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Bacterial DNA is enriched in unmethylated CpG motifs that have been shown to activate the innate immune system. These immunostimulatory DNA sequences (ISS) induce inflammation when injected directly into joints. However, the role of bacterial DNA in systemic arthritis is not known. The purpose of the present experiments was to determine whether ISS contributes to the development of adjuvant arthritis in Lewis rats after intradermal injection of heat-killed Mycobacterium tuberculosis (Mt). The results showed that Mt DNA was necessary for maximal joint inflammation in adjuvant arthritis but could be replaced by synthetic ISS oligodeoxynucleotides. The arthritis-promoting effect of the Mt DNA or of the ISS oligodeoxynucleotides correlated with an increased Th1 response to Mt Ags, as measured by the production of IFN-γ and increased production of the osteoclast differentiation factor, receptor activator of NF-κB ligand (RANKL). The Mt DNA did not enter the joints but dispersed to the bone marrow and spleen before the onset of systemic joint inflammation. Thus, adjuvant arthritis is a microbial DNA-dependent disease. In this model, we postulate that massive and prolonged activation of macrophages, dendritic cells, and osteoclast precursors in the bone marrow may prime the joints for the induction of inflammatory Th1 immune responses to Mt Ags. The Journal of Immunology, 2002, 168: 51–56.

Mycobacterial DNA, compared with mammalian DNA, is enriched in palindromic sequences containing unmethylated CpG dinucleotides that can activate the innate immune system (1). Such immunostimulatory DNA sequences (ISS)4 stimulate the expression of costimulatory molecules (2) and the production of cytokines such as IL-12, TNF-α, and IFN by macrophages, dendritic cells, B lymphocytes, and NK cells (1). Consequently to this effect on innate immunity, ISS skew adaptive immune responses toward a strong and prolonged Th1 type of immunity (3, 4).

Recently, Tarkowski and coworkers (5) were able to link joint damage in a model of septic arthritis to the presence of ISS. They showed that direct injection of bacterial DNA or oligodeoxynucleotides containing ISS into the joints led to arthritis, whereas injection of mammalian DNA did not induce inflammation. An influx of monocytes and macrophages, and only a minority of CD4-positive T lymphocytes, characterized the resulting arthritis. Locally, in the arthritic joint, an increased expression of mRNA was found for TNF-α, IL-1α, and IL-12, and for the chemokines RANTES and monocyte chemoattractant protein-1 (6). Altogether, these results indicated that bacterial DNA was sufficient to provoke joint inflammation in septic arthritis, and raised the question as to whether ISS might also play a role in the pathogenesis of other forms of arthritis (6). To explore this issue, we turned to a T cell-mediated model of experimental autoimmune arthritis, namely adjuvant arthritis (AA). AA is an extensively studied form of chronic arthritis with a close histopathological resemblance to rheumatoid arthritis (7, 8). AA can be induced in susceptible animals such as Lewis rats by injection of heat-killed Mycobacterium tuberculosis (Mt) in IFA in the base of the tail. Because the immunostimulatory properties of bacterial DNA were first discovered in Mt (9), we reasoned that ISS might play a role in this model of autoimmune arthritis. In this study, we show that, indeed, ISS are crucial for the induction of maximal joint inflammation in AA and strongly stimulate both a Th1 response and the production of the osteoclast differentiating factor, receptor activator of NF-κB ligand (RANKL), to mycobacterial heat shock protein 65 (hsp65). Surprisingly, days after immunization with heat-killed Mt, residual mycobacterial DNA was detected in the spleen and bone marrow, but not the synovium, of arthritic rats.

Materials and Methods

Animals

Male inbred Lewis rats (RT1.B1) of 6–9 wk of age and female BALB/c mice of 6–8 wk of age were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME), respectively. All animals were maintained in the University of California at San Diego Animal Facility (La Jolla, CA), which is accredited by the American Association for the Accreditation of Laboratory Animal Care. All experiments were approved by the Animal Subjects Committee in accordance with U.S. Department of Agriculture guidelines.
Reagents
Heat-killed Mtb (strain H37Ra) and IFA were obtained from Difco (Detroit, MI), Hsp65 and β-galactosidase (β-gal) were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada) and Sigma-Aldrich (St. Louis, MO), respectively. Phosphorothioate ISS-containing oligodeoxynucleotide (ISS-ODN) and control ODN were purchased from Trilink Biotechnologies (San Diego, CA). The sequence of the ISS-ODN was 5′-TGAGCTGAGCGGCTCAGGAT-3′ and the sequence of the control ODN was 5′-TGAGCTGAGTTGATAGAGATG-3′. Endotoxin levels of the ISS-ODN and control ODN were below the limit of detection (<1 ng/mg DNA) as measured by the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Induction and clinical assessment of experimental arthritis
AA was induced by a single intradermal (i.d.) injection at the base of the tail with 0.3 mg of Mtb suspended in 100 μl of IFA (CFA). Rats were examined daily for clinical signs of arthritis in a blinded fashion. Severity of arthritis was assessed by weight loss and scoring each paw from zero to four based on degree of swelling, erythema, and deformation of the joints (maximum score, 16).

DNase treatment of Mtb
Mtb was incubated overnight at 37°C with 5 μg/ml DNase I (Sigma-Aldrich) in the presence of 5 mM MgCl₂. After extraction, DNA depletion and frozen at −80°C, the bone marrow of the tibia, synovium, kidney, and liver was harvested (maximum score, 16). The seminested PCR assay consisted of two rounds with each reaction performed as per AdvanTaq Plus PCR kit instructions. The amplification parameters included 20 cycles each of denaturation at 94°C for 30 s, followed by a single annealing and extension step at 72°C for 30 s.

Histology
Excised rat joints were stained with H&E. A synovial histology score was determined on the stained sections using a semiquantitative scale that measures synovial inflammation (0–4), bone and cartilage erosions (0–4), marrow infiltration (0–4), and extra-articular inflammation (0–4) (maximum score, 16).

Results
Characterization of Mtb DNA treated with DNase
Mtb extract was treated with DNase at 37°C overnight. The digest product, untreated Mtb DNA, and a 10-bp ladder standard were electrophoresed on a 20% polyacrylamide gel and stained with cyver green. The DNase treatment was complete, as no digest products were observed (<10 bp, data not shown).

DNase treatment inhibits the Th1-skewing properties of CFA
CFA has been reported to polarize immune responses to coadministered Ags toward a Th1 type. Before turning to the model of AA, we questioned whether it is possible to abolish the Th1-skewing capacities of CFA by DNase treatment and subsequently restore it with oligonucleotides containing ISS sequences. Heat-killed Mtb was treated with DNase and subsequently suspended in IFA to make DNase-CFA. To test immunogenicity of DNase-CFA in vivo, we immunized BALB/c mice with β-gal and the following adjuvants: IFA, CFA, DNase-CFA, DNase-ODN, DNase-CFA mixed with ISS-ODN, and IFA mixed with ISS-ODN. The immunization consisted of a single i.d. injection of β-gal emulsified with one of the mentioned adjuvants. Four weeks later, blood and spleens were harvested. The Ab isotype responses to β-gal were assessed (Fig. 1, upper panel). Compared with the Ag-CFA combination, mice immunized with β-gal and DNase-treated CFA had reduced IgG2a levels (p = 0.05). However, when DNase-treated CFA was mixed with ISS-ODN, anti-β-gal IgG2a production was restored (p < 0.0001).
In addition, we measured the Ag-specific production of IFN-\(\gamma\) and IL-5 by splenocytes after immunization with \(\beta\)-gal and the different adjuvants. The results mimicked the Ab response (Fig. 1, lower panel). Mice immunized with \(\beta\)-gal and DNase-treated CFA showed reduced \(\beta\)-gal-specific IFN-\(\gamma\)-levels, compared with the CFA-positive control (\(p = 0.08\)). In contrast, after immunization with \(\beta\)-gal and DNase-treated CFA and ISS-ODN, a strong increase in IFN-\(\gamma\)-production was found, in comparison with \(\beta\)-gal DNase-treated CFA alone or with control ODN (\(p < 0.02\)), indicative of a Th1-like response. A similar response was induced when \(\beta\)-gal was coadministered with IFA mixed with ISS-ODN, whereas \(\beta\)-gal coadministered with DNase-CFA and control ODN did not lead to increased production of IFN-\(\gamma\) (\(p < 0.03\)). Thus, DNase treatment of Mtb reduced its Th1-polarizing adjuvant effect, which could be completely restored with ISS-ODN alone.

**ISS determine the severity of AA**

Next, we determined whether the presence of Mtb DNA is necessary to induce AA. We first treated heat-killed Mtb with DNase until no high-m.w. DNA was detectable by electrophoresis and ethidium bromide staining. We injected Lewis rats with either emulsified heat-killed Mtb, DNase-treated Mtb, DNase-treated Mtb supplemented with ISS-ODN (3, 10, 30, and 100 \(\mu\)g), or DNase-treated Mtb with a control ODN. The results are shown in Fig. 2. DNase treatment of Mtb led to a delay in the onset and a marked reduction in the severity of arthritis (mean maximum arthritis score, 5) compared with the positive control (mean maximum arthritis score, 12; \(p < 0.0001\)). The addition of ISS-ODN (100 \(\mu\)g) to DNase-treated Mtb restored the severity of arthritis completely (mean maximum arthritis score, 12), whereas the addition of control ODN was devoid of efficacy (\(p < 0.0001\)). The clinical findings were also reflected in the histological scores (Fig. 3). These experiments showed that the severity of joint inflammation in AA depends on the presence of Mtb DNA.

Systemic immunization with ISS, mixed with IFA alone, was not sufficient to induce arthritis in Lewis rats. Rats immunized with ISS and IFA showed normal weight curves (data not shown) and did not display signs of arthritis up until 60 days after immunization (Figs. 2 and 3). These results suggest that mycobacterial Ags, or other factors besides DNA, also are required for arthritis induction.

**Immunostimulatory DNA induces Th1 responses and the production of soluble RANKL to Mtbg Ags**

To determine the effects of Mtb DNA on Ag-specific cytokine synthesis, we cultured ILN at day 55 after arthritis induction with medium alone, Mtb, DNase-treated Mtb, or purified Mtbg hsp65. After a 72-h culture, supernatants were collected, and IFN-\(\gamma\) and RANKL levels were assayed. Fig. 4 illustrates the results of hsp65 stimulation. Similar results were found with Mtb extracts (data not shown). Lymph node cells from rats immunized with whole heat-killed Mtb produced IFN-\(\gamma\) and soluble RANKL after in vitro activation with hsp65. In contrast, lymph node cells from rats immunized with DNase-treated Mtb produced significantly less IFN-\(\gamma\) and RANKL in vitro stimulation with hsp65 (\(p < 0.05\)). However, lymph node cells from rats immunized with DNase-treated Mtb produced significantly less IFN-\(\gamma\) and RANKL in vitro restimulation with hsp65 (\(p < 0.05\)). Thus, primed lymph node cells from rats immunized with DNase-treated Mtb, mixed with a control ODN, produced IFN-\(\gamma\) and RANKL at levels comparable with those produced by cells from rats immunized with DNase-treated Mtb, which had been supplemented with ISS-ODN, produced high levels of IFN-\(\gamma\) and RANKL after in vitro activation with hsp65, whereas lymph node cells from rats immunized with DNase-treated Mtb, mixed with a control ODN, produced IFN-\(\gamma\) and RANKL at levels comparable with those produced by cells from rats immunized with DNase-treated Mtb (\(p < 0.05\), DNase-CFA/ISS-ODN vs DNase-CFA/control ODN). We could not detect Ag-specific IFN-\(\gamma\) and RANKL production in naive rats. Other experiments demonstrated no significant production of IL-4 or IL-10 in any of the lymph node cultures. Thus, primed lymph node cells from rats immunized with a mixture containing ISS (either Mtb DNA or ISS-ODN) produced significantly higher levels of IFN-\(\gamma\) and RANKL, compared with primed lymph node cells from rats immunized with Mtb without ISS. The levels of IFN-\(\gamma\) and RANKL production by Ag-restimulated lymph node cells correlated with the severity of arthritis found in the different treatment groups.

**FIGURE 1.** Th1-skewing capacities of CFA are dependent on the presence of ISS. BALB/c mice were immunized with \(\beta\)-gal alone or mixed with the following adjuvants: IFA, CFA, DNase-treated CFA, DNase-treated CFA mixed with ISS, DNase-treated CFA mixed with control ODN, and IFA mixed with ISS-ODN. Four weeks later, blood and splenocytes were harvested to assess the anti-\(\beta\)-gal IgG2a (A), anti-\(\beta\)-gal IgG1 (B), anti-\(\beta\)-gal IFN-\(\gamma\) (C), and anti-\(\beta\)-gal IL-5 (D) response. Results are the means \(\pm\) SEM for four mice per group. Cont., Control.

**FIGURE 2.** ISS present in CFA determine the clinical severity of AA. A, Lewis rats were immunized with CFA, CFA containing DNase-treated Mtb, CFA containing DNase-treated Mtb mixed with ISS-ODN (100 \(\mu\)g), CFA containing DNase-treated Mtb mixed with control ODN, or IFA mixed with ISS. The ordinate shows the mean arthritis scores (\(\pm\) SEM) at the indicated times after injection (\(n = 16\) for all groups except for CFA/DNase/control ODN (\(n = 6\), IFA/ISS-ODN (\(n = 4\)), and naive (\(n = 8\)). Results are means \(\pm\) SE. CFA vs DNase, \(p < 0.0001\); CFA/DNase vs CFA/DNase plus ISS, \(p < 0.0001\); CFA/DNase plus ISS vs CFA/DNase plus control, \(p < 0.0001\); and CFA/DNase plus ISS vs CFA, \(p = 0.038\). B, Dose response. Three doses of ISS-ODN (3, 10, and 30 \(\mu\)g) were tested (\(n = 5\) per group). There was no significant difference in the arthritis scores between CFA vs CFA/DNase/ISS-ODN (3, 10, and 30 \(\mu\)g) at day 22.
Disposal of Mtb DNA

Because intra-articular injections of ISS induce joint inflammation, it was important to determine whether Mtb DNA was present in the joints. At 1, 3, 7, 10, 14, 17, 21, 29, and 36 days after injection of Mtb into the tails of Lewis rats, PCR analyses were performed on tissue samples taken from kidney, liver, spleen, bone marrow, base of the tail, inguinal lymph node, and synovium (Fig. 5). Mycobacterial DNA was detected at the site of injection, in the spleen up until day 36, in the draining (inguinal) lymph nodes at day 10, and in the bone marrow at days 3, 7, 10, and 14. No Mtb DNA was detected in the liver, kidney, or synovium. Thus, after immunization with CFA, mycobacterial DNA disperses to bone marrow and lymphoid tissues, but not to the synovium. Hence, the arthritogenic effects of Mtb cannot be attributed to a local effect on the synovium, as was the case in the model of septic arthritis (5).

We then explored the presence of ISS-ODN in the affected synovial tissues (ankles). Rats were injected with CFA/DNase/ISS-ODN (100 μg) and killed at days 1 and 14 (two rats per time point). DNA was extracted from the injected area (base of the tail) and from the inflamed ankles, and was subjected to Southern blot analysis using 32P-labeled complementary ODN as a probe. As shown in Fig. 5B, ISS-ODN was detected in the area of injection but not in the affected synovium.
Discussion

Our experiments show that the severity of AA in rats depends upon the presence of ISS in heat-killed Mtb DNA. The ISS polarize the immune response to mycobacterial Ags toward a Th1 phenotype. Indeed, the established Th1-stimulatory properties of CFA in mice are largely attributable to Mtb DNA. In AA, the Th1 response to Mtb Ags correlates with disease intensity. However, the combination of ISS and IFN-γ is not sufficient to induce arthritis. Collectively, the data suggest that ISS amplifies the course of arthritis by massive and prolonged activation of the innate immune system, especially in the bone marrow. This, in turn, may stimulate adaptive Th1 responses to various Mtb Ags (e.g., hsp65).

Once established, AA is clearly a T cell-mediated process, because the passive transfer of CD4+ T cells from affected rats to irradiated syngeneic recipients induces disease, whereas serum transfer has no deleterious effects (10). Moreover, arthritis can be triggered by a cross-reactive CD4 T cell clone that recognizes an epitope present on bacterial heat shock proteins and cartilage proteoglycans (11, 12). However, the early induction of AA requires stimulation of innate immunity, as indicated by the ability of cyclooxygenase inhibitors, TNF-α antagonists, and chemokine blockers to prevent disease (13–15).

One way to explain these data is to propose that the activation of innate immunity by ISS primes the joints for the subsequent development of a Th1 autoimmune response. In this context, Sato et al. (16) recently reported that the prior injection of ISS-ODN into mice exacerbated the severity of arthritis induced by immunization with collagen in CFA. Unlike most other bacterial products that activate innate immunity, ISS are poorly immunogenic and can persist for long periods within transfected cells. Tarkowski et al. (5) found that the direct injection of bacterial DNA into the articular cavity of rats induced severe inflammation. Both Ag-Ab complexes and systemically administered Ags have been shown to traffic to the joints. Thus, the systemic dispersal of Mtb DNA, and its subsequent trapping in the synovium, could explain the potentiating effects of ISS in AA. However, from 1–36 days after injection of heat-killed Mtb in the tail, we were unable to detect Mtb DNA sequences or ISS-ODNs within the joints. Instead, the DNA dispersed primarily to the bone marrow, the lymph nodes, and the spleen.

The activation of innate immune responses within the bone marrow may be particularly relevant to the induction of arthritis. In the collagen arthritis model, which requires Freund’s adjuvant, the normally small channels between the bone marrow and synovial cavity enlarge before the onset of joint swelling (17). The resorption of subchondral bone is an established early event in AA. Bone resorption requires the activation of osteoclast precursors within the marrow. The osteoclast activation, in turn, depends upon the increased production of RANKL (osteoprotegerin ligand/TNF-related apoptosis-inducing ligand) by T lymphocytes. Kong et al. (18) discovered that inhibition of RANKL signaling prevented bone and cartilage destruction in the AA model, although leukocyte infiltration into the joints was still present. Our experiments showed that increased RANKL production by T cells is part and parcel of the immune system activation induced by ISS and Ag.

The deposition of Mtb DNA in the bone marrow not only argues against the direct involvement of Mtb DNA in the pathogenesis of AA, but also suggests that other Mtb-related compounds (e.g., hsp65) could follow the same course. Activation of T cells in the bone marrow by Mtb-related Ags might induce the local secretion of RANKL, which activates osteoclasts and thus makes the joint permeable and more susceptible to the influx of inflammatory cells and the subsequent development of arthritis.

Recently, there has been a revival of interest in the role of innate immunity in the pathogenesis of human rheumatoid arthritis and other inflammatory forms of arthritis of unknown etiology. The products of activated macrophages are abundant in inflammatory synovial fluids. Both bacterial DNA and peptidoglycans have been detected in the joints (19, 20). Th1-type responses to bacterial heat shock proteins are readily measurable in synovial fluid T lymphocytes, whereas responses to other Ags are often weak or absent (21, 22). Moreover, endogenous hsp65, as expressed in inflamed synovial tissue (23), can trigger innate immune responses through the LPS receptor Toll-like receptor (TLR)4 and CD14 (24).

The nine known TLRs regulate the response of the innate immune system to bacterial products (25). Knockout experiments have demonstrated that the murine response to ISS-ODN requires TLR9 (26). In addition to stimulation through TLR9, various Mtb products such as lipopeptide or hsp65 also can interact with TLR2 and TLR4, respectively (25, 27). Thus, the activation of innate immunity by Mtb is multifactorial. Consistent with this interpretation, DNA digestion of mycobacteria did not totally abolish its arthritis-promoting properties. Future experiments will need to determine the role of different TLRs in the human response to microbial DNA, the expression of TLRs in cells from the bone marrow and joints, the fate of bone marrow-derived macrophages exposed to bacterial products, and the interactions between the TLRs and various cytokines.

In general, phosphothioate ODNs are much more potent than phosphodiester ODN or intact DNA. Thus, the lower efficacy of ISS-ODN to induce AA could suggest that intact Mtb DNA is much more effective than the comparable amount of ISS-ODN. This could reflect some intrinsic property of Mtb. Alternatively, the DNase treatment may have destroyed some other factor that plays a proinflammatory role in this system.

Bacterial DNA and ISS-ODN are much more potent activators of the innate and adaptive immune system in rodents, compared with primates (1). However, various ILs have been demonstrated to synergize with ISS in the activation of human NK cells (28). The confined synovial spaces of patients with inflammatory arthritis can act as a sink for cytokines and other inflammatory mediators. In this setting, bone marrow-derived macrophages, lymphocytes, and NK cells could be more readily stimulated by bacterial DNA. Thus, although ISS are unlikely to be a direct cause of rheumatoid arthritis, they could play a significant role in disease progression and severity. Therefore, inhibitors of ISS binding to TLR9 or of ISS-induced signal transduction might be useful for the treatment of affected patients.

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