Determinants of Synoviocyte Clearance of Arthritogenic Bacteria

ROBERT D. INMAN and URSULA PAYNE

ABSTRACT. ***Objective.*** Persistence of intracellular organisms may play a critical role in the initiation and perpetuation of synovitis in reactive arthritis (ReA). We investigated factors that may influence local clearance of arthritogenic pathogens in ReA.

Methods. We studied 11 HLA-B27 positive patients with spondyloarthropathies and contrasted these patients with 6 HLA-B27 negative control patients with rheumatoid arthritis or osteoarthritis. We employed an *ex vivo* system in which human synoviocytes derived from patients with ReA are cocultured with arthritogenic pathogens, and intracellular clearance is measured by quantitating colony-forming units over time.

Results. The clearance kinetics of the organisms bore no relationship to the HLA-B27 status of the patient. Clearance of *S. typhimurium* over a 10 day period was accompanied by a progressive rise in nitric oxide (NO) production, but this appeared not to be rate-limiting, since (1) clearance kinetics were comparable between high versus low NO-producing synoviocytes; and (2) L-NMMA inhibition of NO production did not alter clearance kinetics of *S. typhimurium*. Interferon-γ (IFN-γ) was observed to have a small but measurable effect on bacterial clearance. In certain patients with ReA there was a paradoxical stimulatory response to IFN-γ, in which the addition of IFN-γ was accompanied by an *increase* in intracellular bacteria. This effect was found to be attributable to IFN-γ mediated suppression of NO production in these cells. This pattern was not observed in B27 negative synoviocytes.

Conclusion. Intracellular persistence of arthritogenic organisms may contribute to the cellular basis of ReA, but the molecular basis of the bacteriocidal pathways in synoviocytes has not been fully resolved. Our findings indicate that a direct effect of HLA-B27 on these events is unlikely, but that alterations in cytokine response profiles may play a contributory role. Characterizing these mechanisms holds the promise of more specific therapeutic interventions in this disease. (J Rheumatol 2003;30:1291–7)

Key Indexing Terms:
HLA-B27 REACTIVE ARTHRITIS SPONDYLOARTHROPATHY SYNOVIOCYTE

Reactive arthritis (ReA) refers to a nonseptic inflammatory joint disease that follows an extraarticular infection. The antecedent infection frequently involves the gastrointestinal (GI) tract, with pathogens such as *Salmonella* spp, *Yersinia enterocolitica*, or *Shigella flexneri*. The pathogenesis of the synovitis remains unresolved. It is evident that the inflammatory process may persist for years after the inciting infection. In our 5 year followup of patients with post-*Salmonella* ReA we observed that up to 30% of patients had persistent joint inflammation at this time, although the synovial fluid cultures were negative throughout[1]. In terms of formulating a testable hypothesis of possible mechanisms there are several pertinent details from clinical and experimental studies. First, there is an association with the class I HLA allele B27, implicating a genetic susceptibility to the disease[2]. Second, there is evidence for intraarticular persistence of microbial antigens, notably lipopolysaccharide of *Salmonella* or *Yersinia*, after the respective infections[3], yet the source of these local antigens is obscure. It may be that there is a transient bacteremia with seeding of the joints. We have experimental evidence that septic arthritis can indeed evolve into a chronic aseptic arthritis, and the same principle may obtain in chronic ReA[4]. Observation of these antigens locally has generally employed immunofluorescence techniques, whereas polymerase chain reaction (PCR) analysis of joint tissues from similar tissues has largely been negative for pathogens in the case of post-dysenteric ReA. Third, the arthritis is generally resistant to antibiotic treatment, even when quinolones effective against gram-negative enteric pathogens have been used[5]. Finally, numerous studies of T cell responses have revealed enhanced synovial fluid T cell responses to candidate microbes, in contrast to T cell responses of concurrent peripheral blood lymphocytes. These different lines of investigation have all provided indirect support for the concept that in ReA there may be a

From the Arthritis Center of Excellence, Toronto Western Hospital Research Institute, and Departments of Medicine and Immunology, University of Toronto, Toronto, Ontario, Canada.

Supported by The Arthritis Society and the Canadian Arthritis Network.

R.D. Inman, MD, U. Payne, BSc.

Address reprint requests to Dr. R.D. Inman, Arthritis Center of Excellence, ECW 8-005, Toronto Western Hospital, 399 Bathurst Street, Toronto, Ontario M5T 2S8. E-mail: robert.inman@uhn.on.ca

Submitted July 15, 2002; revision accepted November 28, 2002.

failure to clear the pathogens from the joint, and chronic inflammation sustained by antigen persistence is the result. The basis for this purported defective host response to the pathogen has remained unresolved, and the cell biology of this interaction is not fully characterized.

MATERIALS AND METHODS

The study included 11 HLA-B27 positive patients with spondyloarthropathy (SpA) who met the European Spondylarthropathy Study Group criteria[6]. This group consisted of 9 men and 2 women, with a mean age of 35.9 years. The clinical diagnoses were ankylosing spondylitis (n = 4), undifferentiated SpA (n = 4), and reactive arthritis (n = 3). None had an identified pathogen antecedent to the onset of arthritis. All patients had active synovitis at the time they were studied, indicated by the presence of joint effusion. Controls consisted of 6 patients diagnosed with rheumatoid arthritis or osteoarthritis, and all were HLA-B27 negative. Synovial tissues were obtained at the time of arthroscopy or joint arthroplasty in 2 SpA patients. Synovial fluids were obtained from all patients at the time of arthrocentesis performed for diagnostic or therapeutic reasons.

Synovial cells were plated in 75 cm^2 flasks and cultured in modified Eagle's medium with 10% fetal calf serum (FCS). The medium was changed weekly. When a synoviocyte monolayer was formed (after 2–3 weeks), the cells were trypsinized for passaging. While there was some heterogeneity with respect to growth rate and cell morphology in culture, neither bore a clear relationship to the B27 status of the cells.

Quantitative invasion assays were performed as described[7]. Briefly, bacteria were grown to mid-log phase, washed in phosphate buffered saline (PBS), and resuspended in Dulbecco's modified Eagle's medium (Gibco) with 5% FCS. Then 0.1 ml aliquots were added to the PBS-washed cell monolayer containing 0.3 ml media. To make immediate contact between cells and bacteria, the plate was spun for 10 min at 1800 rpm. The bacteria were incubated with the cells (at a bacteria:cell ratio of 2:1 to 10:1) for 2 h at 37°C, and washed with PBS. Gentamicin 100 µg/ml was added to the cell monolayer for a 1 h incubation to kill the extracellular bacteria. Cells were harvested at different time points and colony-forming units (CFU) were determined after cell lysis (with 0.2% Triton-X-100) to calculate the total intracellular bacteria load. Gentamicin 10 µg/ml was maintained in the medium for the duration of the clearance studies.

The organisms used were arthritogenic strains of *Salmonella typhimurium* and *Yersinia enterocolitica* 0:3, recovered respectively from patients with ReA during an epidemic of salmonellosis and from a patient with post-Yersinia ReA. IFN-γ was obtained from Endogen Inc. (Cambridge, MA, USA).

Nitric oxide (NO) production by the synoviocytes was measured as nitrite in the supernatants of the infected cells in the presence or absence of IFN-γ. The accumulation of nitrate was determined with the diazotization reaction with Griess reagent (0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). Cell-free supernatants of the infected cells with or without IFN-γ were collected at days 0, 1, 2, 3, 7, 10, and 13. The samples (100 µl) were mixed with equal volumes of Griess reagent and incubated at room temperature for 10–13 min. Absorbance was measured at 570 nm and nitrite concentrations were interpolated from standard curves prepared with $NaNO_2$. Results are reported for the infection related NO production by subtracting the amount of NO produced by uninfected synoviocytes at the respective time point from the amount of NO produced by infected synoviocytes.

To define the role of NO in bacterial clearance the following inhibitors of NO generation were utilized: LNMMA (N-monomethyl-L-arginine) and LNNA (N-nitro-L-arginine) from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

B27 and invasion. Quantitation of invasion after 4 h incubation with *S. typhimurium* was compared in synoviocytes derived from B27 postive and B27 negative patients. Figure 1 depicts the representative clearance kinetics from 3 B27 positive and 2 B27 negative patients. Measurements of intracellular bacterial recovery after 4 h post-invasion revealed no consistent relationship to the B27 status of the cells. Similarly, when longer culture periods were studied to address late clearance of intracellular organisms, there was no consistent pattern correlating with the B27 status of the synoviocytes (Figure 2). Of note, the clearance kinetics were stable for a given patient. Figure 3 illustrates clearance profiles for synoviocytes obtained from a B27 positive patient on 2 different knee aspirations.

NO and bacterial clearance. NO has been implicated in playing an important role in eradication of intracellular organisms. We did observe that as CFU decreased there was an incremental production of NO following bacterial invasion of synoviocytes (Figure 4). This inverse relationship of CFU to NO held for both *S. typhimurium* (Figure 4, IA, IB) and *Y. enterocolitica* (Figure 4, IIA, IIB). We noted, however, that comparing a strong NO producer with a weak NO producer there was no significant difference in clearance kinetics (data not shown). To address more rigorously whether these events reflected any causal relationships we examined the effect of adding NO inhibitors to the system. Examining cultures at 9 days post-invasion, we observed that LNMMA 0.2 nM completely abolished production of NO by the infected synoviocytes (Figure 5a). This was true in the absence or the presence of IFN-γ. LNNA did not inhibit NO production to a significant degree. However, when we examined the effect of NO inhibition by LNMMA on intracellular clearance of *S. typhimurium*, there was no effect on clearance profiles at 1 day or 8 days in culture (Figure 5B). This was not influenced by the addition of IFN-γ to the system (B panels in Figure 5b). These results indicated that NO cannot be playing a rate-limiting role in eradication of the organism.

IFN-γ and bacterial clearance. IFN-γ has been found to play a role in host response to several intracellular pathogens, and the success of the host Th1 response to such pathogens has been attributed to this mechanism. We evaluated the effect of adding IFN-γ (0.1, 1.0, or 10.0 ng) to the *Salmonella*-infected synoviocytes. IFN-γ had a measurable but small suppressive effect on intracellular organisms, with a representative clearance profile shown in Figure 6.

In some B27 positive patients, a paradoxical *stimulatory* effect of exogenous IFN-γ was observed, most distinctly at Day 1 post-invasion (Figure 7a). This pattern was never seen in B27 negative synoviocytes. This suggests the possibility that an appropriate Th1 host response might have an inappropriate effect on local clearance of the pathogen from the joint microenvironment. As illustrated in Figure 7b, this IFN-γ stimulatory response bore an inverse relationship with production of NO. This suggests that IFN mediated suppression of endogenous NO may account for this para-

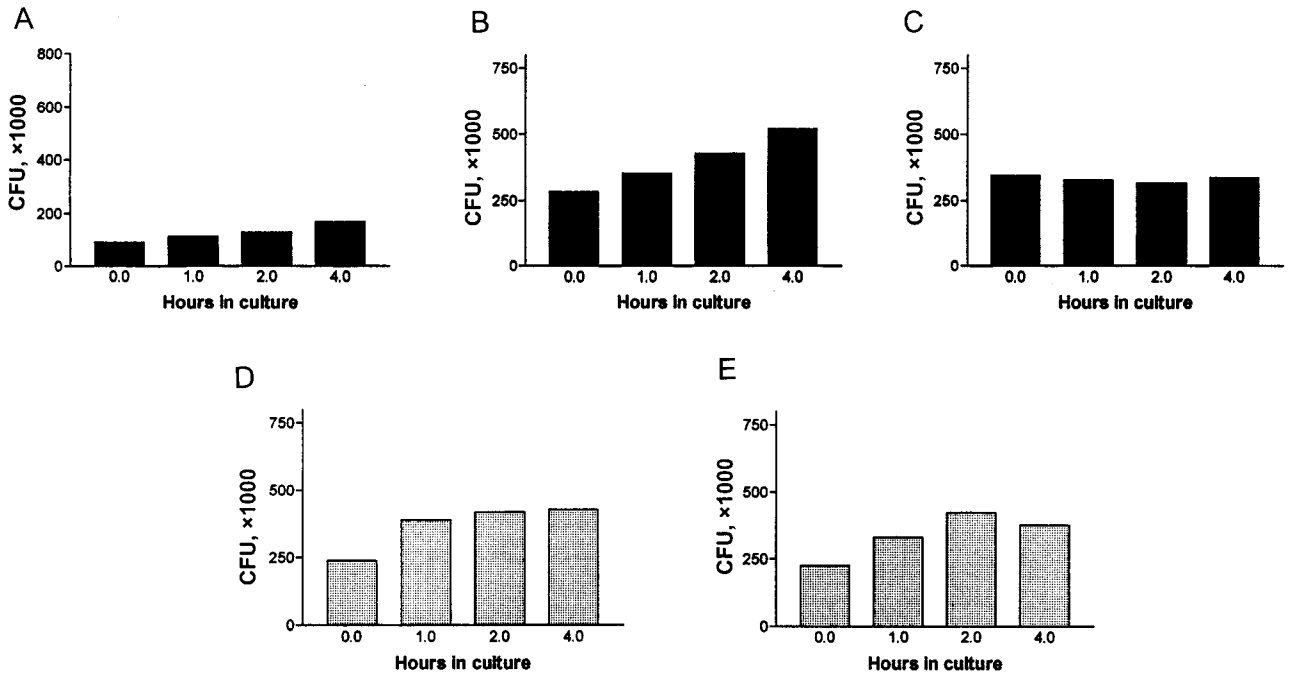

Figure 1. Kinetics of intracellular *S. typhimurium* in the first 4 h after invasion of synoviocytes. CFU are shown on the vertical axis. Data from 5 representative patients are shown: black bars represent B27 positive SpA patients, shaded bars B27 negative control patients. The invasion profiles bear no consistent relationship to the B27 status of the synoviocytes.

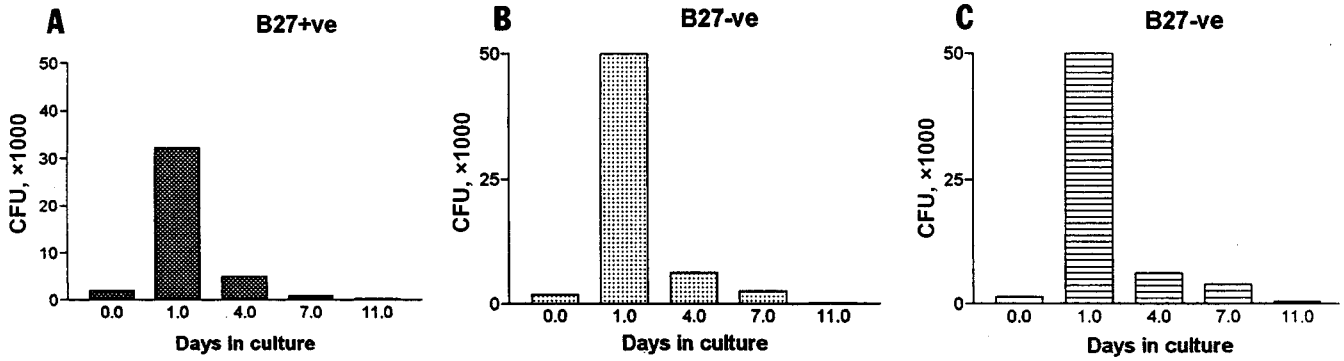

Figure 2. Kinetics of intracellular clearance of *S. typhimurium* from synoviocytes over 11 days in culture. Data from representative patients are depicted: the clearance profile of a B27 positive SpA patient (A) did not differ from that of B27 negative control patients (B, C).

doxical response pattern. This stimulatory pattern was observed in 3 of the 11 B27 positive patients. There was no clinical pattern of disease that discriminated these 3 patients with respect to clinical course.

Addition of exogenous interleukin 4 had no effect on clearance kinetics of *S. typhimurium* in either B27 positive or B27 negative cells (data not shown).

DISCUSSION

The pathogenesis of ReA is not resolved at present. There has been a recurrent notion that there may be an autoimmune basis for the chronic arthritis, but the identity of the autoantigen, if any, remains elusive. Invoking molecular mimicry as a pathway linking infection to autoimmunity has also been a persistent hypothesis, citing possible antigenic cross-reactivity of microbial determinants and host factors such as HLA-27 itself. We recently reviewed the evidence for molecular mimicry, and it is evident that there are a number of important unanswered questions in this theory[8]. The most prevalent hypothesis for the pathogenesis of ReA cites persisting microbial antigens in the joint as the culprits underlying the chronic aseptic synovitis. It has been recognized that immunofluorescence studies of synovial tissues in post-*Yersinia* and post-*Salmonella* ReA have revealed local persistence of the respective microbial antigens in the joint[3]. In the case of post-*Chlamydia* ReA, electron microscopic observation of Chlamydia structures, and more recently evidence by PCR for persisting intraarticular Chlamydia, has been reported[9]. This line of investigation has supported the notion that ReA may be indicative of defective host

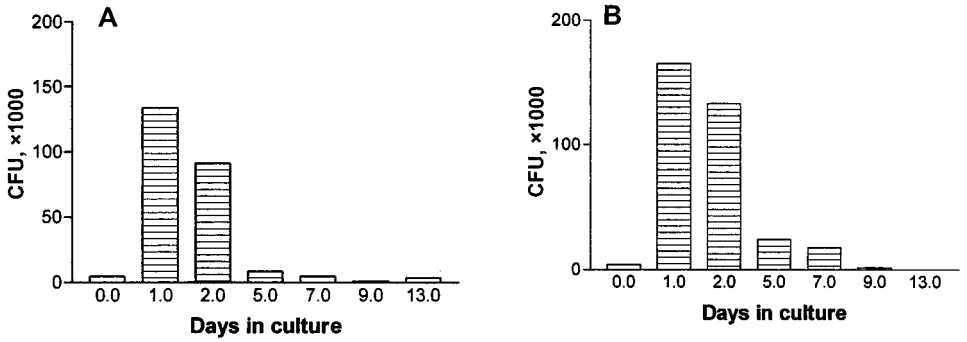

Figure 3. Clearance kinetics were stable within a patient. Data show intracellular clearance kinetics of *S. typhimurium* in synoviocytes obtained from joint aspirations on 2 different occasions from the same patient.

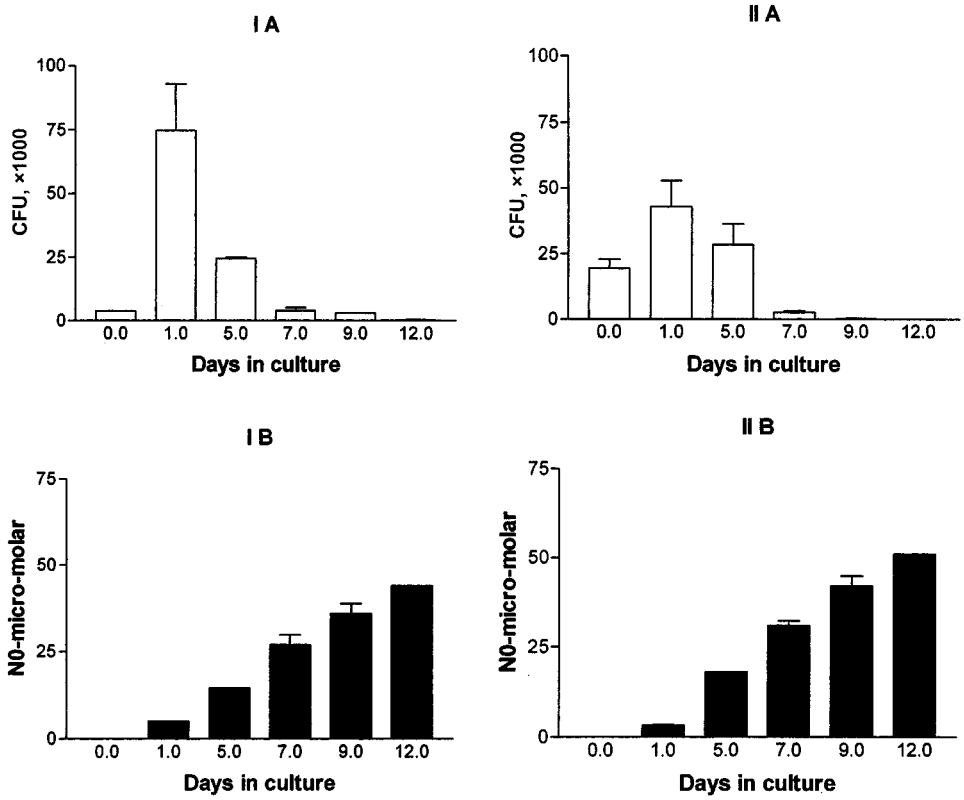

Figure 4. Relationship between NO production and intracellular clearance of *S. typhimurium* (I) and *Y. enterocolitica* (II). NO production (IB, IIB) rises over 12 days in culture as the organisms are cleared over the same time period (IA, IIA). Synoviocytes are from a B27 positive patient with SpA.

clearance of the pathogen, with persisting synovitis developing as a consequence.

Studies to investigate host-microbial interactions specifically addressed the possible contribution of HLA-B27 to altered host response to arthritogenic pathogens. The advantage of using transfected cells in these studies was that it allowed a specific analysis of the following question: since HLA-B27 is a genetic risk factor for ReA, and ReA itself reflects an alteration in host-pathogen interactions, perhaps B27 contributes in a direct way to this interaction. Using class I-transfected L cells we observed that surface expression of B27, in contrast to all other B locus alleles examined, was associated with a decrease in invasion of arthritogenic pathogens[10]. In the study of Huppertz, *et al*[11] invasion of B27-transfected L cells by *Salmonella* was decreased in comparison with non-transfected L cells[11]. Other investigators did not identify a B27-specific modulation of invasion in transfected target cells[12,13], and there are a number of methodological differences in these studies that may contribute to the heterogeneity in the findings[14]. It is evident, however, that this system was an artificial construct that may or may not accurately reflect the real biology occurring in the patient. For one thing, these cells were murine fibroblasts, with the repertoire of endogenous murine genes still intact. Second, almost all patients are heterozygous at the respective A, B, and C loci of class I

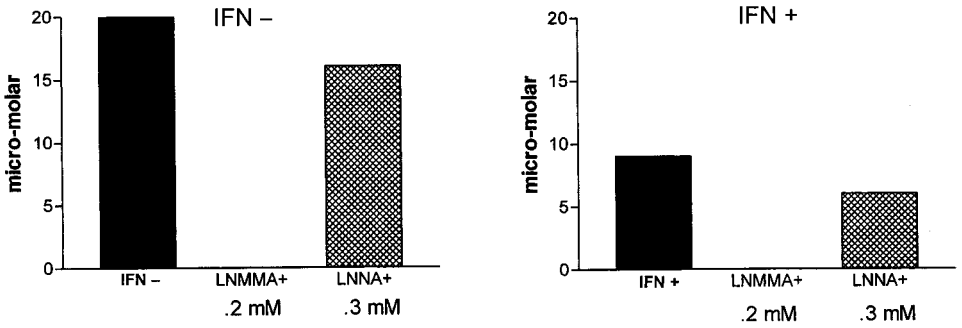

Figure 5a. LNMMA is a potent inhibitor of NO production by synoviocytes in the absence (IFN–) or the presence (IFN+) of IFN-γ, 1 ng/ml. In contrast, neither IFN-γ nor LNNA directly suppressed NO production. Data on synoviocytes at Day 8 post-invasion with *S. typhimurium*. Vertical axis depicts NO production in µM.

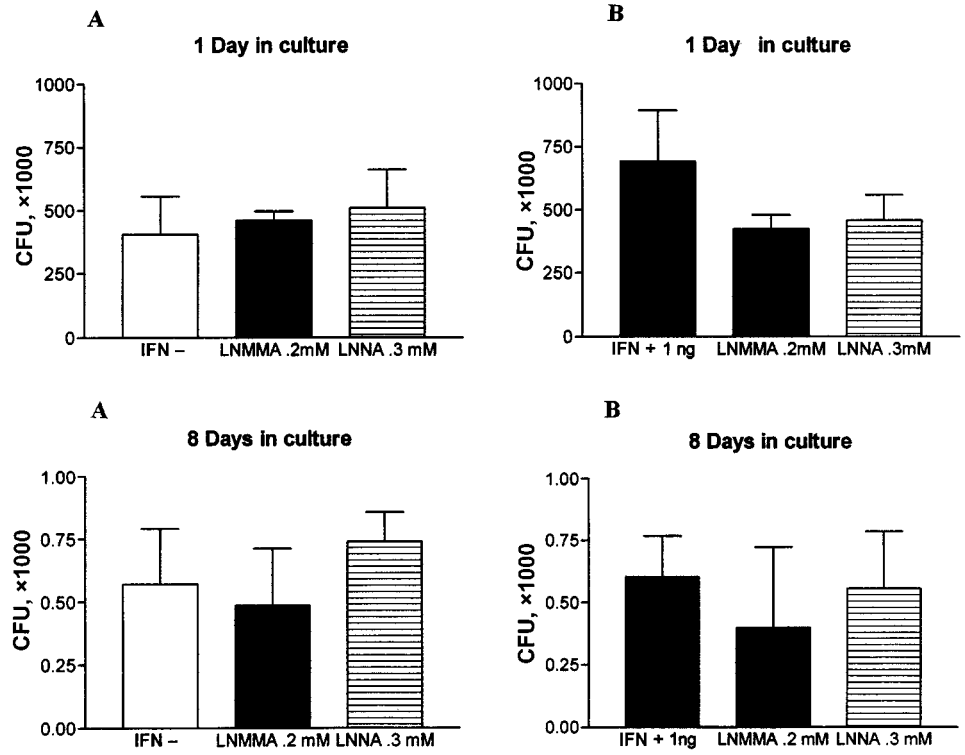

Figure 5b. LNMMA has no measurable effect on clearance kinetics of *S. typhimurium*, in either the absence of IFN-γ (A panels, left) or the presence of IFN-γ (B panels, right). Data are shown for Day 1 in culture and Day 8 in culture.

HLA genes, and the transfectant cell lines oversimplify any possible interaction of codominant expression of different class I alleles. Finally, the appropriate host cell to address these questions has not been fully resolved. We have recently shown in an experimental model for ReA that the synoviocyte is an appropriate host cell for invasion by arthritogenic pathogens, and that the synoviocyte can function as a reservoir of microbial antigens sufficient to sustain a chronic inflammatory response in the joint[4].

Ultrastructural studies document that both *Salmonella* and *Yersinia* can successfully invade synovial fibroblasts, replicate in the intracellular space, and result in the persistence of LPS-containing "ghosts" that could constitute a local source of arthritogenic antigens[15]. Primary human skin fibroblasts and human dendritic cells have both been shown to be suitable target cells for the analysis of invasion and intracellular clearance[16,17].

Using synoviocytes from inflamed joints of patients with SpA, the B27-related rheumatic disease in question has allowed us to avoid some of the issues of generalizability that accompanied studies using transfected cell lines. Using these native cells from the target organ, with no transfection or manipulation, we observed that the expression of B27 on the surface of target cells bore no direct relationship to the degree of invasion by arthritogenic pathogens. This, then, contrasts to some of the earlier studies using transfected L

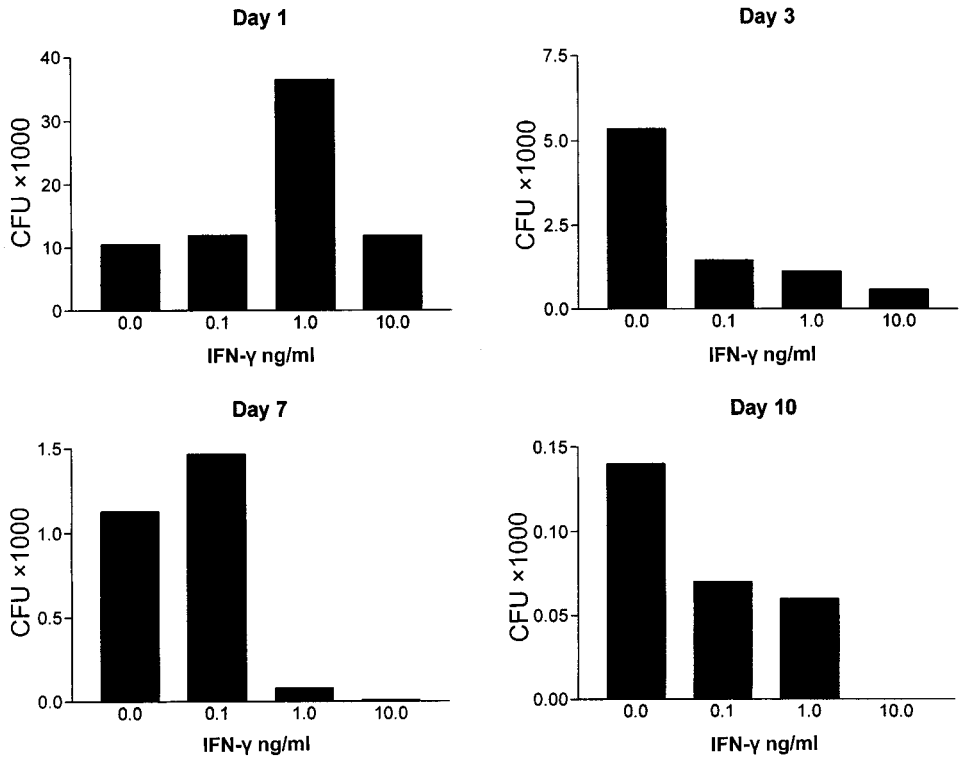

Figure 6. IFN-γ has a measurable but small suppressive effect on intracellular *S. typhimurium* when assessed at different time points post-invasion.

cells, but is in accord with studies using skin fibroblasts as target cells[11]. The additional advantage of studying cells from patients with ReA is that it is inclusive of gene effects in addition to HLA-B27, since genetic studies have pointed toward a polygenic process with additional genes acting in concert with HLA-B27.

We did not observe any relationship between the B27 status of the host cells and intracellular clearance kinetics. This is still an important issue, since it remains an attractive hypothesis that defective host clearance of arthritogenic organisms plays an important role in setting the stage for ReA following a GI or genitourinary infection. Using B27-transfected U937 cells, a human monocyte line, Laitio, *et al* observed an impaired clearance profile for those cells expressing B27 in contrast to HLA-A2-transfected control cells[18]. The basis for altered clearance of the pathogen in these tranfectants is not resolved. There appears to be no defect in NO production in the B27 positive U937 cells[19], in contrast to the findings in B27 positive L cells[12], which had suggested that a B27-related decrease in NO production may account for delayed microbial clearance in the transfected L cells. Recent studies further suggest that the clearance kinetics in the transfected U937 cells cannot be attributed to simple alteration in endogenous production of tumor necrosis factor-α or interleukin 10[20].

There are several technical aspects to the analysis of *in vitro* host-microbial interactions that are relevant to some of the discrepancies in the studies published in this area. The bacteria:cell ratio influences quantitative invasion assays, with a higher percentage invasion seen at the lower ratios and vice versa. Confluency and adherence may vary widely between transfected cell lines and primary synoviocyte cultures, although confluency has been maintained in all our assays and differential elutability of target cells bore no relationship to invasion data in our experience. Similarly, the number of cell passages and the time in culture for cells bore no relationship to the invasion profile.

Polyclonality of target cells may account for variability between laboratories, and systems that utilized clonal populations may represent a more homogeneous target population. Finally, the specific strain of microorganism influences the biology of host-pathogen interactions in a fundamental way, and varying bacteria in these studies may influence the variability in results.

ReA remains a paradigm for a rheumatic disease that reflects an interaction of host genetic susceptibility and infectious triggers, and may serve as a model for other diseases at this interface. While the identity of the arthritogenic pathogens can often be established in these patients, the genetic elements acting in concert with HLA-B27 remain to be characterized. Genes that influence host resistance to infection constitute an important focus of interest in these studies. Improved understanding of host-microbial interaction in the joint may point the way to important candidate genes in this search.

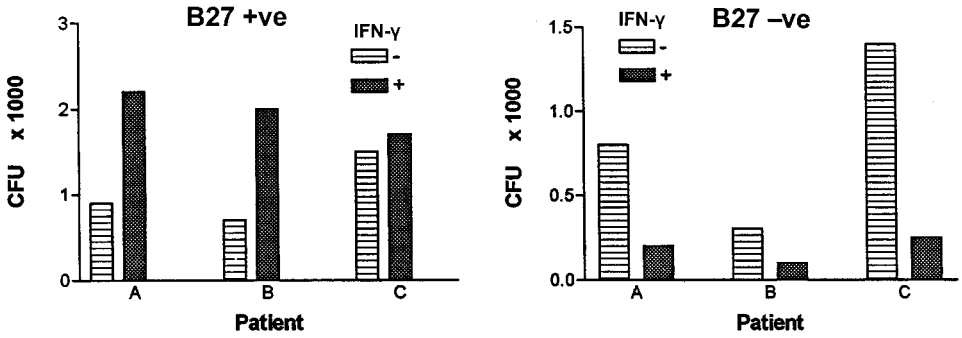

Figure 7a. Paradoxical stimulatory CFU response to IFN-γ (1 ng/ml) is seen in a B27 positive patient, but not observed in a B27 negative patient. Data presented at 6 days post-invasion with *S. typhimurium*.

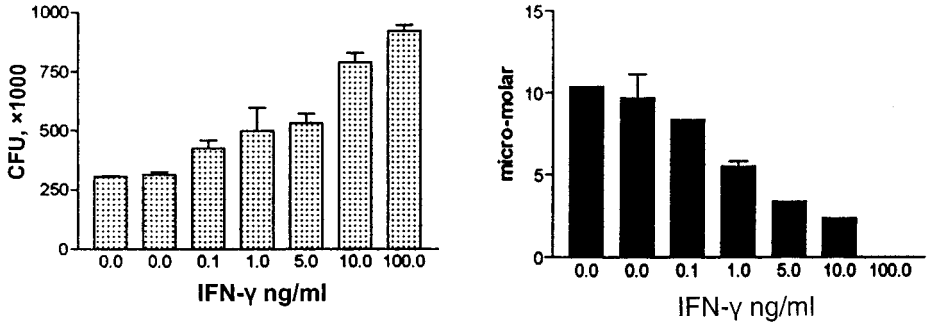

Figure 7b. IFN-γ-mediated suppression of NO production (vertical axis) seen in the stimulatory response subset. Data presented at Day 1 post-invasion with *S. typhimurium* in a B27 positive patient with SpA.

REFERENCES

1. Thomson GTD, Inman RD. Post-Salmonella dysentery reactive arthritis. Late sequelae in a point source cohort. Am J Med 1995;98:13-21.
2. Inman RD, Chiu B, Johnston MEA, Vas S, Falk J. HLA class I-mediated impairment in IL-2 production and lymphocyte response to microbial antigens in reactive arthritis. J Immunol 1989:142:4256-60.
3. Granfors KS, Jalkanen R, von Essen R, et al. Yersinia antigens in synovial fluid cells from patients with reactive arthritis. New Engl J Med 1989;320:216-21.
4. Inman RD, Chiu B. Synoviocyte-packaged Chlamydia trachomatis induces an aseptic chronic arthritis. J Clin Invest 1998; 102:1776-82.
5. Sieper J, Fendler C, Laitko S, et al. No benefit of long-term ciprofloxacin treatment in patients with reactive arthritis and undifferentiated oligoarthritis. Arthritis Rheum 1999;42:1386-96.
6. Dougados M, van der Linden S, Juhlin R, et al. The European Spondylarthropathy Study Group preliminary criteria for the classification of spondylarthropathy. Arthritis Rheum 1991;34:1218-27.
7. Kapasi K, Inman RD. HLA-B27 modulates invasion by gram-negative bacteria in transfected L cells. J Immunol 1992;148:3554-9.
8. Albert LJ, Inman RD. Molecular mimicry and autoimmunity. N Engl J Med 1999;341:2068-74.
9. Gerard HC, Branigan PJ, Schumacher HR, Hudson AP. Synovial Chlamydia trachomatis in patients with reactive arthritis/Reiter's syndrome are viable but show aberrant gene expression. J Rheumatol 1998;25:734-42.
10. Kapasi K, Inman RD. The ME1 epitope of HLA-B27 confers class I-mediated modulation of gram-negative bacterial invasion. J Immunol 1994;153:833-40.
11. Huppertz HI, Heesemann J. Invasion and persistence of Salmonella in human fibroblasts positive or negative for endogenous HLA-B27. Ann Rheum Dis 1997;56:671-6.
12. Virtala M, Kirveskari J, Granfors K. HLA-B27 modulates the survival of Salmonella enteritidis in transfected L cells, possibly by impaired nitric oxide production. Infect Immun 1997;65:4236-42.
13. Ortiz-Alvarez O, Yu DTY, Petty RE, Findlay BR. HLA-B27 does not affect invasion of arthritogenic bacteria into human cells. J Rheumatol 1998;25:1765-71.
14. Granfors K. Host-microbe interaction in reactive arthritis: does HLA-B27 have a direct effect? J Rheumatol 1998;25:1659-61.
15. Meyer-Bahlburg A, Brinkhoff J, Krenn V, Trebesius K, Heeseman J, Huppertz HI. Infection of synovial fibroblasts in culture by Yersinia enterocolitica and Salmonella enterica serovar enteritidis: ultrastructural investigation with respect to the pathogenesis of reactive arthritis. Infect Immun 2001;69:7915-21.
16. Huppertz HI, Heeseman J. The effect of cytokines on invasion and survival of Yersinia in primary human fibroblasts. Med Microbiol Immunol 1999;187:157-64.
17. Schoppet M, Bubert A, Huppertz HI. Dendritic cell function is perturbed by Yersinia enterocolitica infection in vitro. Clin Exp Immunol 2000;122:316-23.
18. Laitio P, Virtala M, Salmi M, Pelliniemi LJ, Yu DTY, Granfors K. HLA-B27 modulates intracellular survival of Salmonella enteritidis in human monocytic cells. Eur J Immunol 1997;27:1331-8.
19. Ekman P, Saarinen M, He Q, Virtala M, Salmi M, Granfors K. Human monocytic U937 cells kill Salmonella in vitro by NO-independent mechanisms. Infect Immun 1999;67:3670-3.
20. Ekman, P, Saarinen M, He Q, et al. HLA-B27-transfected (Salmonella permissive) and HLA-A2-transfected (Salmonella nonpermissive) human monocytic U937 cells differ in their production of cytokines. Infect Immun 2002;70:1609-14.