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Arthritogenic Properties of Double-Stranded (Viral) RNA 1

Fariba Zare, 2,* Maria Bokarewa, 2 Nancy Nenonen, † Thomas Bergström, † Lena Alexopoulou, ‡ Richard A. Flavell, ‡§ and Andrej Tarkowski*

Viral infections often lead to arthralgias and overt arthritic states. The inflammagenic compound of the viruses giving rise to such an outcome has to date not been identified. Because expression of dsRNA is a common feature of all viruses, we decided to analyze whether this property leads to the induction of arthritis. Histological signs of arthritis were evident already on day 3 following intra-articular administration of dsRNA. Arthritis was characterized by infiltration of macrophages into synovial tissue. It was not dependent on acquired immune responses because SCID mice also raised joint inflammation. NF-κB was activated upon in vitro exposure to dsRNA, indicating its role in the induction/progression of arthritis. Importantly, we found that dsRNA arthritis was triggered through IL-1R signaling because mice being deficient for this molecule were unable to develop joint inflammation. Although dsRNA is typically recognized by Toll-like receptor 3, Toll-like receptor 3 knockout mice developed arthritis, indicating that some other receptors are instrumental in the inducing of inflammation. Our results from in vitro experiments indicate that proinflammatory cytokines and chemokines stimulating monocyte influx were readily triggered in response to stimulation with dsRNA. These findings demonstrate that viral dsRNA is clearly arthritogenic. Importantly, macrophages and their products play an important role in the development of arthritis triggered by dsRNA. The Journal of Immunology, 2004, 172: 5656–5663.

Various viruses have been implicated in the pathogenesis of reactive arthritis (1). In addition, participation of various viruses in the etiology of rheumatoid arthritis has been suggested (1). However, the nature of the inflammagenic stimuli in the induction of inflammation by viruses has not been identified.

The existence of viral infection is signaled by the production of dsRNA, which is formed during replication and transcription of all viruses. In contrast, dsRNA is normally not found in uninfected host cells (2). Viral infection often results in the destruction of infected cells, which is mediated either by virus itself or by immune responses. Intracellular mechanisms that inhibit virus replication and enable viral clearance and cell survival seem to exist (3, 4). Most viral infections are associated with strong Th1 immune responses (5). This usually results in the production of different cytokines (TNF-α, IFNs, and IL-6) and chemokines (macrophage-inflammagenic protein-1α (MIP-1α) 3 and monocyte chemotactic protein-1 (MCP-1)) activating innate immune responses (6). In clinical setting, it leads to arthralgias and overt arthritic states. The inflammagenic compound of the viruses giving rise to such an outcome has not been identified.

Infection by virus giving rise to endogenous exposure to dsRNA results in recognition of this molecule by Toll-like receptor 3 (TLR3) (6, 7). Ligation of TLR3 leads to the activation of various transcription factors including NF-κB (8–11). Such an activation results in production of proinflammatory cytokines from macrophages through a signaling pathway dependent on an adaptor protein called MyD88 (6).

In the present study, we investigated the possible role of viral dsRNA in arthritis by injecting genuine viral dsRNA and synthetic dsRNA (polynosinic-polycytidylic acid (poly(IC))) mimicking viral dsRNA into murine knee joints. Our results indicate that viral dsRNA is arthritogenic, because it induces joint inflammation in a healthy host.

Materials and Methods

Mice

Naval Medical Research Institute (NMRI) and BALB/c mice were purchased from B&K Research Universal AB (Stockholm, Sweden). C57Bl6/J and C3H/HeJ Hen mice as well as SCID mice and their congenic strain CB17 were purchased from M&B (Bomholtvej, Denmark). IL-1R-deficient mice (B6129S7-111r1tm1Imx 5) as well as their wild-type controls (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR3-deficient (TLR3−/−) mice as well as their wild-type controls (TLR3+/+) were originally generated at Yale University School of Medicine. All mice were housed in the animal facility of Department of Rheumatology and Inflammation Research, University of Göteborg. Female mice 6–8 wk of age were used in all the experiments.

RNA preparations

Synthetic and viral dsRNA and ssRNA. Synthetic (viral) dsRNA consisted of double-stranded copolymer, poly(IC), and polynosinic-polycytidylic acid. Synthetic ssRNA polynosinic acid (poly(I)) was purchased from Sigma-Aldrich (Stockholm, Sweden). Each strand contained dI, dC, and dU in precise alternating sequences. dsRNA and ssRNA molecules were dissolved in 1 ml of sterile water and further in PBS to obtain a stock concentration of 1 mg/ml, which was kept in a freezer at −20°C until use. The LPS concentration was 33 pg/10 μg poly(IC). This amount of LPS has been previously documented to not to cause joint inflammation (12).

To ensure a double-stranded configuration, dsRNA was kept in a water
bath at +50°C for 30 min and cooled down to room temperature thereafter. Finally, it was diluted with PBS to the required concentration.

Viral dsRNA was purified from Rotavirus and was used as the source of genuine dsRNA. Growing of virus and purification procedure is described briefly below.

**Virus culture.** The MA104 cell line of fetal rhesus monkey kidney cells was used for virus cultivation. Monolayers were grown to confluence in flat-bottom plastic flasks (125 cm²) in the presence of Eagle’s MEM supplemented with 5–10% FCS. Virus suspension was pretreated with trypsin (10 μg/ml inoculum) for 30 min at 37°C, to activate the virus inoculum before cultivation. MA104 monolayers were washed twice with serum-free Eagle’s MEM before adsorption of trypsin-activated Rotavirus for 1 h at 37°C. After the adsorption step, the medium was removed and replaced by serum-free Eagle’s MEM containing 1 μM trypsin. Cell cultures were held at 37°C and inspected daily for development of cytopathogenic effects.

**Virus purification.** When cytopathogenic effects were observed, the cell cultures were harvested for viral purification by gentle scraping and dispersal of the cells into the medium. Equal volumes (20 ml) of dispersed infected cells and Freon (trichlor-fluorothane; Sigma-Aldrich) were added to plastic Falcon centrifuge tubes, and shaken vigorously for 10 min. The supernatant was pipetted over to another centrifuge tube and treated once more with an equal volume of trichlor-fluorothane with vigorous shaking and centrifuging, as above. Supernatants were pooled and divided into 2 vol. One volume was inoculated as 1-ml aliquots into 9-ml aliquots of nuclisens lysis buffer (bioMerieux, Durham, NC) before storage at −70°C. The second volume of supernatant was subjected to ultracentrifugation at 45,000 rpm, for 21/4 h, to pellet virus (4 tubes of 13 ml). Each pellet was subsequently resuspended in 9 ml of nuclisens lysis buffer for storage at −70°C.

**RNA extraction.** The semiautomated nuclisens extractor (bioMerieux) was used to prepare viral RNA from the nuclisens lysated according to Booms silica gel method. RNA concentration and purity were assessed by OD spectrophotometry.

**Injection protocol**

dsRNA and ssRNA were injected intra-articularly at different concentrations in a volume of 20 μl into knee joints of mice of different strains. The contralateral knee joints were always used as a negative control and were injected with PBS alone.

**Histopathologic examination**

Histopathologic examination of joints was performed after routine fixation, decalcification, and paraffin embedding. Sections were cut and stained with H&E. All the slides were coded and evaluated blindly. Specimens were evaluated with regard to synovial hyperthrophy, pannus formation, and cartilage and subchondral bone destruction (13). The extent of synovitis was judged on an arbitrary scale from 0 to 3. No signs of inflammation represented 0 grade; grade 1 was characterized by mild inflammation with hyperplasia of synovial lining layer. Grades 2 and 3 represented different degrees of inflammation characterized by influx of inflammatory cells scattered throughout the synovial tissue.

**Immunohistochemical examination**

The knee joints from five NMRI mice were removed and deminerallized by enzymatic procedures detailed previously (14). The deminerallized specimens were mounted on cryostat chucks, frozen in isopentane that had been prechilled in liquid nitrogen, and kept at −70°C until cryosectioned. Serial cryosections, 5-μm thick, were stained with a rat mAb to mouse CD11b (Mac-1, clone M1/70), CD3 (clone 17A2; BD PharMingen, San Diego, CA), and followed by incubation with biotinylated secondary Abs, avidin-biotin-peroxidase complexes, and 3-amoio-9-ethyl-carbazole containing H₂O₂. All sections were counterstained with Meyer’s hematoxylin.

**Depletion of immunocompetent cells**

**Monocyte depletion.** Etoposide (Vepesid; Bristol-Myers Squibb, Bromma, Sweden) is a cytotoxic drug known to selectively deplete the monocyte population in mice (15). BALB/c mice were injected with etoposide (12.5 mg/kg, in a volume of 125 μl, s.c., into the groin) on 3 consecutive days before and after intra-articular injection of dsRNA. Control mice received the same volume of PBS.

**Neutrophil depletion.** mAb RB6-8C5 is a rat IgG2b Ab that selectively binds to and depletes mature mouse neutrophils (16). BALB/c mice were injected i.p. with 1 mg of mAb RB6-8C5, or the IgG rat anti-OVA mAb as a control 2 h before intra-articular injection with dsRNA.

In certain experiments, BALB/c mice were injected with both etoposide and mAb RB6-8C5 to obtain simultaneous depletion of monocytes and neutrophils. All mice from single and double depletion experiments were killed on day 3 after intra-articular injection with poly(IC), and their knee joints were histopathologically analyzed.

**Nuclear extract preparation**

Cultures of spleen cells (10⁶) were stimulated with different concentrations of poly(IC), or LPS, as described above. After 2 h, the stimulation was stopped with ice-cold PBS, and cells were washed, resuspended in 2 ml of hypotonic buffer (pH 7.9, containing 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermin, 1 M dithio-reitol, and proteinase inhibitors (Complete MiniTab; Boehringer Mannheim, Indianapolis, IN)), and homogenized. Following centrifugation at 14,000 × g at 4°C for 10 min, the supernatant was removed. The pellet was resuspended in the ice-cold extraction buffer (pH 7.9, 20 mM HEPES, 0.42 M NaCl, 1 M EDTA, 1 mM EGTA, 25% glycerol, 1 M DTT, and proteinase inhibitors).

**FIGURE 1.** Photomicrographs showing the histopathologic and immunohistochemical features of viral dsRNA-induced arthritis. A, Normal histologic appearance of a mouse knee joint following injection with control PBS. B, Histology of an arthritic knee joint of a mouse 3 days after intra-articular injection with 10 μg of poly(IC). Immunohistochemical analysis of an arthritic knee joint, showing synovial expansion of Mac-1-expressing cells (C) and of CD3-expressing cells (D) 3 days after intra-articular injection with 10 μg of poly. JC = joint cavity; V = blood vessel; C = cartilage; ST = synovial tissue. Arrow indicates inflammatory cells in the synovium.
Extraction proceeded at 4°C under continuous rotation for 60 min. The superna-
tants containing nuclear extracts were collected after centrifugation at
14,000 × g for 1 h at 4°C. Protein concentration in the extracts was deter-
rmined using Bradford reagent (Sigma-Aldrich). Nuclear extracts were aliquoted and
stored at −70°C until use.

**EMSA**

EMSA was performed, as described elsewhere (17), with minor modi-
fications. The sequences for oligonucleotides used for the assay were as follows: NF-
κB sense, 5′-GGCTTACACAGGGGGCTTTCCCCTTCAATAT-3′, and anti-
sense, 5′-GGATTTGGAGGGAAGGCCTTGTGGAG-3′; AP-1 sense, 5′-GGCTTCTC
CACATGAGATCATGGTTTTCT-3′, and anti-
sense, 5′-GGAGAAAACCATGATCTCATGTGGAGGAAG-3′. Oligonu-
cleotides were annealed at 56°C. The double-stranded product was purified
by elution from the electrophoretic gel. Double-stranded oligonucleotides were labeled
with [α-32P]deoxyxynucleotide (Amersham Pharmacia Biotech, Uppsala,
Sweden) using Klenow polymerase (5 U/ml; Roche Diagnostic Systems,
Somerville, NJ). Binding reactions were performed at room temperature for 20
min. The reaction mixture contained nuclear extract (5 μg), 1 mM dithiotreitol, and 1 μl
of [32P]-labeled double-stranded oligonucleotides (0.1 μg/μl) dissolved in the binding buffer (pH 7.9, 20 mM
Tris-HCl, 30 mM NaCl, 5 μg/ml EGTA, 50% glycerol), and supplemented with
0.2 μg/ml BSA.

For competition studies, a 100-molar excess of unlabeled double-
stranded oligonucleotides was added to the reaction mixture and incubated
for 20 min before the introduction of the 32P-labeled probe. For supershift
assays, antiserum to NF-kB p50 (clone C-19; Santa Cruz Biotechnology, Santa
Cruz, CA) was incubated with nuclear extracts for another 20 min at
room temperature.

Samples containing equal amount of protein were loaded directly onto
2.5% polyacrylamide gel prepared in Tris-borate-EDTA buffer (0.25×),
and electrophoresis was performed at 200 V at room temperature. The gel
was vacuum dried and exposed to x-ray film for 48 h at −70°C.

**Cytokine and chemokine assay**

To assess release of cytokines and chemokines in response to dsRNA,
murine spleens were obtained aseptically and passed through a nylon mesh.
Erythrocytes were depleted by hypotonic lysis. The resulting single-cell
suspension was resuspended in Iscove’s complete medium (10% FCS, 50
μM 2-ME, 4 mM L-glutamine, and 20 mg/ml gentamicin). Subsequently,
1 × 10^6 cells/ml were incubated with different concentrations of dsRNA
and ssRNA. The cultures were maintained in 24-well plates (Nunc, Rosk-
ilde, Denmark) at 37°C in 5% CO2 and 95% humidity. The supernatants
were collected after 3 days for analysis of IL-6, TNF-α, MIP-1α, and
MCP-1.

Levels of cytokines and chemokines in supernatants were determined
using TNF-α, MIP-1α, and MCP-1 enzyme-linked immunosorbent assay
kits from R&D Systems (London, U.K.). The assays were performed as
recommended by the manufacturer. The values below the detection limit
were considered as zero.

IL-6 levels were measured by a bioassay with cell clone B13.29, sub-
clonal B9, which is dependent on IL-6 for growth, as previously described
(18). B9 cells were harvested from tissue culture flasks, seeded into mi-
cro-titer plates (Nunc) at a concentration of 2.5 × 10^3 cells/ml in Iscove’s
complete medium. Supernatants of spleen cells stimulated with dsRNA
were added. [3H]Thymidine was added after 2 h of culturing, and the cells
were harvested on glass-fiber filter (AB Ninolab, Upplands Väsby, Swe-
den) after 4–5 h and counted in a beta counter. The samples were tested in
2-fold dilutions and compared with standard curve obtained using mouse
rIL-6 (Genzyme, Kent, U.K.). B9 cells were previously shown not to react
with several recombinant cytokines, including IL-1α, IL-1β, IL-12, IL-3,
IL-5, GM-CSF, TNF-α, and IFN-γ. There was only weak reactivity with
IL-4 (18).

**Statistical analysis**

Statistical comparisons were made by using the χ² with Yates’ corrections.
All values are reported as the mean ± SEM. Values of p < 0.05 were
considered significant.

**Results**

**Induction of arthritis by viral RNA**

Intra-articular injection of synthetic dsRNA (poly(IC)), but not
PBS, directly into murine knee joints led to development of ar-
thritis (Fig. 1, A and B). It was important to confirm that the highly
purified viral dsRNA is also able to give rise to joint inflammation.
Five micrograms of viral dsRNA was injected intra-articularly.

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Injected IC</th>
<th>No. of Mice, n</th>
<th>Frequency of Arthritis</th>
<th>Severity Grade, Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMRI</td>
<td>Poly(IC)</td>
<td>14</td>
<td>9/14b</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>NMRI</td>
<td>Poly(I)</td>
<td>14</td>
<td>4/14</td>
<td>0.5 ± 0.8</td>
</tr>
<tr>
<td>NMRI</td>
<td>PBS</td>
<td>14</td>
<td>1/14</td>
<td>0.1 ± 0.4</td>
</tr>
<tr>
<td>SCID (CB17)</td>
<td>Poly(IC)</td>
<td>7</td>
<td>4/7</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>Non-SCID (CB17)</td>
<td>Poly(IC)</td>
<td>9</td>
<td>5/9</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>IL-1R⁻/⁻ (C57BL/6)</td>
<td>Poly(IC)</td>
<td>9</td>
<td>1/9</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>IL-1R⁻/⁻ (C57BL/6)</td>
<td>Poly(IC)</td>
<td>10</td>
<td>5/10</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>C57/H-HeJ</td>
<td>Poly(IC)</td>
<td>9</td>
<td>2/9</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>C57/H-HeK</td>
<td>Poly(IC)</td>
<td>9</td>
<td>2/9</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>Etoposide (BALB/c)</td>
<td>Poly(IC)</td>
<td>9</td>
<td>2/9</td>
<td>0.3 ± 0.7</td>
</tr>
<tr>
<td>RB6–8C5 (BALB/c)</td>
<td>Poly(IC)</td>
<td>9</td>
<td>6/9</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Control IgG + PBS</td>
<td>Poly(IC)</td>
<td>9</td>
<td>5/9</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>Etoposide (BALB/c) + RB6–8C5 (BALB/c)</td>
<td>Poly(IC)</td>
<td>10</td>
<td>3/10</td>
<td>0.4 ± 0.7</td>
</tr>
</tbody>
</table>

⁺⁺ Mice were killed 3 days after a single intra-articular injection with 20 μg of dsRNA (poly(IC); 10 μg/knee), ssRNA (poly(I); 10 μg/knee), and PBS (20 μg/knee). BALB/c mice were injected with etoposide (12.5 mg/kg, in a volume of 125 μl s.c. into the groin) or PBS as a control on 3 consecutive days before and after intra-articular injection of
dsRNA. BALB/c mice were injected i.p. with 1 mg of mAb RB6–8C5, or the IgG rat anti-OVA mAb as a control 2 hrs prior to intra-articular injection with dsRNA.

⁺⁺⁺ Value of p < 0.05, as compared with mice injected with PBS.
The mice were killed 3 days after the injection. Just as in the case of synthetic dsRNA, viral dsRNA gave rise to arthritis (Fig. 2).

Histologically, the arthritis was characterized by synovial hyperplasia and infiltrating mononuclear cells in the synovial lining cell layer, deep in the sublining space, as well as around surrounding synovial vessels. Immunohistochemical examination resulted in good morphologic preservation of synovial and articular tissue. In sections of arthritic joints, a large proportion of synovial cells was stained with Mac-1, which recognizes granulocytes and macrophages, and with CD3-specific Ab. The Mac-1-stained cells, having a morphology of macrophages, were found both within the thickened synovial lining layer and within the deeper synovial tissue. The predominance of Mac-1 cells was evident at day 3 after intra-articular injection with poly(IC), but also CD3+ T lymphocytes were found in these arthritic joints (Fig. 1, C and D).

To assess the optimal amount of poly(IC) for triggering arthritis, different doses of poly(IC) (0.1, 1, 10, 20 μg/knee) were used in one experiment. We found that 10–20 μg of poly(IC) was the optimal dose because it triggered arthritis in the great majority of animals.

To exclude contribution of LPS contamination in the induction of arthritis, synthetic dsRNA was injected intra-articularly into knee joints of LPS-nonresponder C3H/HeJ mice. No difference in frequency and severity of arthritis was observed upon injection of dsRNA to LPS-nonresponding mice (strain C3H/HeJ) and congenic LPS-responding mice (strain C3H/HeN) (Table I). This indicates that the induction of arthritis was due to dsRNA rather than LPS contamination in the injected vehicle.

Five different mouse strains were assessed for their susceptibility to poly(IC)-induced arthritis, including NMRI, C3H, BALB/c, CB17, and C57BL/6. We found that arthritis was inducible by dsRNA in all of these strains (Table I).

To study the effects of repeated exposure of dsRNA on joint pathology, we injected 10 μg of poly(IC) intra-articularly on days 0 and 7. On day 10, the mice were killed and their joints were analyzed. The severity of arthritis was not significantly different with repetitive injections than with a single injection (results not shown).

We wanted also to assess the time point for the development of maximal frequency and severity of arthritis induced by a single injection of poly(IC). We found that histological signs of arthritis were most pronounced on day 3 after the injection of poly(IC), and its frequency and intensity diminished on days 7, 14, and 28 (Fig. 3).

Synthetic ssRNA did not give rise to the same frequency or severity of arthritis when provided intra-articularly (Table I). In analogy, intra-articular injection of polyinosinic-uridylic acid did not give rise to arthritis. Indeed, only one of five mice developed histological signs of joint inflammation.

**Systemic in vivo inflammatory response following intra-articular exposure to dsRNA**

Because viral dsRNA can activate macrophages to release IL-6, we measured its level in sera from mice injected intra-articularly with viral dsRNA (poly(IC)) at day 0, and then they were killed at days 3, 7, 14, and 28. Serum IL-6 levels peaked 7 days after injection of dsRNA and decreased with time to baseline levels at day 28 (Fig. 4).

Interestingly, depletion of monocytes almost totally abrogated the dsRNA-triggered arthritis (Fig. 5). In contrast, control mice developed joint inflammation at the expected frequency. These results strongly indicate that monocyte/macrophage population is responsible for the induction of dsRNA-triggered arthritis.

For analysis of the role of neutrophils, which are typically the earliest cells, first to migrate into tissues in response to inflammatory stimuli, we used neutrophil-depleting RB6-8C5 Ab. We found...
that neutrophil-depleted mice did not differ from control mice in the development of arthritis triggered by dsRNA (Fig. 5). This indicates that neutrophils are not mandatory in the development of this condition. Simultaneous depletion of neutrophils and monocytes did not further decrease the frequency of dsRNA-induced arthritis more than etoposide treatment alone.

Next, we assessed the role of T and B lymphocytes using SCID mice lacking these cell populations, but having an intact population of monocytes/macrophages. Histologic results from SCID mice and their congenic littermates (CB17) demonstrated that the severity and incidence of arthritis were similar (Fig. 6). This finding proves that T and B cells are not pivotal for the development of dsRNA-triggered arthritis. Taken together, these studies provided strong evidence of the role of macrophages in initiating arthritis triggered by dsRNA.

**Important regulatory role of NF-κB in dsRNA-triggered arthritis**

What controls and regulates the macrophage activity in dsRNA-triggered arthritis? To answer this question, we investigated the transcription factors in the spleen cell cultures was assayed in vitro. The binding of NF-κB and AP-1 to oligonucleotides containing NF-κB and AP-1 sense and antisense in the presence or absence of dsRNA (poly(IC)) was assessed by EMSA (Fig. 7). Increasing concentration of dsRNA (50, 5, 0.5 μg/ml) gave rise to a dose-dependent DNA binding to NF-κB (Fig. 7A). Specificity of DNA binding to NF-κB oligonucleotides was proved by a competitive inhibition of the reaction by introduction of unlabeled (cold) NF-κB oligonucleotides to the reaction mixture. In addition, incubation of nuclear extracts with Abs to NF-κB p50 subunit resulted in the formation of an additional band (supershift) in the gel (Fig. 7B). Similar pattern was observed when nuclear extracts from spleen cells stimulated with dsRNA were assessed for AP-1 protein (Fig. 7C). Intensity of AP-1 binding increased in parallel with increasing concentrations of stimulated dsRNA. Introduction of an excess of unmarked AP-1 oligonucleotides prevented the reaction between nuclear extracts and 32P-marked AP-1 oligonucleotides.

**FIGURE 7.** The activation of NF-κB and AP-1 in response to dsRNA was tested in cultures of spleen cells stimulated with increasing concentrations of synthetic dsRNA (poly(IC)). Nuclear extracts were prepared after 2 h of stimulation. Ns = Nonstimulated; mm = mastermix. A, EMSA was performed using specific probe to NF-κB binding site, and after 20 min at room temperature the complexes were resolved by electrophoresis through a 2.5% polyacrylamide gel. B, For competition studies, a 100-molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min at room temperature before the introduction of the 32P-labeled probe. To identify the NF-κB subunits, we performed Ab-mediated supershift assays. Specific Abs to p50 subunits of NF-κB were added to nuclear extracts and incubated for another 20 min at room temperature. C, EMSA was performed using specific probe to AP-1 binding site, and after 20 min at room temperature the complexes were resolved by electrophoresis through a 2.5% polyacrylamide gel. For competition studies, a 100-molar excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min at room temperature before the introduction of the 32P-labeled probe.

**Does TLR3 exert any role in dsRNA-triggered arthritis?**

It has been previously reported that the mammalian TLRs recognize a variety of microbial components and activate NF-κB and other transcription factors in the spleen cell cultures was assayed in vitro. The binding of NF-κB and AP-1 to oligonucleotides containing NF-κB and AP-1 sense and antisense in the presence or absence of dsRNA (poly(IC)) was assessed by EMSA (Fig. 7). Increasing concentration of dsRNA (50, 5, 0.5 μg/ml) gave rise to a dose-dependent DNA binding to NF-κB (Fig. 7A). Specificity of DNA binding to NF-κB oligonucleotides was proved by a competitive inhibition of the reaction by introduction of unlabeled (cold) NF-κB oligonucleotides to the reaction mixture. In addition, incubation of nuclear extracts with Abs to NF-κB p50 subunit resulted in the formation of an additional band (supershift) in the gel (Fig. 7B). Similar pattern was observed when nuclear extracts from spleen cells stimulated with dsRNA were assessed for AP-1 protein (Fig. 7C). Intensity of AP-1 binding increased in parallel with increasing concentrations of stimulated dsRNA. Introduction of an excess of unmarked AP-1 oligonucleotides prevented the reaction between nuclear extracts and 32P-marked AP-1 oligonucleotides.

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**FIGURE 5.** Frequency of arthritis after a single intra-articular injection of 10 μg of poly(IC). BALB/c mice were depleted of neutrophils using mAb against RB6-8C5 (n = 9). BALB/c mice were depleted of monocytes using etoposide treatment (n = 9). BALB/c mice were depleted simultaneously of both neutrophils and monocytes (n = 10).

**FIGURE 6.** Incidence of arthritis in mice being deficient for T and B cells (n = 9) in comparison with their congenic strain (CB17) (n = 9). Three days after a single intra-articular injection of 10 μg of poly(IC), all the mice were killed.
Furthermore, we assessed the role of LPS contamination in vitro by using spleen cell cultures from LPS-nonresponder strain C57BL/6J mice and their congenic LPS-responder C57BL/6N mice. The results from this experiment demonstrated no differences between C57BL/6J mice and their congenic littermates regarding the production of IL-6, TNF-α, MIP-1α, and MCP-1 upon the exposure to dsRNA. This finding proves that the inflammation is dependent on dsRNA rather than contaminating LPS (results not shown).

Discussion
This is the first study presenting the central role of viral dsRNA in induction of arthritis. Our results indicate that synthetic dsRNA, mimicking viral dsRNA as well as purified viral dsRNA itself, are able to induce arthritis. dsRNA may, therefore, play an important pathogenic role in virally triggered inflammatory joint diseases. This conclusion is supported by our finding that the proinflammatory effect exerted by viral dsRNA is not caused by endotoxin contamination. It is based on: 1) a low LPS concentration in the dsRNA preparation; and 2) similar frequency and severity of arthritis between the LPS-nonresponder and their congenic LPS-responder mice.

What mechanism triggers induction of dsRNA-mediated arthritis? Because joint inflammation occurs already on day 3 following intra-articular injection with poly(IC), it is strongly suggestive for activation of the innate rather than acquired immunity. Indeed, dsRNA-triggered arthritis occurs in SCID mice lacking T and B cells, almost at the same frequency and magnitude as in their congenic counterparts, supporting mechanisms operating in innate immunity. We believe that monocytes/macrophages are the main cell population that mediates arthritis caused by dsRNA. This is based on abundance of Mac-1+ mononuclear cells in the inflamed synovial tissue. Further support for the role of macrophages is clearly decreased frequency of arthritis upon in vivo depletion of monocyte/macrophage population in mice intra-articularly exposed to dsRNA. In contrast, neutrophils, despite their rapid influx into tissues in response to inflammatory stimuli, are not mandatory in the development of dsRNA-triggered arthritis. Lysis of this cell population in vivo does not affect natural course of the disease.

Some of the major intracellular mediators of inflammatory response in macrophages are NF-κB and AP-1. The activation of NF-κB and AP-1 in dsRNA-exposed macrophages was examined using EMSA. Our findings indicate that dsRNA activates dose dependently NF-κB and AP-1, especially with respect to its p50 subunit. Such an activation is a prerequisite for subsequent induction of proinflammatory cytokine and metalloproteinase production. Indeed, in vitro analysis showed that dsRNA induces both cytokine and chemokine release by leukocytes. Also, a single intra-articular injection of dsRNA leads to systemic production of inflammatory mediators. Finally and importantly, expression of joint inflammation upon exposure to dsRNA is completely abrogated in mice deficient for IL-1R expression.

TLRs are a family of molecules that recognize danger signals associated with microbial pathogens, and induce antimicrobial immune responses (29, 30). It has been shown that TLR3 is an important recognition molecule specific for dsRNA (6). Our data demonstrate that mice lacking TLR3 were still able to develop arthritis, indicating that some other recognition systems for dsRNA may also be operative. One of such systems might be dsRNA-dependent protein kinase (PKR) interaction with its ligand (i.e., dsRNA) giving rise to NF-κB activation in a direct way, as described by Yang et al. (31). In agreement with the previous study (6), dsRNA was more efficient in vitro to give rise to cytokine and chemokine responses in controls as compared with TLR3KO mice. Altogether, our results suggest that dsRNA is arthritogenic and
FIGURE 9.  A, Levels of IL-6, TNF-α, MCP-1, and MIP-1α in vitro by spleen cell cultures from mice being deficient for TLR3 (n = 4) or their congenic littermates (n = 3) after 2 days of stimulation with poly(IC) (0, 50, 150 μg/ml).  B, Levels of IL-6, TNF-α, MCP-1, and MIP-1α produced in vitro by spleen cell (obtained from NMRI mice, n = 2) cultures after 3 days of stimulation with poly(IC) or poly(I) (0, 50, 150 μg/ml).  C, Levels of IL-6, TNF-α, MCP-1, and MIP-1α produced in vitro by spleen cell cultures from mice being deficient for IL-1R (n = 3) or their congenic littermates (n = 3) after 3 days of stimulation with poly(IC) (0, 50, 150 μg/ml).
that its arthritogenic properties are mediated by NF-κB activation and signaling involving IL-1R interaction. This finding is the first molecular evidence of inflammatogenic property of viral constituents once deposited in the joint cavity.

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References