Experimental induction of rheumatoid factor and joint lesions in rabbits after intravenous injections of killed bacteria

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SUMMARY Rabbits receiving repeated intravenous injections of killed bacteria (*Escherichia coli* or *Bacillus subtilis*) developed IgM rheumatoid factor which reacted with autologous heat-aggregated IgG. In addition, 5/7 'Old English' and 7/8 'Sandy Lop' rabbits receiving killed *E. coli* developed rheumatoid-like synovial lesions. 'Old English' rabbits developed lesions of a more severe nature. Three of eight 'Sandy Lop' rabbits injected with killed *B. subtilis* had high levels of rheumatoid factor but only mild joint lesions.

Key words: arthritis, autologous heat-aggregated IgG, *Escherichia coli*, *Bacillus subtilis*.

Rheumatoid factor-like substances (RFLS) have been described in rabbits after the administration of a variety of bacterial¹ or protein^{2,3} antigens. However, joint lesions were not reported in any of these models. In this paper we report the induction of both rheumatoid factor (RF), reacting with autologous heat-aggregated IgG, and rheumatoid-like synovial lesions in rabbits injected with either killed *Escherichia coli* or *Bacillus subtilis*.

Materials and methods

RABBITS

'Old English' rabbits were purchased from Cheshire Rabbit Farms Ltd, Dudden Lodge, Cheshire, UK. 'Sandy Lop' rabbits were purchased from the Medical Research Council. All animals received food (R14 diet, K K Greeff Ltd, Croydon, UK) and water ad libitum.

PREPARATION OF *Escherichia coli* AND *Bacillus subtilis*

Escherichia coli (*E. coli* NCTC 9016) and *Bacillus*

subtilis (*B. subtilis* NCTC 3610) were grown in 1 litre volumes in 3 litre Erlenmeyer flasks at 37°C for 48 hours in brain/heart infusion broth (Difco). Formalin (final concentration of 0.6%) was then added to the broth cultures, which were left for 24 h at 20°C in order to kill the bacteria. The bacteria were harvested by centrifugation, and the deposit was washed three times in phosphate-buffered saline (PBS). The bacteria were resuspended in PBS and standardised by opacity testing to 6×10^9 bacteria/ml. The bacteria were finally sterilised by steaming for 1 h and thereafter stored at -20°C.

INJECTION PROTOCOL

'Old English' and 'Sandy Lop' rabbits received repeated intravenous injections of killed *E. coli* (2×10^9 bacteria/0.3 ml injections three times weekly) over a period of 12 weeks. In addition, another group of 'Sandy Lop' rabbits were injected with killed *B. subtilis* under an identical regimen. Control 'Old English' and 'Sandy Lop' rabbits did not receive any bacteria. Rabbits were bled at monthly intervals from the marginal vein of the ear. Sera were frozen and stored at -20°C.

ASSESSMENT OF ARTHRITIS

Rabbits were killed by lethal administration of sodium pentobarbitone (May and Baker Ltd). Syno-

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vial fluids were recovered as previously described,⁴ centrifuged at 200 g for 15 min at 4°C, and the supernatants removed and stored at -20°C. Synovial fluid cells were resuspended in balanced salt solution (BSS, Flow Laboratories) supplemented with 1% heat-inactivated fetal calf serum (Flow Laboratories).

Enumeration of nucleated cells in synovial fluid

The number of viable nucleated cells in the synovial fluids of rabbits was estimated with a mixture of acridine orange and ethidium bromide.⁵ Cells were usually more than 95% viable.

Enumeration of T cells in synovial fluids

The percentage of T cells in synovial fluids was determined by rosetting with papain-treated rabbit erythrocytes as previously described.⁶

Dissection of joints and histological procedures

Both knee joints from each rabbit were dissected and the synovia divided into infrapatellar, interpatellar, and lateral synovia before fixing in 10% neutral buffered formalin and embedding in paraffin. 5 µm sections were cut, stained with haematoxylin and eosin, and graded for inflammatory lesions as previously described.⁴

Kidney histology

Kidney samples were obtained after death, fixed in formalin, and embedded in paraffin. 3 µm sections were cut and stained conventionally with haematoxylin and eosin and with the standard periodic acid silver technique.

MEASUREMENT OF IgM RHEUMATOID FACTOR

In order to overcome the problem of naturally occurring anti-allotypic antibodies in rabbits⁷ serum RFs were measured by their ability to bind to autologous heat-aggregated IgG. Before the start of the experiment IgG was prepared from the serum of each rabbit by the caprylic acid method.⁸ Rabbit IgG (1 g/l in normal saline) was aggregated by heating at 70°C for 10 min and stored at -20°C. RF was measured in the Mrs PAH test (mixed reverse (solid phase) passive antiglobulin haemadherence) described by March *et al.*⁹ Briefly, microtitre plates (U shaped) (Sterilin, Teddington) were coated overnight at 4°C with 60 µl of a 10 mg/l solution of heat-aggregated rabbit IgG diluted in carbonate coating buffer (0.05 M, pH 9.6). Sera and synovial fluids were serially diluted in PBS+0.08% Tween 20 diluent, and 60 µl of each dilution was added to the coated plates. After incubation for 90 min at 4°C the plates were washed three times with cold PBS/

Tween and subsequently three times with PBS. IgM RF binding to autologous IgG was detected with a goat antirabbit µFc (a gift from Dr A Feinstein) coupled by chromic chloride¹⁰ to trypsin-treated sheep red blood cells. One drop of a 1% suspension of these indicator cells was added to each well of the microtitre plates. The plates were agitated and read for haemagglutination after the erythrocytes had settled.

MEASUREMENT OF SERUM ANTIBODIES TO *E.coli* AND *B.subtilis*

Serum antibodies to *E.coli* and *B.subtilis* were measured in a solid phase binding assay. Briefly, a soluble bacterial antigen was prepared by sonication of a suspension of killed *E.coli* or *B.subtilis* (6×10^9 bacteria/ml) in a cup-horn sonicator (Heat Systems 10375) by pulsing until visible disruption of the bacterial suspension occurred (5–10 min at 50% duty cycle). The bacterial extract was centrifuged (11 500 g) and the supernatant adjusted to 100 mg protein/l (absorbance read at 280 nm) in disodium carbonate buffer (50 mM, pH 9.6 containing 0.002% sodium azide). Each well of a polyvinyl U well microtitre plate (Falcon Microtest III flexible assay plate) was coated with 50 µl of the bacterial preparation by incubating overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and then coated with blocking buffer (PBS containing 1% bovine serum albumin, 1% normal sheep serum, and 0.1% sodium azide) by incubating at 37°C for 60 min. After washing 50 µl of serial dilutions of test sera were added to the plate and incubated for two hours at 20°C. After further washing antibodies to *E.coli* or *B.subtilis* were detected by the addition of 50 µl of a 1% suspension of trypsin-treated sheep erythrocytes coupled to IgG goat antirabbit γFc by the chromic chloride method (anti-γFc a gift from Dr A Feinstein). The plates were agitated and read for haemagglutination after the erythrocytes had settled.

STATISTICAL ANALYSIS

This was performed by the Student's *t* test on log₂ transformed data.

Results

DETECTION OF SERUM IgM RHEUMATOID FACTORS AND IgG ANTIBACTERIA ANTIBODIES

Measurement of serum IgM rheumatoid factor

The highest titres of serum IgM RF were detected in 'Sandy Lop' rabbits injected with killed *B.subtilis* (mean titre $\pm$ SD 42 ± 3) compared with either 'Sandy Lop' or Old English' rabbits injected with killed

Fig. 2 Correlation between serum IgM RF levels and IgG antibody titres to injected bacteria: IgM RF and IgG antibody titres to injected bacteria were measured in experimental (x) and control (●) rabbits: (a) 'Old English' rabbits injected with *E.coli*; (b) 'Sandy Lop' rabbits injected with *E.coli*; (c) 'Sandy Lop' rabbits injected with *B.subtilis*.

(71%) 'Old English' and 7/8 (88%) 'Sandy Lop' rabbits (Table 1). However, all the lesions observed in 'Sandy Lop' rabbits were only mild in nature, whereas 3/7 (43%) of the 'Old English' rabbits developed lesions classified as moderate to severe. Three out of eight 'Sandy Lop' rabbits injected with killed *B.subtilis* developed joint lesions which were all classified as 'mild'. One untreated female 'Sandy Lop' rabbit developed a slight infiltration of lymphocytes in one synovium. This reflects the low incidence of spontaneous arthritis in this strain of 14% (1/7). None of the control 'Old English' rabbits used in this series of experiments developed synovial changes, though previous work has shown the spontaneous incidence of joint lesions in this strain to be 4%⁴

Description of joint lesions

A continuous spectrum of synovitis was observed which was arbitrarily divided into mild, moderate and severe categories (Table 2).

'Mild' lesions were characterised by light focal subsynovial infiltration by polymorphs, lymphocytes, and occasional plasma cells. The synovial lining layer remained flat in many cases, while in others mild cuboidal hyperplasia was evident (Fig. 3b).

'Moderate' lesions were characterised by a denser and more confluent inflammatory infiltrate containing greater numbers of lymphocytes and plasma cells. Synovial cells showed cuboidal hyperplasia and a degree of multilayering. Mild increases in vascularity were sometimes apparent. Inflammatory villous configuration started to become a feature.

'Severe' lesions were characterised by a dense inflammatory infiltrate, with the formation of a small germinal centre in one instance. Inflammatory villus formation was conspicuous as was multilayering of the synovial cells. In some instances fibrin was incorporated into the synovial layer and vascularity

Table 1 Incidence of synovial lesions in rabbits injected intravenously with killed bacteria

Strain of rabbit	Killed bacteria (injected IV) [†]	Incidence of joint lesions (%)	Incidence of moderate to severe lesions (%)
'Old English'	<i>E.coli</i>	71 (5/7)*	43 (3/7)
	Control	0 (0/7)	0 (0/7)
'Sandy Lop'	<i>E.coli</i>	88 (7/8)	0 (0/8)
	<i>B.subtilis</i>	38 (3/8)	0 (0/8)
	Control	14 (1/7)	0 (0/7)

* Actual number of rabbits used is shown in parentheses.

[†] IV=intravenously.

Table 2 Histological assessment of synovial lesions in rabbits injected intravenously with killed bacteria

Strain of rabbit	Bacteria (injected IV)	Rabbit No and sex	Overall classification	Neutrophils	Plasma cells	Lymphoid cells	Germinal centres	Fibrosis	Cuboidal/flat synovial lining cells	Villous	Fibrin	Number of layers of synovial lining cells	Unilateral or bilateral lesions
'Old English'	<i>E.coli</i> 5/7 injected	6510 ♂	+	+	-	-	-	-	F	-	-	1	U
		6511 ♀	+++	+	+	+++	-	+	C	++	+	4	B
		6512 ♀	+++	+	+	+++	+	+	C	++	-	3	B
		6619 ♂	+++	-	+++	+++	-	+	C	++	++	4	B
		6620 ♂	+	-	-	+	-	-	F	-	-	1	B
'Sandy Lop'	<i>E.coli</i> 7/8 injected	6561 ♀	+	+	+	-	-	-	F/C	-	-	1	U
		6562 ♀	+	+	-	-	-	-	F/C	-	-	2	U
		6563 ♂	+	-	-	+	-	-	F	-	-	1	B
		6564 ♀	+	+	-	-	-	-	C	-	-	1	U
		6565 ♂	+	-	-	+	-	-	C	-	-	1	B
		6566 ♀	+	-	-	+/-	-	-	F	-	-	1	U
		6571 ♂	+	-	-	+	-	-	C	-	-	1	U
	<i>B.subtilis</i> 3/8 injected	6572 ♀	+	-	-	+	-	-	C	-	-	1	B
		6575 ♂	+	+	-	+	-	-	C	-	-	2	U
		6579 ♂	+	-	-	+	-	-	F	-	-	2	U
	Control 1/7	6558 ♀	+	-	-	+	-	-	C	-	-	2	U

The interpatellar, infrapatellar, and lateral synovia were dissected from both knees of each rabbit after death, fixed in formalin, and stained with haematoxylin and eosin as described in the text. Severity of joint lesions is assessed relative to normal rabbit synovia [-]. Joint lesions are scored as either mild [+], moderate [++], or severe [+++]. A similar coding for infiltration by neutrophils, plasma cells, and lymphocytes has been used. Synovial lining cells are classified as either cuboidal (C), flat (F), or focally cuboidal (F/C).

was greatly increased (Figs 3c and 3d). In one animal inflammation spread into the adjacent fibrocartilage which showed evidence of vascularisation (Fig. 3e). Mild lesions were usually unilateral (only one joint affected), while moderate to severe lesions affected both knees to a similar degree.

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

Fig. 3 Histological classification of joint lesions. (a) Normal synovium from a control rabbit. A single layer of flat synovial cells is present on the surface. Beneath lies unremarkable adipose tissue. (Haematoxylin and eosin, $\times 425$). (b) An area of lightly inflamed synovium from a 'Sandy Lop' rabbit injected with *E. coli*. Synovial cells have

Fig. 3e

an increased amount of cytoplasm and lie two to three cells deep. The underlying chronic inflammatory cells are lymphocytes and plasma cells in approximately equal proportions. Capillary blood vessels are prominent, suggesting increased vascularity. (Haematoxylin and eosin, $\times 300$). (c) Heavily inflamed synovium from an 'Old English' rabbit injected with *E. coli*. Synovial cells have a considerably increased amount of cytoplasm and lie four to five cells deep on the surface. There is a dense chronic inflammatory cell infiltrate immediately beneath the hyperplastic synovial cells. (Haematoxylin and eosin, $\times 300$). (d) Adjacent inflammatory villi from an 'Old English' rabbit injected with *E. coli*. This was classed as severe inflammation. Synovial cells lie two to three deep and have abundant cytoplasm; occasional binucleate forms are present. The cores of the villi are rich in plasma cells and, at higher magnification, appear rather vascular. (Haematoxylin and eosin, $\times 120$). (e) Fibrocartilage from the severely inflamed joint of an 'Old English' rabbit injected with *E. coli*. At the top of the figure (slightly crushed) lies part of the inflammatory infiltrate of lymphocytes and plasma cells. A few of these cells extend more deeply into the fibrocartilage and are associated with tiny capillaries which have plump endothelial cells (arrows), the appearance being those of inflammatory neovascularisation. (Haematoxylin and eosin, $\times 375$).

Lesions in 3/7 'Old English' rabbits were classified as moderate to severe and in 2/7 as mild.

In contrast, all the lesions observed in the 'Sandy Lop' rabbits injected with either killed *E.coli* or *B.subtilis* were mild. There was no detectable qualitative difference between the nature of the lesions in 'Old English' and 'Sandy Lop' rabbits.

Kidneys were available for study from all but one animal. In no case was there evidence of glomerulonephritis.

ANALYSIS OF CELLS RECOVERED FROM SYNOVIAL FLUIDS

Absolute numbers of nucleated cells

'Old English'. The mean number of viable nucleated cells recovered after death from the synovial fluids of untreated 'Old English' rabbits was 8240 (range 6000–15 360) (Fig. 4). Increased numbers were recovered from those rabbits injected with *E.coli* that developed joint lesions (mean 304 600, range 16 300–816 000).

'Sandy Lop'. The mean number of nucleated cells

recovered from the synovial fluids of 'Sandy Lop' rabbits, injected with either *E.coli* or *B.subtilis* did not exceed 25 000. There was no significant increase over control values, which ranged from 1000 to 25 000 (mean 10 700) (data not shown).

T cells levels in synovial fluids

The mean absolute number of T cells recovered from the synovial fluids of untreated 'Old English' rabbits was 370 (range 100–1075). These values reflect a mean of 4% of total nucleated cells (range 1–7%). *E.coli* injected rabbits with joint lesions had increased absolute numbers and percentage of T cells in the synovial fluids, i.e., mean value of 77 670 (range 2610–179 520). The percentage of T cells within the synovial fluid ranged between 1 and 60% (mean 23%) (Table 3).

HIGH TITRES OF SERUM RF WERE NOT ASSOCIATED WITH THE DEVELOPMENT OF JOINT LESIONS

Although the highest levels of serum RF were

Table 3 Absolute number of T cells recovered from the synovial fluids of 'Old English' rabbits injected with killed *E.coli*

Treatment	Rabbit number and sex	Knee joint	Severity of joint lesion	T cells (%)	Absolute number of T cells recovered from synovial fluid	Mean value $\pm$ SD
<i>E.coli</i> injected with joint lesions	6510 ♀	R	+	5	8 480	77 670 $\pm$ 74 520
		L	+	1	6 220	
	6511 ♀	R	+++	60	165 600	
		L	+++	41	132 600	
	6512 ♀	R	++	45	57 690	
		L	+++	35	161 700	
	6619 ♂	R	+++	22	179 520	
		L	+++	28	58 860	
<i>E.coli</i> injected without joint lesions	6620 ♂	R	+	16	2 610	1750 $\pm$ 500
		L	+	15	3 390	
	6509 ♂	R	—	72	1 460	
		L	—	8	2 500	
	6617 ♀	R	—	17	1 500	
		L	—	19	1 540	
Untreated controls	5633 ♀	R	—	7	730	370 $\pm$ 340
		L	—	ND	ND	
	5635 ♂	R	—	2	220	
		L	—	5	480	
	5638 ♀	R	—	7	1 075	
		L	—	3	320	
	5641 ♀	R	—	ND	ND	
		L	—	ND	ND	
	6618 ♂	R	—	7	100	
		L	—	4	<100	
	6621 ♀	R	—	1	<100	
		L	—	3	150	

T cells in synovial fluids (from both right [R] and left [L] knee joints) were identified by their ability to form rosettes with papain-treated rabbit erythrocytes. Severity of joint lesions was assessed relative to normal rabbit synovia [—]. Joint lesions are scored as either mild [+], moderate [++], or severe [+++].

Fig. 4 Correlation between the severity of joint lesions and total nucleated cells recovered from the synovial fluids of 'Old English' rabbits injected with killed *E. coli*. The total number of nucleated cells recovered from the synovial fluids of *E. coli* injected (×) and control (●) animals has been plotted against the severity of joint lesions. Joint histology has been classified as either normal (–), mild lesions (+), moderate lesions (++), or severe lesions (+++).

detected in 'Sandy Lop' rabbits injected with killed *B. subtilis*, only 3/8 of these rabbits developed joint lesions, all of which were classified as mild. Indeed, the rabbit with the highest titre (256) did not show any joint inflammation. In contrast, the most severe joint changes were seen in the 'Old English' rabbits injected with killed *E. coli* which did not show high titres of serum IgM RF (Fig. 5).

Discussion

Repeated intravenous injections of killed bacteria stimulate the production of IgM RF and rheumatoid-like synovial lesions in rabbits. These RFs are true autoantibodies reacting with IgG from the same rabbit. Several other groups have described the development of rheumatoid factor-like substances (RFLS) in rabbits after stimulation with protein,³ bacterial^{11 12} or parasitic¹³ antigens and after injections of ammonium sulphate precipitated autologous rabbit IgG.¹⁴ In all these systems RFLS were measured by their reactivity to either heat-aggregated human IgG or pooled heat-aggregated rabbit IgG. Therefore, they neither necessarily measure true autoantibodies to autologous IgG nor eliminate antiallotypic antibodies. However, joint lesions were not described in any of these other reports; joint inflammation could easily have been overlooked without histological examination. In our experimental rabbits with moderate to severe lesions no impairment of movement or gross joint swelling was observed.

In addition, we have observed that injections of killed *B. subtilis* induced higher levels of RF than did *E. coli* (Fig. 1). This is in contrast to the observations of Abruzzo and Christian¹ who found *B. subtilis* to be a poor stimulator of RF compared with *E. coli*. However, this discrepancy is probably attributable to the differences in assay systems employed for measuring RF and differences in breeds of rabbits used. High levels of IgM RF were associated with high titres of IgG directed against the injected bacteria (Fig. 2). This is consistent with Abruzzo and Christian's hypothesis that RF is directed against specific IgG present in immune complexes containing bacterial antigens.

The mechanisms resulting in the development of synovitis in rabbits injected repeatedly with killed bacteria are not understood. The development of chronic erosive polyarthrititis in rats injected intraperitoneally with group A streptococcal cell walls is thought to depend upon the deposition and persistence of bacterial cell walls in synovial tissues.¹⁵ If a similar mechanism were operating in the present study we would have expected a similar incidence of joint lesions in the 'Sandy Lop' rabbits injected with either *E. coli* or *B. subtilis* and this was not the case.

It is possible that the RF itself may be initiating or contributing to the development of joint lesions. Reactivity against self IgG has been postulated to be an important mechanism initiating joint inflammation in human rheumatoid arthritis.¹⁶ However, we found that although *B. subtilis* injected rabbits developed the highest titres of serum RF, only 3/8 animals developed mild synovitis. In contrast, mod-

Fig. 5 Lack of correlation between high IgM RF titres and joint lesions in rabbits injected with killed bacteria. Serum IgM RF titres in (a) 'Old English' rabbits injected with *E.coli*; (b) 'Sandy Lop' rabbits injected with *E.coli*, and (c) 'Sandy Lop' rabbits injected with *B.subtilis*. Joint histology in control (●) and injected (×) animals has been classified as either normal (—), mild lesions (+), moderate lesions (++), or severe lesions (+++).

erate to severe lesions were seen in the 'Old English' rabbits injected with *E.coli* which had the lowest titres of RF. This result may be a consequence of the strain differences, since 'Old English' rabbits show increased susceptibility to experimentally induced arthritis, e.g., serum sickness¹⁷ and the Glynn-Dumonde model of arthritis (Hunneyball, personal communication). It is also possible that the high titres of RF detected in *B.subtilis* injected rabbits have a protective effect. RF production may be a normal physiological process involved in the clearance of immune complexes and elimination of antigen,^{18 19} as is the production of immunoglobulin, i.e., antibody to iC3b.²⁰ Further evidence to support this hypothesis comes from the work of Nemazee and Sato²¹ who showed that the 129/Sv mouse strain produces a very high RF-like antibody in response to an unknown environmental stimulus in the absence of obvious disease.

Alternatively, high serum levels of IgM RF may not be associated with severe joint lesions, because serum RF is not representative of RF produced elsewhere in the tissues including synovium. Any

RF produced in the synovium and highly avid for IgG may never reach the blood in detectable amounts; instead it may become trapped in the tissue or vessel walls of the synovium as immune complexes are locally precipitated. This could result in the direct initiation of inflammation.²² RF bound to immune complexes could also be removed by the reticuloendothelial system and therefore not be available for measurement. As yet, for technical reasons, we have only measured serum IgM RF. Abruzzo and Christian,¹ whose protocol for RF induction we have followed, claimed to have measured an IgG RFLS. IgG anti-IgG may make a more direct contribution to the development of arthritis.

This study is, to the best of our knowledge, the first report of experimentally induced arthritis in rabbits concurrent with true RF production, measured by reactivity with self-IgG, without the use of Freund's complete adjuvant or injection of antigen directly into the joint. The synovial lesions are rheumatoid like with the synovium infiltrated by mixed chronic inflammatory cells. Germinal centres and villi formation were also observed in the most

severe lesions, and T cells were present in increased number in the synovial fluids of affected rabbits (Table 3). Glomerulonephritis was not observed in any of these animals.

E. coli has been implicated in human arthritis development as a consequence of intestinal bypass operations for morbid obesity.²³ It is a member of the *Enterobacteriaceae* which includes yersinia, shigella, salmonella, and klebsiella species, organisms implicated in HLA-B27 associated reactive arthritis and ankylosing spondylitis, though reactive arthritides are not associated with IgM RF production.²⁴ Further studies of this bacterially induced model of arthritis may give an understanding to the development of joint lesions after minimal but repeated bacteraemic episodes which could arise (amongst other situations) through an alteration in gut flora, perhaps as a consequence of dietary changes. For example, erosive joint lesions have been described in piglets fed on a protein rich diet.²⁵ This arthritis was associated with the development of an abnormal intestinal flora with high levels of *Clostridium perfringens* type A which produce alpha toxins, increasing gut permeability.

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