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Reaction of bacterium-primed murine T cells to cartilage components: a clue for the pathogenesis of arthritis?

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SUMMARY

Although different models for rheumatoid arthritis have been studied, the pathogenesis in humans remains unknown. A possible mechanism is the crossreactivity between bacterial components and the target-tissue, the cartilage. The existence of this crossreactivity is supported by various data from clinical and experimental studies. Here we provide direct evidence that priming *in vivo* with cell wall fragments of *Streptococcus pyogenes* or *Escherichia coli* can induce a cellular and humoral anti-cartilage response in Balb/c mice *in vitro*. T cells isolated from these mice can be stimulated *in vitro* to proliferate by a variety of antigens among which are the priming bacterium, an unrelated bacterium, small bacterial components and diverse antigens of cartilagenous origin. In bacterium-primed mice antibodies were also detected that displayed a reactivity to cartilage extract besides the reactivity to bacteria. A crossreactive response occurred *in vivo* in certain circumstances: a delayed type hypersensitivity reaction could be elicited in cell-wall-primed mice by challenge with cartilage extract. For the expression of this crossreactive response *in vivo* however, it was obligatory to attenuate the mouse's suppressor-circuit. In this paper we would suggest a mechanism for the pathology of chronic arthritis, based on repeated challenges with different bacterial stimuli.

Keywords bacteria arthritis crossreactivity to cartilage

INTRODUCTION

A single injection of streptococcal cell wall fragments into susceptible rats results in a chronic polyarthritis (Cromartie *et al.*, 1977) which can also be induced with cell wall fragments of *Lactobacillus casei* (Lehman *et al.*, 1983; 1984). The reason for the chronicity and the localization in the joints is not completely understood. It has been suggested that retention of bacterial material in the joint (Cromartie *et al.* 1977; Lehman *et al.*, 1983; 1984) is fully responsible for both features, but the presence of cell wall fragments of *L. casei* in the early, but more important also in the later, stages of the disease is not restricted to the joints. The distribution of cell wall material in diverse organs, including the joint, is very similar in susceptible (Lew/N) and resistant (F334/N) rats (Lehman *et al.*, 1984). Whether rats with a different genetic background could mount different immune responses to certain bacterial antigens is at present unclear.

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This study was undertaken to investigate an alternative mechanism, which can provide arguments for both the chronicity and the localization in the joints. The basis of this mechanism is the possible crossreactivity of the immune system between bacterial cell walls and cartilage components. This study has been carried out to test the hypothesis that any bacterial stimulus from the environment has the capacity to induce an anti-cartilage response which may in certain situations lead to an arthritis. Here we present clear evidence for the ability of different bacteria to induce a cellular and humoral anti-cartilage response in Balb/c mice.

MATERIALS AND METHODS

Priming of mice

Female Balb/c mice were primed by a subcutaneous injection of cell wall fragments of *Streptococcus pyogenes, Escherichia coli* RR1 or ovalbumin emulsified in incomplete Freunds adjuvant. The respective doses of antigen were 1·0 mg glucose-equivalents, 0·2 mg glucose-equivalents or 0·1 mg.

Preparation of bacterial cell wall components
Cell wall fragments of *S.pyogenes* were prepared as described previously (Lehman *et al.*, 1983). Cell wall fragments of *E.coli* RR1 were prepared as follows: bacteria were grown in 1 litre flasks for 24 h at 37°C, formalin was added to a final concentration of 0·6% and the bacteria were fixed for 24 h at room temperature. Cells were harvested and washed with PBS. The pellet was transferred to a mortar on ice and Alcoa-powder (Alcoa Chemicals, Aluminium Company of America, no A301) was added in portions of 0·5 g to a total of twice the weight of the bacterial pellet. After adding each portion, the mixture was thoroughly pounded. When all the Alcoa-powder was added, twice the volume of PBS was added in portions of 1 ml. The Alcoa-powder and the non-disrupted cells were removed by centrifugation. The material was subsequently centrifuged for 15 min, 25,000 ***g***. The pellet was discarded, the supernatant was treated with 250 μg/ml trypsin type III (Sigma Chemical Company, St Louis, Missouri, USA) for 4 h at 37°C. The resulting material was incubated with bovine serum albumin (BSA) to check whether there was no proteolytic activity left. If this was not the case, the preparation was used for stimulation. The amount of carbohydrate in both cell wall preparations was determined as described by Dubois *et al.* (1956), glucose being used as a standard.

T cell proliferation assay
Twelve days after priming, spleens of the mice were removed aseptically and a single cell suspension was made. The erythrocytes were lysed and T cells were isolated by adherence to plastic and nylonwool. The eluted cells consisted of 95% thy1·2 positive cells (NEN, UK). These cells were resuspended in RPMI 1640 (Dutch modification) supplemented with 5% heat inactivated fetal calf serum, 10 mM pyruvate, 20 mM glutamine and 50 μg/ml gentamycin (all Flow Labs.). Cells (10^5) were placed in the wells of a 96-well round-bottomed microtitreplate with variable concentrations of different cartilage components, cell walls or control stimuli in a final volume of 0·2 ml. After 72 h 1 μCi ^{3}H-thymidine was added per well and 18 h later the incorporated ^{3}H-thymidine was counted. All cultures were done in triplicate and pools of four spleens were always used. The results of a representative experiment are listed in Table 1.

Stimuli for T cell proliferation in vitro
The stimuli were prepared as follows: dermatansulphate-proteoglycan (dspg) was prepared as described by van Kuppevelt *et al.* (1988) and was kindly provided by Dr A. van Kuppevelt (Department of Chemical Cytology, Faculty of Science, University of Nijmegen, The Netherlands); N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide) was obtained as synthetic MDP from Sigma Chemical Company (St Louis, Missouri, USA); chondroitin sulphate (CS) was obtained from BDH Chemicals Ltd (Poole, UK); heparan sulphate (Heps) was obtained from Seikaguku Kogyo Ltd, Tokyo, Japan; bovine cartilage extract (BCE) was prepared by incubating cartilage slices for 18 h at room temperature with 8 M guanidiniumchloride and a subsequent dialysis versus PBS of the supernatant. The material was further isolated by gelfiltration (Sephadex G200, Pharmacia Fine Chemicals). Extraction of cartilage according to this method results in a preparation containing proteoglycans and glycosaminoglycans, but not collagen. The amount of glycosaminoglycans (gags) was determined by measuring the metachromatic shift of dimethyl methylene blue (Farndale, Sayers & Barrett, 1982); rheumatoid synovial fluid (SF-RA) was obtained by puncture of a knee joint of a patient with definite rheumatoid arthritis, the amount of gags being determined as mentioned above; the culture supernatant of fresh bovine chondrocytes (chondrocyte sup) was prepared as follows: cartilage slices from a fresh bovine knee joint were incubated at 37°C for 18 h with 3 mg/ml collagenase in RPMI 1640 (Dutch modification). The washed cells were put into culture for 6 days in a CO_2-incubator at a density of 5×10^5/ml in RPMI + 5% fetal calf serum + 10 mM pyruvate + 20 mM glutamine + 50 μg/ml gentamycin (all Flow Labs). The amount of gags was determined as mentioned above and the hexosamine content was measured as described (Lane Smith & Gilkerson, 1979); ovalbumin (OA) was obtained from Sigma Chemical Company (St Louis, Missouri, USA); collagen type II (CII) was prepared from chicken sternum by salt extraction; lipopolysaccaride (LPS-B) from *E.coli* 055:B5 was obtained from Difco Labs (Detroit, MI USA); concanavalin A (Con A) was obtained from Flow Labs.

Enzyme linked immunosorbent assay
Polystyrene flatbottom microtitre plates (Costar, Cambridge, MA) were coated overnight with per well 150 μl BCE or CS (gag content = 10 μg/ml) or cell walls of *S.pyogenes* (carbohydrate = 0·4 μg/ml). To avoid nonspecific binding, the plates were subsequently coated with 150 μl 1/160 ethanolamine per well for 2 h. After three washes with washbuffer (PBS + 0·05% (v/v) Tween-20) the sera were diluted in 1% (w/v) bovine serum albumin (BSA) in PBS in 2-fold dilutions with a final volume of 100 μl per well. After 1 h of incubation the plates were washed five times with washbuffer and were incubated with 100 μl rabbit anti-mouse Ig (Miles, Elkhart, Ind.) diluted 1/500 in 0·5% (w/v) BSA in PBS for 1 h. After five washes with washbuffer, 100 μl peroxidase-conjugated goat anti-rabbit Ig (Miles, Elkhart, Ind.) diluted 1/500 was added. After 1 h, the plates were washed and incubated with 100 μl substrate per well: 0·8 mg/ml 5-aminosalicylic acid, 0·8 μg/ml 30% H_2O_2 in 50 mM sodium phosphate buffer, PH 6·0. The extinction at 450 nm was read after 30 min in a Titertek Multiskan (Flow Laboratories). All incubations were carried out at room temperature.

Delayed type hypersensitivity reaction
To test the existence of T cells crossreactive to BCE *in vivo* due to bacterial priming, we immunized mice with cell walls of *S.pyogenes* emulsified in incomplete Freund's adjuvant (IFA). The dose of antigen used was 0·2 mg glucose-equivalents. As a control, mice were primed with 0·2 mg OA in IFA. After 14 days the mice were boosted with the same material. Seven days after the booster injection, mice were challenged by injection of BCE (5 μg gag-equivalent) in PBS into the right ear. The ear thickness was measured blindly with an industrial micrometer and expressed as right over left ratios. To weaken the suppressor circuit (suppressor T cells), mice were treated 1 day before the first antigenic stimulus in one of the following ways: total body irradiation (dosis 250 or 400 rad = 2·5 or 4 gray) or intraperitoneal injection of cyclophosphamide (2 or 20 mg/kg body weight) (Gill & Liew, 1978).

Statistics

DTH reactions were compared statistically by the one tailed Mann–Whitney *U*-test.

RESULTS

T cell response in vitro

Table 1 shows the cellular response of Balb/c mice to different cartilage components, cell wall preparations or an irrelevant antigen (ovalbumin). Mice were primed with cell walls of *Streptococcus pyogenes* or *Escherichia coli* or with ovalbumin. In the case of priming with cell walls, the data were the same regardless of the used vehicle; this was either incomplete Freunds adjuvant (Table 1) or PBS (phosphate buffered saline) (data not shown).

T cells from mice, primed with cell wall preparations of *S. pyogenes* or *E.coli* displayed a clear response to a variety of cartilage components, both to pure and to crude preparations. This indicates that reaction of a heterogeneous population of bacterium-primed T cells with a wide range of cartilage components is possible. When these cells were stimulated with the priming bacterium, a clear proliferative response was seen. A response of the same magnitude was also seen when the T cells were stimulated with an unrelated bacterial species. The stimulatory properties of both cell wall preparations were similar regardless of the priming bacterium. Two very small components present in all (MDP) or in Gram-negative (LPS) bacterial cell walls induced a response in bacterium-primed T cells. The cartilage components or extracts: dermatansulphate-proteoglycan, bovine cartilage extract, heparansulphate, chondroitinsulphate and supernatant of a bovine chondrocyte culture are all clear stimuli for bacterial cell wall primed T cells, their effectiveness decreasing in the order listed. Collagen type II did not induce a proliferative response of bacterium-primed T cells. T cells from mice primed with ovalbumin showed no distinct proliferation in response to any of the used stimuli with the exception of ovalbumin itself. A slight, but a significant, response occurred to the synovial fluid. This nonspecific stimulation might be due to an IL-2 or IL-1 activity. The latter is certainly present in the tested fluids (data not shown). LPS also induced a response of OA-primed cells, although again significantly less than that of bacteria-primed cells. The nonspecific response might be caused by monokines released upon LPS stimulation. Thus it is clear that none of the tested stimuli is mitogenic or has other nonspecific effects on the proliferative response with the exception of the above mentioned ones.

Humoral response

Mice primed with cell walls were bled 21 days after priming. Sera obtained from mice primed with cell walls displayed an antibody response to BCE, whereas sera from OA-primed mice did not (ELISA Fig 1). The same results were obtained when CS was the coated antigen (data not shown). *Streptococcus pyogenes* as well as *E.coli* primed mice showed a humoral response to cell walls of *S.pyogenes*, indicating that at the level of antibodies a crossreactive response between different, non-related bacteria exists.

Crossreactive DTH reaction

A crossreactive response *in vivo* can also occur as a result of priming with bacterial components in certain circumstances. Mice primed with cell walls of *S.pyogenes* did not display a

Table 1. Proliferative response of primed murine T cells to various bacterial and cartilage derived antigens

	H-TdR × 10^3 ct/min incorporated by T cells primed with:		
Antigen (μg/ml)	*Streptococcus pyogenes* cell wall	*Escherichia coli* cell wall	OA
*Streptococcus pyogenes**			
2	1·2	1·2	1·0
6	2·0	2·2	0·9
20	2·6	2·9	0·9
60	5·5	5·1	0·9
*Escherichia coli**			
2	1·6	1·9	0·6
6	2·4	3·9	0·8
20	3·1	4·5	0·8
60	5·0	6·5	0·6
MDP			
3	1·5	1·2	0·9
10	1·9	2·0	1·0
30	3·0	3·2	1·2
LPS			
0·001	1·4	1·2	0·6
0·01	1·9	2·0	2·1
0·1	3·4	2·2	2·1
1	5·5	5·0	2·2
BCE†			
3	1·7	1·2	0·5
10	2·5	2·1	0·6
30	3·0	2·6	0·7
chondrocyte sup‡			
6	1·6	1·1	0·6
20	2·8	1·9	0·6
dspg			
3	1·5	1·6	0·7
10	2·6	3·0	0·8
30	6·5	4·9	0·8
CS			
10	1·7	1·7	0·8
30	3·0	2·5	0·8
HepS			
10	1·6	1·6	0·8
30	2·1	1·9	0·7
CII			
100	0·9	0·7	0·4
300	0·7	0·7	0·5
SF-RA§			
1/216	1·3	1·0	0·7
1/72	2·6	1·9	1·0
1/24	2·9	3·0	1·0
1/8	3·5	4·1	1·3
OA			
100	0·9	0·6	2·9
300	0·7	0·8	6·5
Medium	0·8	0·6	0·6
Con A	15·4	15·0	16·5

* With respect to total carbohydrate.

† With respect to gag content.

‡ With respect to gag content; the amount of hexosamine in undiluted material was 2 μmol/ml.

§ Dilution, the amount of gag in undiluted SF was 30 μg/ml.

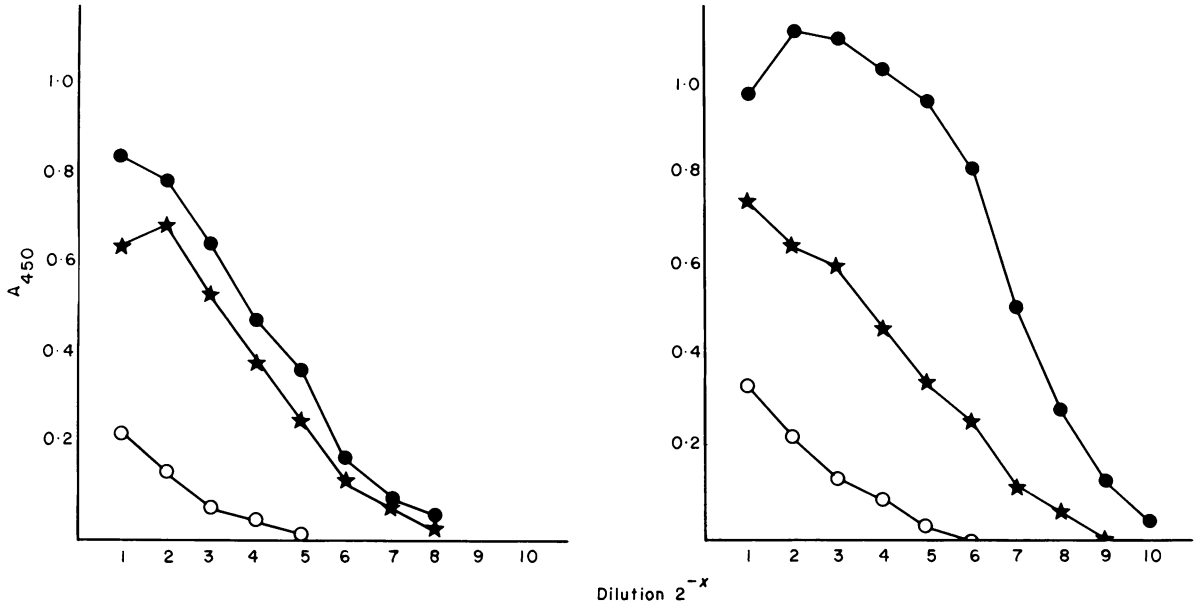

Fig. 1. (a) Humoral anti-BCE response in mice primed 21 days before with cell walls of *S. pyogenes* (●——●) and *E.coli* (*——*) or with ovalbumin (○——○). (b) Humoral response against cell walls of *S. pyogenes* in mice primed 21 days before with cell walls of *S. pyogenes* (●——●) and *E.coli* (*——*) or with ovalbumin (○——○).

crossreacive DTH reaction under normal conditions, but when the suppressor cells were attenuated by low doses of cyclophosphamide (2 mg/kg) (Gill & Liew, 1978) or total body irradiation (250 rad), a crossreactive response occurred in cell wall-primed, but not in ovalbumin-primed, mice. The blindly measured ratios did not show a big difference between the treated group ($1{\cdot}11 \pm 0{\cdot}05$ for cyclophosphamide and $1{\cdot}12 \pm 0{\cdot}08$ for irradiation) and the control group ($1{\cdot}04 \pm 0{\cdot}04$), but the difference was statistically significant ($P < 0{\cdot}03$). A higher dose of cyclophosphamide or irradiation suppressed the DTH reaction again, probably by an effect on helper circuits.

DISCUSSION

Here we show that bacteral stimuli can induce an unwanted crossrective autoimmune response in addition to the normal defensive anti-bacterium response. This crossreacting anti-cartilage response occurred both at the cellular and at the humoral level as was measured *in vitro* by an antigen-induced proliferative response of isolated T cells and an ELISA, respectively. For expression of the crossrecting response *in vivo* however, it was obligatory to attenuate the suppressor-circuit of the mouse before priming. That attenuation of this circuit is not necessary when this T cell response *in vitro* is considered may indicate that the system *in vitro* is more sensitive, or that it lacks cells or mediators which are present *in vivo*. For instance macrophages, which have been removed before T cell stimulation *in vitro*, are able to mediate suppression of T cell responses. This effect can either be mediated by release of prostaglandins or by activation of suppressor cells (Allison, 1978; Ptak *et al.*, 1978). Moreover, activation of suppressor cells may display other kinetics *in vivo* and *in vitro*.

Expression of anti-cartilage response *in vivo* is not enough to induce an arthritis however: mice which developed an anti-cartilage DTH response after bacterial priming (with foregoing attenuation of the suppressor-circuit) did not display any abnormality in the joints at all. It is possible that, in addition to an immunological anti-cartilage response, a sensitization of the joints is a prerequisite for arthritis. This is in all probability a potentiating factor in one of the animal models for arthritis, the streptococcal cell wall induced arthritis. In this model, bacterial material has been demonstrated in the joints after an intraperitoneal injection of it (Cromartie *et al.*, 1977; Lehman *et al.*, 1983; 1984). In our study however, the amount of bacterial cell walls injected was 10 times less (corrected for the animal weight) than in the rat model, so this could be a limiting factor when sensitization of the joints is considered. Another difference with the rat model is the route of administration: for the DTH reaction, cell wall material was given to mice emulsified in incomplete Freund's Adjuvant, so the immune system is continuously triggered by relatively low doses of antigen, while in the rat model the immune system is bombarded with enormous amounts of antigen at one time. The latter causes serious systemic effects (Hunter *et al.*, 1980; Ridge *et al.*, 1986), probably also affecting the immunoregulatory properties. This could then lead to a more vehement expression of the inflammatory reaction.

If mice are more effectively protected against autoreactive responses than rats—by a better suppressor-circuit for instance— it could be necessary to eliminate suppressor cells by stronger means for arthritis to occur due to the presence of anti-cartilage responses. Factors which influence the expression of arthritis in this model are currently subjected to further study in our laboratory.

In this study we provide direct evidence for the suggestion that bacterial stimuli can result in an anti-cartilage response. Pointers to a role for bacteria in human arthritis can amply be found. First, streptococcal infections have been shown to give rise to acute rheumatic fever (Cantazarro *et al.*, 1954). Second, the reported cases of postdysenteric Reiter's syndrome (Catter-

all, 1976) and arthritis associated with jejunal bypass surgery for obesity (Utsinger, 1980) suggest a role for gastroenteric bacteria in the induction of arthritis. Third, in patients with rheumatoid arthritis, T cell reactivity to *M.tuberculosis* has been reported (Holoshitz *et al.*, 1986). This T cell reactivity was specific for mycobacteria and was merely found in the synovial fluid during the first year of disease, between the second and tenth year mainly in the peripheral blood and after ten years of disease not in either compartment.

The widely used model for rheumatoid arthritis, the adjuvant arthritis in rats (Pearson, Waksman & Sharp, 1961), provides evidence for the crossreactivity between bacteria and cartilage. This disease can be transferred with lymphocytes (Pearson & Wood, 1964) and even by a T cell clone, reactive to *Mycobacterium tuberculosis* (Holoshitz *et al.*, 1983; 1984). The clone only induces an arthritis in recipient rats after irradiation of the animal; the same condition of the recipient is necessary for crossreactivity between the clone and osteoarthritic synovial fluid or chondrocyte supernatants to occur *in vivo* (van Eden *et al.*, 1985). This illustrates the importance of crossreactivity: an arthritis can only be accomplished if the conditions are suitable for crossreactivity. Also in streptococcal cell wall induced arthritis, T cells are of utmost importance: it has been shown that only the acute but not the chronic phase of the disease can be induced in nude rats (Ridge *et al.*, 1985). The chronic phase of the disease is dependent on (crossreacting?) T cells, whereas the acute response is not and thus is possibly a nonspecific reaction. These facts may imply that cell-wall-induced arthritis and adjuvant arthritis share similar pathogenic pathways, the only difference being the bacterium used as a stimulus. That different bacteria can lead to arthritis in experimental animals has also been shown in the rabbit with *E.coli* and *Bacillus subtilis* (Hanglow *et al.*, 1986), and in the mouse with *Nocardia* ssp., *Lactobacillus plantarum, Staphylococcus epidermis* and *Streptococcus mutans* (Koga *et al.*, 1985).

Also on the level of antibody responses reactivity to bacterial components is demonstrated in patients with various rheumatic diseases (Pardo *et al.*, 1984; Burgos-Vagas *et al.*, 1986; Johnson *et al.*, 1984). The antibody levels to peptidoglycans were most obvious at the time of onset of the disease (Pardo *et al.*, 1984; Burgos-Vags, Howard & Ansell 1986), which might be a pointer in the direction of bacteria or bacterial components as an inductory agent in the pathogenesis of rheumatic diseases.

Additional facts of evidence for the existence of crossreacting epitopes on bacteria and proteoglycans or glycosaminoglycans are provided by the following observations: sera from patients with post-streptococcal glomerulonephritis have been shown to contain antibodies reactive with glomerular basement membrane heparan sulphate proteoglycan (Fillit *et al.*, 1985). Support for the possibility that this could be due to cross reactive antibodies is given by the finding that murine hybridomas reactive to streptococcal cell walls crossreact with glomerular basement membrane (a structure in the kidney, like the joint very rich in glycosaminoglycans) *in vivo* and *in vivo* (Fritzsimons, Weber & Lange, 1986).

Taken together, our data and the studies of others provide suggestive evidence for a mechanism for the pathology of arthritis. If an individual passes through a (subclinical) bacterial infection, he will mount an anti-bacterium immune response in defence. As becomes clear from our data, this anti-bacterium response can display an autoimmune character by reacting with various cartilage components both at cellular and at humoral level. This crossreacting response will in all probability need some aberrant conditions (defective suppression or feedback mechanisms) to come to full expression and to give rise to joint lesions.

We also show that a response induced by one bacterial species can be reactivated by another non-related species or even by small common bacterial components like LPS (a cell wall structure of Gram-negative bacteria) and MDP (a cell wall structure of almost all bacteria). The latter can have important consequences for the maintenance of the arthritis: once an anti-bacterium response—and thus an anti-cartilage response—is induced by one bacterium, any other invading species or its degradation product is able to reactivate not only the anti-bacterium but also the anti-cartilage response. These reactivations of the anti-cartilage response by (subclinical) infections with bacteria from the environment or the individual itself (gastrointestinal tract) can give rise to (subclinical) exacerbations of the inflammation. Exacerbations could also be mediated by auto-antigen, released as a result of cartilage damage. Repeated flares of the arthritis thus can lead to chronicity.

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