dilution for 30 min at 20°C followed by the addition of $2.5 \mu\text{l}$ of rabbit complement and incubation for a further 60 min. Cells were stained with $1 \mu\text{l}$ of 0.25% trypan blue after excess complement and serum had been removed with a peristaltic pump, and viability was assessed by examination under an inverted microscope.

ALS and human anti-B27 tissue typing sera were used as controls in all the assays. Cell death greater than 50% was taken as a definite positive result and ALS was positive for all the PBL tested at all the five serum dilutions, while the B27 tissue typing sera were positive for at least the first three serum

dilutions for PBL from the B27 positive patients and controls.

ELISA

The assays were carried out with rigid flat bottomed, 96 well microELISA plates (Dynatech) which had been coated with 0.1 mg/ml poly-L-lysine by incubation at 37°C for one hour. The plates were washed three times with phosphate buffered saline (PBS) and 100 µl samples of PBL at a concentration of 5×10^6 cells/ml were incubated in appropriate wells for one hour at 37°C after centrifugation of the plates at 100 g for five minutes to sediment the cells. The wells were washed three times with PBS and incubated with 100 µl 0.3% gelatine in PBS (PBS-Gel) to saturate any remaining protein reactive sites. After three washes with PBS 100 µl samples of four rabbit anti-klebsiella sera diluted 1:500 with PBS-Gel and each of five dilutions of ALS (1:300, 1:1000, 1:3000, 1:10 000, and 1:30 000) were added to the appropriate wells. After incubation at 37°C for one hour the wells were washed three times with PBS and then 100 µl of horseradish peroxidase labelled, swine antirabbit immunoglobulins (Mercia Brocades Ltd) diluted 1:1000 with PBS-Gel was added to each well. The plates were incubated and washed as described previously and 100 µl of fresh substrate solution was added to each well. The substrate solution was prepared by dissolving 10 mg of *o*-phenylenediamine in 25 ml of phosphate citrate

buffer (pH 5.0; 9.15 g disodium hydrogen orthophosphate dihydrate and 5.11 g citric acid monohydrate/l distilled water) and adding 10 µl of hydrogen peroxide immediately before use. The substrate was allowed to react with the contents of the wells for five minutes at room temperature before the reaction was stopped by the addition of 100 µl 4 M H₂SO₄. The optical density (OD) was determined at a wavelength of 492 nm by a vertical light spectrophotometer (Titertek Multiscan, Flow Laboratories Ltd).

The tests were all carried out in duplicate wells and the corrected OD for each test sample was calculated by subtracting the mean OD of the control with the highest value (i.e., wells with PBL, enzyme-antibody conjugate, and substrate but no serum) from that of the test sample. A standard curve was plotted for each PBL suspension from measurements on five dilutions of ALS and the binding capacity of each anti-klebsiella serum for a particular PBL suspension was calculated from this standard curve: a serum which produced an OD that corresponded with an ALS dilution of 1:500 was assigned a binding capacity of 100 units and one which corresponded with a dilution of 1:5000 a binding capacity of 10 units.

STATISTICAL ANALYSIS

The results of the lymphocytotoxicity assays and the ELISAs were analysed by the Mann-Whitney U

Table 1 *Lymphocytotoxic activity of antisera raised against local isolates*

Serum No	Source	Capsular serotype	Animal immunised	No > 25% ⁵¹ Cr release/No tested [†]		
				AS ⁺ B27 ⁺	AS ⁻ B27 ⁻	AS ⁻ B27 ⁺
AK6	RI‡	K39	Rabbit	1/18	0/8	0/8
AK11	RI	K46	Rabbit	0/14	0/8	0/1
AK12	RI	K2	Rabbit	0/14	0/8	0/4
AK16	RI	NT‡	Rabbit	4/30	0/15	0/8
AK23	RI	K68	Mouse	0/10	0/8	0/1
AK26	RI	K43	Rabbit	2/24	0/14	0/5
AK41	AS‡	K41	Mouse	0/6	0/1	0/3
AK42*	RI	K21	Mouse	11/11	5/6	3/3
AK43	AS	K52	Mouse	0/6	0/1	0/1
AK44	AS	K29	Mouse	0/6	0/1	0/3
AK45	RI	K43	Mouse	0/6	0/1	0/3
AK46	RI	K43	Mouse	0/6	0/1	0/3
AK47	RI	K43	Mouse	0/6	0/1	0/3
AK48*	RI	K21	Mouse	0/12	0/4	0/2
AK49*	RI	K21	Rabbit	0/16	0/13	0/2
AK50*	RI	K21	Rabbit	0/16	0/11	0/2

Sera were raised in Liverpool against local klebsiella isolates and tested against PBL by the chromium release assay described in 'Patients and methods'.

*Four antisera prepared with the same klebsiella K21 isolate.

†Number of PBL suspensions that each gave greater than 25% chromium release out of a total that were tested for each group of AS⁻ and AS⁺ individuals as described in 'Patients and methods'.

‡RI=random klebsiella isolates; AS=klebsiella strains isolated from the faeces of AS patients; NT=non-typable.

test, which is the non-parametric equivalent of the *t* test.²⁰

Results

LYMPHOCYTOTOXIC ACTIVITY OF ANTI-KLEBSIELLA SERA

Determination by a chromium release cytotoxicity assay

Antisera were raised against 13 local klebsiella strains, three of which were isolated from the faeces of three AS patients. Levels of cytotoxicity rarely exceeded 25% chromium release when these antisera were tested against PBL from AS⁺B27⁺ patients, AS⁻B27⁻ individuals, and AS⁻B27⁺ individuals (Table 1). The exception was antiserum AK42, an anti-klebsiella K21 serum raised in mice, which was cytotoxic for PBL from all the groups tested. A typical example of the cytotoxic pattern obtained with this antiserum is shown in Fig. 1. When a further group of mice and two rabbits were immunised with this klebsiella K21 isolate the resultant antisera (AK48, AK49, and AK50) failed to show similar lymphocytotoxic activity (Table 1).

By comparing the distribution of percentage chromium release produced by the various antisera in the AS⁺B27⁺ group with that of the two control groups using the Mann-Whitney test, no statistically significant difference was found.

Antisera raised in Liverpool against the 'cross reactive' klebsiella K43 isolate provided by Geczy were tested together with antisera from Geczy and Ebringer. Chromium release exceeded 25% for PBL from only one AS⁺B27⁺ patient out of a total of 21 AS⁺B27⁺ patients, 18 AS⁻B27⁻, and two AS⁻B27⁺ individuals tested (Table 2). In these tests postimmunisation sera rarely showed greater lymphocytotoxic activity than that of the corresponding preimmunisation sera (Fig. 2). Statistical analysis of the data again showed no significant difference between the distribution of chromium release in patients compared with that in controls.

Determination by a dye exclusion microlymphocytotoxicity assay

As shown in Table 3 the results of these assays confirmed those of the chromium release lymphocytotoxicity assay.

Fig. 1 Lymphocytotoxicity pattern obtained when testing PBL from an AS⁻B27⁻ individual. Lymphocytotoxicity of the various antisera was tested as described in 'Patients and methods'. Details of the antisera are given in Table 1.

Table 2 Lymphocytotoxic activity of antisera against the 'cross reactive' klebsiella isolates

Serum code*	No > 25% ⁵¹ Cr release/No tested†		
	AS ⁺ B27 ⁺	AS ⁻ B27 ⁻	AS ⁻ B27 ⁺
EAK	0/21	0/4	0/2
GAK	0/8	—	0/2
FGAK	1/10	0/4	—
AK51	1/15	0/18	0/2
AK52	1/17	0/18	0/2
AK57	1/14	0/18	0/2
AK60	1/14	0/18	0/2

Sera were tested against PBL by the chromium release assay described in 'Patients and methods'.

*EAK=anti-klebsiella antiserum from Ebringer; GAK=anti-klebsiella K43 serum from Geczy; FGAK=reconstituted freeze dried GAK from Geczy; AK51, 52, 57, and 60=antisera raised in Liverpool against the 'cross reactive' klebsiella K43 isolate from Geczy.

†Number of PBL suspensions that each gave greater than 25% chromium release out of a total that were tested for each group of AS⁻ and AS⁺ individuals as described in 'Patients and methods'.

Table 3 Lymphocytotoxic activity of antisera determined by microlymphocytotoxicity assays

Serum code*	No > 50% cell killing/No tested†		
	AS ⁺ B27 ⁺	AS ⁻ B27 ⁻	AS ⁻ B27 ⁺
AK42	20/20	14/14	2/2
AK43	4/20	3/14	1/2
AK44	2/20	2/14	0/2
AK50	0/20	0/14	0/2
RAB534	0/20	0/14	0/2
AK60	0/20	0/14	0/2
FGAK	0/20	0/14	0/2
A-B27	20/20	0/14	2/2
ALS	20/20	14/14	2/2

*A-B27=human anti-HLA-B27 tissue typing serum. For other sera refer to legends to Fig. 2 and Tables 1 and 2.

†Number of PBL suspensions that each gave greater than 50% cell killing out of a total that were tested for each group of AS⁻ and AS⁺ individuals as described in 'Patients and methods'.

Fig. 2 Lymphocytotoxic activity of preimmunisation and postimmunisation sera. Rabbit No 534 was immunised with the Australian klebsiella K43 isolate to produce antiserum AK60 and both the sera were tested for lymphocytotoxicity against PBL from 14 AS⁺ B27⁺, 18 AS⁻ B27⁻, and two AS⁻ B27⁺ individuals by the chromium release assay described in 'Patients and methods'.

phocytotoxicity assays in that antiserum AK42 was the only anti-klebsiella serum that was cytotoxic, and that its cytotoxic activity was directed against all the PBL samples tested.

BINDING CAPACITY OF ANTI-KLEBSIELLA SERA FOR PBL

The results of the ELISAs to determine the binding capacity of anti-klebsiella sera for PBL are shown in Fig. 3. There was no statistically significant difference in the binding capacity for AS⁺ B27⁺ PBL compared with AS⁻ B27⁻ PBL for any of the antisera, indicating that each antiserum bound equally well to both groups of PBL.

Discussion

There is good evidence for the participation of HLA linked genes in the pathogenesis of many chronic disorders, but in no case has the mechanism of the action of such genes been as yet elucidated. Thus the evidence provided by Geczy and his colleagues,¹⁶ and Ebringer and coworkers,⁸ for the involvement of klebsiella in AS is of major importance. The difficulty has been that no other group of workers has been able to reproduce either set of experimental findings, namely the cross reaction of klebsiella and B27 or the presence on AS⁺ B27⁺ cells of a klebsiella derived component.

Archer, with a ⁵¹Cr release assay, tested eight anti-klebsiella sera, including two provided by Ebringer, against PBL from both B27⁻ and B27⁺ individuals, the latter including both AS⁻ and AS⁺ subjects.²¹ There was no significant cytotoxicity

number of rabbits, however, have now been used in the various reported studies without this resulting in antibodies with the specificity sought. Furthermore, antisera provided by Geczy have also failed to show specific lymphocytotoxicity for AS⁺B27⁺ PBL. In regard to the assays used a puzzling feature is that the specific cytotoxicity of certain anti-klebsiella sera could only be shown by the ⁵¹Cr release method and not by an immunofluorescence or a dye exclusion cytotoxicity assay.²⁸ In the present study although the ⁵¹Cr release method differs from that used by Seager *et al.*,¹² high levels of cytotoxicity were obtained against the lymphocytes in control tests with antilymphocytic serum, and also in dye exclusion microlymphocytotoxicity assays with anti-B27 alloantisera. A notable feature in the data reported by Geczy and his colleagues is that the level of cytotoxicity by ⁵¹Cr release assays was consistently lower for anti-B27 alloantisera, i.e., 41, 44, and 65%, than that observed for the anti-klebsiella sera, i.e., 74, 69, and 80%.^{12 15 29} Yet when dye exclusion methods were used, which might be expected to give approximately 100% cytotoxicity for isoantibodies, negative results were apparently obtained when anti-klebsiella sera were tested. Comparison of the methods used in different laboratories suggests that it is doubtful that variation in the source of the complement used in the cytotoxicity assays could be important, or that the final concentration of complement matters, as it has been shown that the lymphocytotoxic effect is apparent at a lower complement concentration.³⁰ The investigations reported here also include a series of experiments in which assays independent of complement (ELISA) were used, and the results again were negative.

In conclusion, we, like others, including Georgopoulos *et al.*³¹ have failed to find evidence that antisera raised against klebsiella organisms have any particular ability to react with B27⁺ lymphocytes, and the suggestions that klebsiella organisms have a key role in the pathogenesis of ankylosing spondylitis remains difficult to support until the original observations are independently confirmed.

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