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Immunostimulatory DNA Sequences Influence the Course of Adjuvant Arthritis

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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.

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.
散热条件下的Mtb（strain H37Ra）和IFA都被来自Difco（Detroit, MI）的Hsp65和β-galactosidase (β-gal) 被来自Stressgen Biotechnologies (Victoria, British Columbia, Canada) 和Sigma-Aldrich (St. Louis, MO), 分别。通过将含有oligodeoxynucleotide (ISS-ODN) 和control ODN都被从Trilink Biotechnologies (San Diego, CA) 购得。序列为的ISS-ODN是5’-TGAAGTGGAGACCCCGTTCGATG-3’和序列控制ODN是5’-TGAAGTGGAGACCCCGTTCGATG-3’。Endotoxin levels of the ISS-ODN and control ODN were below the limit of detection (<1 ng/mg DNA) 作为Limulus amoebocyte lysis 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 1 (Sigma-Aldrich) in the presence of 5 mM MgCl₂. After extraction, DNA depletion was confirmed by agarose gel electrophoresis and ethidium bromide staining. Mtb (0.3 mg) was suspended in 100 μl of IFA (DNase/CFA).

Immunization protocols

Rats received a single i.d. injection with either Mtb, DNase-treated Mtb, or DNase-treated Mtb supplemented with either ISS-ODN (3, 10, 30, and 100 μg) or control-ODN (100 μg) in a final volume of 100 μl of IFA. A total of 3 μg of ISS corresponds to the estimated concentration of DNA in 0.3 mg of heat-killed Mtb. The animals were examined daily for clinical signs of arthritis, as described above. Animals injected with ISS-ODN (100 μg) suspended in 100 μl of IFA served as a control.

Mice received a single i.d. injection of β-gal (10 μg) emulsified with either Mtb (50 μg), DNase-treated Mtb (50 μg), or DNase-treated Mtb supplemented with either ISS-ODN (10 μg) or control ODN (10 μg) in a final volume of 50 μl of IFA. Injections of β-gal alone or β-gal with either IFA or IFA/ISS-ODN were used as controls. Four weeks after injection, mice were bled and spleens were harvested for cytokine assays.

Ab and cytokine assays

Mouse β-gal-specific IgG2a and IgG1 Abs were measured by ELISA and are expressed in relative units, as previously described (3). Ag-specific mouse splenocyte cytokine profiles also were assessed by ELISA, as described earlier (3). Mouse spleens were harvested 4 wk after injection, teased to single cell suspensions, and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS. Splenocytes were incubated at 5 × 10⁶ cells/well in 96-well plates in a final volume of 200 μl of complete medium with β-gal added at 10 μg/ml at 37°C and 5% CO₂. Culture supernatants were harvested at 72 h and analyzed for IL-5 and IFN-γ (BD PharMingen, San Diego, CA) by ELISA, according to the manufacturer’s directions.

Rat inguinal lymph node cells (ILN) were harvested for Ag-specific IFN-γ and RANKL assessment 55 days after induction of AA. Briefly, ILN were strained through a 70-μm nylon cell strainer (BD Biosciences, Mountain View, CA) and resuspended at 1 × 10⁶ cells/ml in 96-well plates in a final volume of 200 μl of IMDM supplemented with 10% heat-inactivated FCS. ILN were cultured for 72 h with hsp65 (10 μg/ml) and analyzed by ELISA, as per kit instructions. IFN-γ was obtained from Biosource International, Camarillo, CA; RANKL was obtained from R&D Systems, Minneapolis, MN. The Ab against mouse RANKL cross-reacted with the rat protein.

Detection of Mtb DNA in rat tissues

Mtb in various rat tissues was detected by PCR. Two reactions for each organ for each time point were performed for each animal (n = 4). Lewis rats were sacrificed 1, 3, 7, 10, 14, 17, 21, 29, and 36 days after receiving a single i.d. injection of 0.3 mg of Mtb in IFA. For each time point, approximately 50 mm³ of tail base (site of injection), spleen, inguinal lymph node, bone marrow of the tibia, synovium, kidney, and liver was harvested and frozen at −80°C until analyzed. Samples were boiled for 10 min in 0.5 ml of pyrogen-free sterile water to lyse cells and release DNA.

The seminested PCR assay consisted of two rounds with each reaction (total volume, 30 μl) performed as per AdvanTaq Plus PCR kit instructions (Clontech Laboratories, Palo Alto, CA). The template for the first PCR round consisted of 1 μl of a 1/20 dilution of DNA extract with a 0.3 μM concentration of forward primer (IDT, San Diego, CA) 5’-GATTCCTGC (GAGCCGTAGCGGTGCCTGAG-3’) and reverse primer 5’-GATCTCG GCCGCGCGTTCGGCGGAC-AC3’) (6). This primer pair was designed to amplify a 123-bp region of a Mtb multiplex insertion sequence (Is6110). 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. A second round of PCR amplification used the forward primer and an internal primer 5’-GCCATTCAGCCTGGTGCTGAAG-3’ with identical conditions and one-thirtieth of the amount of the first PCR product as a template. The second round of amplification consisted of 27 cycles and yielded a 100-bp product. All samples were compared against the control gene actin, with an amplification procedure of 35 cycles with identical conditions for each cycle. The PCR products were analyzed by ethidium bromide staining and viewed on a UV transilluminator.

Dot-blot hybridization

Nucleic acids were extracted from the injected areas (tail base) and from affected synovial tissues (ankle) by boiling in 1 ml of Tris-EDTA buffer at 100°C for 10 min. Solid materials were removed by centrifugation at 14,000 × g for 10 min, and 1 μl from each supernatant was loaded on a nylon membrane (NEN Research Products, Arlington Heights, IL). After fixation of the nucleic acids onto the membrane with 0.2 M NaOH, the filter was hybridized with 32P-labeled oligonucleotide complementary to the ISS-ODN (5’-TGACTGTGGAACGTTCGAGATG-3’), washed with 2X SSC containing 0.1% SDS three times, and exposed to x-ray film (Kodak X-Omat Blue X-B1; Kodak, Rochester, NY).

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).

Statistics

Two-tailed unpaired Student t tests were used to compare Ab levels, cytokine levels, clinical arthritis scores, and histology scores using StatView (SAS Institute, Cary, NC) and Mathsoft computer software (Mathsoft, Cambridge, MA).

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 cybr 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, CFA-DNase mixed with ISS-ODN, DNase-CFA mixed with control ODN, and CFA 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 later, blood and spleens were harvested. The Ab isotype responses...
In addition, we measured the Ag-specific production of IFN-γ and IL-5 by splenocytes after immunization with β-gal and the different adjuvants. The results mimicked the Ab data (Fig. 1, lower panel). Mice immunized with β-gal and DNase-treated CFA showed reduced β-gal-specific IFN-γ levels, compared with the CFA-positive control (p = 0.08). In contrast, after immunization with β-gal and DNase-treated CFA and ISS-ODN, a strong increase in IFN-γ production was found, in comparison with β-gal DNase-treated CFA alone or with control ODN (p < 0.02), indicative of a Th1-like response. A similar response was induced when β-gal was coadministered with IFA mixed with ISS-ODN, whereas β-gal coadministered with DNase-CFA and control ODN did not lead to increased production of IFN-γ (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 μg), 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 μg) 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 Mtb 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 Mtb hsp65. After a 72-h culture, supernatants were collected, and IFN-γ 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-γ 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-γ and RANKL after in vitro restimulation with hsp65 (p < 0.05). However, lymph node cells from rats immunized with DNase-treated Mtb produced significantly less IFN-γ 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-γ 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-γ 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-γ 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-γ and RANKL, compared with primed lymph node cells from rats immunized with Mtb without ISS. The levels of IFN-γ and RANKL production by Ag-restimulated lymph node cells correlated with the severity of arthritis found in the different treatment groups.
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 and 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.

FIGURE 5. Trafficking of mycobacterial DNA following arthritis induction. A, PCR analysis. Rats were immunized with Mtb in oil to induce arthritis. At days 1, 3, 7, 10, 14, 17, 21, 29, and 36 following immunization, tissue samples were taken from the base of the tail, spleen, inguinal lymph nodes, bone marrow of tibia, synovium, kidney, and liver to detect the presence of Mtb DNA using a seminested PCR. Actin was used as a control for each tissue. Note that Mtb DNA could be detected after immunization at the site of injection and in the spleen until day 36. Mtb DNA in the inguinal lymph nodes was present 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. Results shown here were reproduced in four rats per organ per time point. B, Southern blot analysis. Rats (n = 2) were injected with CFA/DNase/ISS-ODN (100 μg) and killed at days 1 and 14. Southern blot analysis was performed on DNA samples extracted from the injected area (base of the tail) and from the inflamed ankles using 32P-labeled complementary ODN as a probe. ISS-ODN was detected only in the area of injection but not in the affected ankle synovium. Data represent one experiment of two, which yielded the same results. TB, Tail base; RA, right ankle; LA, left ankle.
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 IFA 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 syngenic 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, phosphorothioate ODN 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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