Experimental arthritis induced by atypical strains of *Streptococcus pyogenes*

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Summary. Experimental arthritis was induced in Sprague-Dawley rats by intra-articular injection of whole-cell sonicates, heat-killed cells and cell-wall preparations of typical and atypical strains of Group-A streptococci (*Streptococcus pyogenes*). The non-haemolytic nitrosoguanidine-derived mutant and the naturally occurring Lowry strain induced a similar but less severe form of arthritis. Direct immunofluorescent staining demonstrated maximum fluorescence in the sections of articular joint taken 60 days after injection. The level of immune complexes increased for up to 90 days after injection of cell walls or whole-cell sonicates and correlated well with the development of the chronic stage of arthritis observed in haematoxylin and eosin and fluorescence staining of thin tissue sections.

Introduction

Whole-cell sonicates of Group-A streptococci and streptococcal cell-wall components have been shown to induce experimental arthritis in rats (Schwab *et al.*, 1967; Cromartie *et al.*, 1977; Anderle *et al.*, 1979; Clark *et al.*, 1979; Wilder *et al.*, 1983). Each of these studies used typical Group-A β -haemolytic streptococci as the arthritis-inducing agent. This paper describes the ability of atypical, non-haemolytic strains of *Streptococcus pyogenes* to induce experimental arthritis in Sprague-Dawley rats. Non-haemolytic strains are more difficult to identify and characterise in routine diagnostic laboratories and their ability to induce arthritis warrants concern about their pathogenicity for man.

Materials and methods

Organisms

S. pyogenes Lancefield SS-95 and the non-haemolytic Lowry strain were obtained from Dr R. Facklam, Center for Disease Control, Atlanta, Georgia. A non-haemolytic mutant of the parent SS-95 strain was isolated by treatment with N-methyl-N'-nitro-nitrosoguanidine as described previously (Owens *et al.*, 1978).

Cell preparations

S. pyogenes strains were grown in Todd-Hewitt broth as previously described (Owens *et al.*, 1978). Cells from 1.5 L of broth were harvested by centrifugation, resuspended and washed three times in sterile phosphate-buffered saline (PBS), pH 7.0. Washed, packed whole cells were resuspended in 30 ml of PBS and ultrasonicated for 90 min (Sonifier Cell Disruptor, Model W185D from Heat Systems-Ultrasonics, Inc., Plainview, L.I., New York). Whole-cell sonic extracts and cell-wall suspensions were isolated and purified according to the procedure of Anderle *et al.* (1979) and the rhamnose content of the cell walls was determined by the method of Dische and Shettles (1948). Heat-killed whole cells were prepared by resuspending the washed, packed cells from 1 L of Todd-Hewitt broth in 15 ml of PBS and heating at 80°C for 30 min. All preparations were checked for sterility by plating on blood agar.

Experimental animals

Outbred male and female Sprague-Dawley rats weighing 150–250 g (Sasco, Inc., Omaha, NB) were divided into nine groups. Each group contained eight animals which were given injections of (1) whole-cell sonicates, (2) heat-killed whole cells, or (3) purified cell walls from the parent β -haemolytic *S. pyogenes*, the non-haemolytic mutant, or the Lowry strain. Doses for injection were calculated on the basis of the rhamnose content of the streptococcal cell wall (Cromartie *et al.*, 1977; Greenblatt *et al.*, 1980) and a single intra-articular injection was made with rhamnose concentrations in the range 3–100 μ g/g body weight. These doses represented approximately 1–33.3 mg of cell-wall material. Control animals

received either an equal concentration of analytical grade rhamnose or an equal volume of PBS. Animals to be used for radiological or histological examination, for studies of immune complex formation, or immunofluorescence received a single intra-articular injection of whole-cell sonicate or cell-wall preparations of the parent or non-haemolytic mutant equivalent to rhamnose 60 µg/g body weight (approximately 20 mg of cell-wall material).

Evaluation of arthritis

Animals were monitored daily for the first 2 weeks and twice weekly thereafter until completion of the experiments. Joint diameter was measured with a steel caliper calibrated to read to 0.05 mm. Severity was scored on a scale of 0–4 based on inflammation, oedema of peri-articular tissue, and enlargement, distortion or ankylosis of the joint (Cromartie *et al.*, 1977; Hunter *et al.*, 1980). Joint lesions were observed by X-ray and histological techniques.

Radiological techniques

Rats were anaesthetised by intra-peritoneal injection of ketamine hydrochloride 100 mg/kg body weight. X-rays were taken with a General Electric X-ray machine (MST62511) with a 200-ma focal spot. Exposures were made for 0.25 s at 32 kv without a filter. The focal film distance was 40 in. with an object–film distance of 6 in. Kodak min-R cassettes with Kodak min-R screen and single coated Kodak min-R film were used. Each radiograph was evaluated for the presence and severity of soft-tissue swelling, joint effusion, joint narrowing, osteoporosis, bone erosion, and subperiosteal new bone formation.

Histology

Joint sections were taken from arthritic rats killed periodically after injection of whole-cell sonicates or cell-wall preparations of the parent, mutant or Lowry strains. Samples were immersion-fixed in 10% neutral buffered formalin. Joint specimens were decalcified in formic acid and sodium citrate solutions for 10–15 days after fixation. Paraffin embedded specimens were stained with haematoxylin-eosin (H & E) or with periodic acid schiff (PAS).

Immunofluorescence

Animals from control and test groups were killed after 0, 3, 15, 30, 60, 90 and 300 days. Ankle and knee joints were collected, fixed in 10% formalin and sections 10–15 µm thick were taken. Sections were stained with fluorescein isothiocyanate conjugated rat anti-IgG and anti-IgM antibodies according to routine procedures for direct immunofluorescence staining. Photographs were taken with a 35 mm camera (Leitz) attached to a fluorescent microscope (E. Leitz, Wetzlar).

Immune complexes

A slight modification of the polyethylene glycol precipitation method of Van der Giessen and The (1986) was used to detect immune complexes. Serum samples (150 µl) from arthritic rats given whole-cell sonicates or cell walls of the parent or mutant strains, were mixed with 450 µl of 0.08 M calcium carbonate and 3 ml polyethylene glycol (PEG) 5% in 0.1 M borate buffer, pH 8.4. The mixture was left at 4°C overnight and centrifuged for 3 min at 3500 g. The supernate was carefully removed and the pellet dissolved in 3 ml of 0.1 M sodium chloride. Optical density at 280 nm was recorded.

Chemicals and growth media

Todd-Hewitt broth was from BBL, Cockeysville, MD, fluorescent labeled rat anti-IgM and anti-IgG were from Serotech, Indianapolis, IN, and ketamine hydrochloride was from Parke-Davis, Morris Plains, NJ. All other chemicals were from Sigma Chemicals, St Louis, MO.

Results

Induction of experimental arthritis

Comparison was made between the typical haemolytic Group-A streptococcus and the two atypical, non-haemolytic strains for their ability to induce experimental arthritis in rats. Development and severity of arthritis induced by the typical and atypical strains showed a dose-dependent response similar to that observed for other species of *Streptococcus* previously tested in this model (Cromartie *et al.*, 1977; Hunter *et al.*, 1980; Eisenberg *et al.*, 1982; Esser *et al.*, 1986). As little as the equivalent of rhamnose 3 µg/g body weight of the whole-cell sonicate or purified cell walls from the parent typical Group-A streptococcus induced the primary inflammatory response in 3 of 8 animals tested. All animals developed inflammation when given doses equivalent to ≥ 20 µg of rhamnose/g body weight. Doses equivalent to 30 µg of rhamnose/g body weight of the mutant or Lowry strains were necessary to induce arthritis. Swelling and erythema were observed in the ankle or knee joint, or both, beginning on day 2 and lasting 5–7 days in animals that had received either parent or mutant whole-cell sonicates or purified cell walls. Those animals receiving the Lowry strain required 4–5 days longer for the primary inflammatory response to appear and they failed to develop the chronic erosive stage with any concentrations. Heat-killed whole cells of the parent or mutant induced swelling and erythema but to a lesser extent than in animals treated with whole-cell sonicates or cell walls.

Controls receiving PBS or analytical grade rhamnose did not exhibit inflammation or other symptoms of arthritis.

Soft-tissue swelling, calcification, bony overgrowth of the joint and joint narrowing could be seen (figs. 1 and 2) during the clinical and chronic stages of the disease.

Histological observations

Sections from the ankle or knee joint revealed the chronic erosive nature of the joint tissue when stained with H & E or PAS. This phase was characterised by hyperplasia of the synovial lining cells, destruction of the cartilage and subchondral bone and replacement of this tissue by fibrous connective tissue. Irregularly shaped islets of osteoid tissue were seen surrounding the area of bone destruction. In some areas, invasion of chondroid and osteoid tissue by fibrous tissue (osteofibrosis), pannus formation and extension of fibrous connective tissue over the articular cartilage could be seen (figs. 3, 4 and 5). Proliferation of connective tissue of the stroma of synovial villi and infiltration of tissue by mononuclear phagocytes and lymphocytes were also observed. Eventually the joint space became filled with connective tissue and the destroyed articular cartilage was replaced by fibrous connective tissue leading to complete joint ankylosis and the loss of normal bone and cartilage architecture. The Lowry strain primarily

induced swelling of the peri-articular tissue such as the ligaments, joint capsule and fibrous tissue around the joint.

Fluorescent antibodies

Ankle and knee joint sections from controls and animals given whole-cell sonicates or cell walls from either the parent or mutant strain were stained with fluorescein isothiocyanate-conjugated anti-IgG and anti-IgM. With uninoculated control rats, there was no pre-existing IgG or IgM in the synovium. Sections from treated groups, stained 3 and 15 days after injection, also showed no fluorescence against anti-IgG or anti-IgM. Fluorescence with anti-IgG was first detected in joint tissues 30 days after injection and reached a peak within 60 days (fig. 6). Most of the fluorescence appeared in the articular cartilage 300 days after injection of the antigen. All fluorescence observed was in response to anti-IgG and fluorescence could not be detected at any stage of the disease when anti-IgM was added to the tissue. These data indicate the presence of IgG in the cells of the synovial membrane or on their surface and suggest the presence of immune complexes at these sites.

Immune complexes

The level of immune complexes increased up to 90 days after injection of whole-cell sonicates or cell

Fig. 1. Radiographs of knee and ankle joints 3 months after intra-articular injection of parent *Streptococcus* whole-cell sonicates (equivalent to rhamnose 60 $\mu\text{g/g}$ body weight). Calcification, bony overgrowth and narrowing of the joint can be seen. Subperiosteal new bone formation is evident in distal extremities of the tibia and proximal tarsus.

Fig. 2. X-ray from a rat 3 months after intra-articular injection of non-haemolytic mutant whole-cell sonicates (equivalent to rhamnose 60 $\mu\text{g/g}$ body weight). Mild soft tissue swelling, calcification, and joint narrowing can be seen. Arthritis is less severe than in animals receiving the parent whole-cell sonicate.

Fig. 3. Joint section from uninoculated control rat. The joint has a smooth delicate articular surface and normal joint space. Animals were maintained under the same conditions as the treated group. Controls receiving PBS or analytical grade rhamnose exhibited identical histological characteristics.

Fig. 4. Section of knee joint from rat 3 months after intra-articular injection with parent Group-A *Streptococcus* whole-cell sonicate (equivalent to rhamnose 60 $\mu\text{g/g}$ body weight). Joint space has narrowed, osteofibrosis and pannus formation over the articular cartilage, plus breakage of the bone surface can be seen (H & E, $\times 125$).

Fig. 5. Knee joint section from rat 3 months after injection of whole cell sonicate of non-haemolytic mutant (equivalent to rhamnose 60 $\mu\text{g/g}$ body weight). Joint space has narrowed due to pannus extension over the articular cartilage and osteofibrosis is evident. (H & E, $\times 125$).

walls (fig. 6) and correlated well with development of the chronic stage of arthritis observed in H & E-stained sections and by immunofluorescent staining.

Discussion

These results, as well as confirming findings of other investigators, establish for the first time a role for atypical non-haemolytic strains of *S. pyogenes* in the induction of experimental arthritis in Sprague-Dawley rats. This animal model has been widely used to study the arthropathic properties of *S. pyogenes*. The atypical strains induced a similar but less severe form of arthritis. It is not surprising to find that these atypical strains can induce arthritis if one assumes that the cell wall peptidoglycan-polysaccharide, or components of this complex, are responsible for its induction (Schwab *et al.*, 1967; Cromartie *et al.*, 1977; Clark *et al.*, 1979; Dalldorf *et al.*, 1980; Lambris *et al.*, 1982; Chetty *et al.*, 1983; Wilder *et al.*, 1983; Esser *et al.*, 1986). The non-haemolytic mutant used in this investigation has been described previously (Owens *et al.*, 1978). It was non-haemolytic on surface and stab cultures and did not revert to β -haemolysis in the presence of reducing agents or in a GasPak atmosphere. The non-haemolytic mutant contained levels of hyaluronidase, DNAase, NADase, and streptolysin O

identical to those of the parent strain. The mutant was sensitive to bacitracin, pathogenic for mice and was identified by the Center for Disease Control in Atlanta, Georgia, as an M-type, T-type 12 *S. pyogenes*, the same as the parent from which it was derived. Both the non-haemolytic mutant and the Lowry strain were deficient in streptolysin S but retained their group characteristics and other cell-wall properties.

The serum concentration of immune complexes reached a peak about the same time that maximum immunofluorescence was observed and correlated well with development of the chronic stage of the disease. Detection of serum immune complexes with PEG is concentration dependent (Van der Giessen and The, 1986). It is not known whether the serum level of immune complexes in animals given streptococci in our study is dose dependent or not. Only those doses of antigen known to induce good arthritic response(s) were used in these experiments. The linear response noted in fig. 4 is a plot of detectable immune complex at specific times during the development of arthritis. Attempts to correlate immune-complex levels with antigen concentration were not made. We did note increased severity of the inflammatory response and chronic erosive stage of the disease with increased dose of antigen administered. A similar dose dependent relationship was observed by Eisenberg

Fig. 6. Right knee joint from animals stained with fluorescent labelled anti-IgG 30, 60 and 90 days after injection of mutant streptococcal cell walls (rhamnose 60 $\mu\text{g/g}$ body weight). Fluorescence is visible in the synovial lining beginning 30 days after injection, reaching a peak in 60 days, as well as in the joint cartilage after 90 days. **A.** Control. Animals received phosphate-buffered saline. Sections of knee joint were taken after 60 days and treated with fluorescein-conjugated rat anti-IgG. No fluorescence is detectable. **B.** Rats received cell-wall preparations of non-haemolytic mutant *Streptococcus* (rhamnose 60 $\mu\text{g/g}$ body weight). Sections were taken from knee joints of arthritic rats 60 days after injection and stained with fluorescein labeled anti-IgG (100 $\times$). Clear areas indicate fluorescence seen against a pale background.

Fig. 7. Immune complex formation at various time intervals after injection of cell walls from the non-haemolytic mutant (equivalent to rhamnose 60 µg/g body weight). Serum was precipitated with 0.08 M calcium carbonate and PEG 5% (in 0.1 M borate buffer, pH 8.4). The precipitate was removed by centrifugation, dissolved in 0.1 M sodium chloride and the optical density at 280 nm recorded.

et al. (1982) and Esser *et al.* (1986). If immune complexes are an integral part of this disease process, one would expect a dose dependent relationship between the antigen and amount of complex formed. The composition of these immune complexes is not known but could be similar to rheumatoid factors formed in man and other experimental animals.

Rheumatoid factors of the IgM and IgG classes

have been shown to form immune complexes in serum or joint fluid by self association (Mannik, 1979) or reaction with native IgG (Carson *et al.*, 1978). The complexes are capable of complement fixation, can activate phagocytic cells and may thereby elicit inflammatory erosive diseases in joints and extra-articular lesions (McDougal and McDuffie, 1985).

Activation of the alternate complement pathway has been suggested to be a significant factor in experimental arthritis induced by streptococcal cell-wall components (Greenblatt *et al.*, 1980; Lambris *et al.*, 1982). The detection of IgG in the joint tissues coupled with the presence of immune complexes in the serum during the chronic stages of experimental arthritis might also depend upon the same bacterial components. The presence of IgG in the synovial tissue, and later in cartilage, could be due to deposits of immune complexes of the IgG-IgG type. Our inability to detect IgM at any stage of the disease would appear to rule out IgG-IgM complexes.

It seems clear from this investigation that atypical strains of *S. pyogenes* can be pathogenic as measured by their ability to induce experimental arthritis *in vitro*. Involvement of atypical strains with naturally occurring disease processes is well documented (James and McFarland, 1971; Chapman, 1972; Bannatyne and Robson, 1974; Drapkin *et al.*, 1976) and as such should be recognised as a group of medically important bacteria.

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