A Mouse Herpesvirus Induces Relapse of Experimental Autoimmune Arthritis by Infection of the Inflammatory Target Tissue

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*J Immunol* 2004; 173:5238-5246; 
doi: 10.4049/jimmunol.173.8.5238
http://www.jimmunol.org/content/173/8/5238
A Mouse Herpesvirus Induces Relapse of Experimental Autoimmune Arthritis by Infection of the Inflammatory Target Tissue

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It is not known how an initial episode of autoimmunity progresses to a self-sustained autoimmune disease with clinical progression or relapses. Possible mechanisms might include antigenic mimicry, the “adjuvant effect,” and epitope spreading (1–4). In this study, we describe a new mechanism: reversible superimposed infection of the target autoimmune tissue. For this purpose, we have used a model of transient arthritis in mice to assess the effects of infection with a mouse gamma-herpesvirus.

Human autoimmune disease frequently manifests itself initially as a transient, poorly defined disease. In large studies of patients with a first attack of autoimmune demyelinating disease, such as optic neuritis, is well recognized, as only 34–74% of cases go on to develop definite multiple sclerosis (8–10). It is not clear yet what causes disease progression in these autoimmune disorders.

Rheumatoid arthritis (RA) is a prototypical human tissue-specific autoimmune disease. Previous work showed that acute polyarthritis was observed in association with EBV infection (11), increased Ab titers were found in RA (12–14), molecular mimicry was entertained (15, 16), and CD8 cell clones specific for lytic and latent EBV gene products were clonally expanded in inflamed synovia (17–20). Staining with MHC class I peptide tetramers showed that EBV-specific CD8 T cells were enriched in RA synovia compared with blood from the same subject (21, 22). The presence in synovium of CD8 cells specific for lytic viral Ags, suggested that EBV latency was interrupted and that this virus was at least intermittently productive. Indeed, a few (but not all) studies provided evidence of productive infection of synoviocytes by EBV in vivo in some RA patients (16, 23, 24). It is important to note that these observations were not RA- or EBV-specific: CD8 clones specific for EBV were also found in the synovia of other chronic inflammatory arthritic diseases and CMV-specific CD8 cells were also observed in RA synovia (18, 20).

K/BxN (KxN) mice are an F1 cross between NOD and KR. They spontaneously develop symmetric small joint arthritis of the limbs at ~4 wk of age, with 100% incidence (25–27). They produce autoantibodies to the ubiquitous autoantigen glucose-6-phosphate isomerase (GPI). A peptide of this protein is presented by IAg7 (from the NOD background) to CD4 T cells bearing a transgenic TCR (from the KRN mouse). These T cells provide help for autoimmune disease. Previous work showed that herpesviruses have long been suspected of contributing to human inflammatory arthritic diseases and CMV-specific CD8 cells were purchased (The Jackson Laboratory, Bar Harbor, ME) and KRN mice were purchased (The Jackson Laboratory, Bar Harbor, ME) and KRN mice were

Materials and Methods

Animals

C57BL/6 (B), NOD (N), and RAG1−/− on a C57BL/6 background were purchased (The Jackson Laboratory, Bar Harbor, ME) and KRN mice were purchased (The Jackson Laboratory, Bar Harbor, ME). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; GPI, glucose 6-phosphate isomerase; MS, multiple sclerosis; MHV-68, murine gamma-herpesvirus 68.
from Diane Mathis. Mice were age and sex matched and 6–10 wk old. Arthritic K/BxN F1 mice were bred locally to generate serum containing anti-GPI Abs (25–27). Control sera were obtained from K/BxN F1 non-transgenic littermates.

**Serum transfer protocol and arthritis scoring**

Arthritis was induced by two i.p injections (day −2 and day 0) of 80–150 μl of pretested arthritic serum. Injections with sera from transgene-negative littermates did not cause arthritis. Fore (wrist) and hind (ankle) paw thickness was measured with calipers. In addition, a clinical index was calculated to assess the number of paws affected. The index was a sum of 1 point for each involved paw if swelling was >0.4 mm over baseline and 0.5 points/paw if the swelling was <0.4 mm over baseline (maximum index = 4).

**Viral infections**

Mice were infected intranasally with 3 × 10⁴ PFU of MHV-68 WUMS strain (American Type Culture Collection, Manassas, VA). Stocks were prepared in OMK cells (ATCC CRL 1566) and titered by plaque assay on NIH 3T3 cells (ATCC CRL 1658) (35). Influenza PR-8 strain was used at 400 hemagglutinin units/infection by the intranasal route. HSV-1 strain 17 was used at 200,000 PFU/infection i.p.

**Drug therapy**

Cidofovir (Vistide; Gilead Sciences, Foster City, CA) was given s.c. every 3 days at a dose of 25 mg/kg (36). Cyclophosphamide (Cytoxan; Mead Johnson, Princeton, NJ) was given i.p. at 200 mg/kg three times over a week.

**FIGURE 1.** Clinical effect of viral infection on transient arthritis. C57BL/6 (B6) or RAG1−/− (RAG) mice were infected with MHV-68 either on day 2 or day 5 as indicated. All mice received serum from arthritic K/BxN mice on days −2 and 0 to induce transient arthritis. All experiments were run with an arthritis control group (no viral infection). No arthritis was observed in controls with “virus only” or with neither serum nor virus. Average ankle thickness is shown for five mice per group. A, B6 mice with transient arthritis were infected with the indicated viruses on day 2. B and C, B6 and RAG1−/− mice with or without virus infection. The second peak of arthritis in C differed significantly from the control group at three time points (p < 0.00006). D and E, Treatment with cidofovir, as indicated by the arrows, to inhibit second peak of arthritis. Statistical significance for group comparisons with or without cidofovir: p < 0.05 at three time points of second peak (D) and p < 0.003 at four time points of second peak (E).
Detection of virus-specific T cells

PBL were stained with PE-labeled MHC class I tetramers corresponding to a lytic phase Ag of MHV-68 (p79, Kβ/TSINFVKI) (37) (Trudeau Institute, Saranac Lake, NY), with anti-CD8-CyChrome (BD Pharmingen, San Diego, CA) or with anti-Vβ4-FTTC Abs and analyzed by flow cytometry. Mice were serially tested over time. Peak tetramer responses occurred on day 21 after infection and Vβ4 CD8 responses after day 21.

Histology and immunohistology

Ankle joints were dissected, fixed in 10% Formalin, decalcified in Decal fix (38), paraffin sections were incubated overnight at 4°C with rabbit hyper-immune serum against MHV-68 (39, 40) followed by biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h. Streptavidin-HRP was used (TSA kit; NEN Life Sciences, Boston, MA) and substrate was added (Nova RED; Vector Laboratories). Nonspecific staining was blocked with 5% normal goat serum prior to the primary Ab. Preimmune rabbit serum was the negative control.

Detection of MHV-68 DNA by nested PCR

DNA was extracted from spleen, ankle joints, and kidneys (QIAmp DNA Blood Minikit; Qiagen, Valencia, CA) and equilibrated to ~0.01 μg/μl. The estimated variability in DNA input for the PCR was <1 log. A nested PCR for open reading frame 50 with a sensitivity of one copy of MHV-68 DNA was used: outer PCR primers were 5'-AACCTGGGAACCTCTTTCTGT GGC-3' and 5'-GGCCGCAGACATTTAATGAC-3' (586 bp); inner PCR primers were 5'-CCCCAATGGTTCATAAGTG-3' and 5'-ATCACAG CAGGCCATAACATC-3' (382bp). Reactions contained 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM nucleotides, 1 ng of each primer, 1 μl of DNA, and 1 U Taq (Promega, Madison, WI) in 20 μl. PCR cycles were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (for 45 cycles). The second PCR was identical, but only 25 cycles. For the second PCR, 1 μl of the first-round product was amplified in 10 μl. For end point dilutions, original DNA concentrations were equilibrated for the different tissues. Nine replicates of each dilution were assayed. The average end point dilutions were calculated for each tissue (n = 5 mice/group).
cells, and fibrosis on day 30 (Fig. 3, B and D). A consistent finding in virus-infected mice was papillary synovial fronds (Fig. 3C), a possible indication of proliferative changes of synoviocytes. However, there was no overall difference in synovial infiltration in immunocompetent arthritic animals with or without virus infection. At the same time point (day 30), MHV-68-infected RAG1−/− mice had extensive synovial infiltrates and bony erosions (Fig. 3, E and F).

**Viral replication required for relapse of arthritis**

MHV-68 replication can be inhibited by the antiviral drug cidofovir (36, 42). A course of cidofovir, begun 2 days after viral infection (day 7), completely abrogated second peak arthritis (Fig. 1D) but did not affect the first peak of arthritis. Even a short course of cidofovir (days 17–26) inhibited the relapse of arthritis (Fig. 1E). In this group of mice, unabated viral replication resumed after cidofovir was halted on day 26, and the mice succumbed from viral infection on days 44–48. Thus, later viral infection (e.g., after day 26) was insufficient to induce arthritis. We conclude that second peak arthritis requires viral replication during a window in time shortly after serum transfer.

**Viral DNA enriched in joint tissues**

Varied tissues were sampled for a quantitative DNA PCR using end point dilutions. On average, viral DNA in ankles was 2–3 logs higher than in spleen and ~4 logs higher than in kidney in infected RAG1−/− mice sacrificed on day 30 (Fig. 4). Even in immunocompetent C57BL/6 mice, viral DNA was enriched in the ankle tissues over kidney by 2 logs. However, these data do not distinguish between lytic and latent virus and do not indicate which cells might be infected in the joint tissue.

**Virus-induced CD8 T cells enriched in joint tissues**

If lytic MHV-68 infection was occurring in synovial tissues, one would expect an influx of virus-specific T cells. Systemic responses to MHV-68 include a massive Vβ4 CD8 response (43, 44) after day 20 and an earlier CD8 response to a peptide of viral P79 measured with Kb tetramers (37). Viral infection alone was not associated with clinical arthritis and it was not possible to obtain sufficient synovial cells for flow cytometry. However, this was feasible in mice with clinical arthritis. Both Vβ4 CD8 cells and tetramer-positive CD8 cells were enriched in synovial T cells (Fig. 5), suggesting that MHV-68 might have infected synovial cells, even in immunocompetent mice.

**Viral Ags in joint tissues**

Tissue sections were stained with a polyclonal rabbit Ab specific for lytic and latent MHV-68 Ags (40). As a positive control, three RAG1−/− mice were infected with 10⁶ PFU i.p. on day 2 of arthritis onset and sacrificed on day 12. In all RAG1−/− mice, viral Ags could easily be detected in the synovial tissues of affected...
Mice. Representative results are shown with averages of three mice. 

Average percent Vβ4/CD8 staining was performed with Vβ4-FITC/CD8-Cy5/p79-Kb-PE tetramer. A. Tissues were harvested on day 29 after infection from infected mice without arthritis. Representative results are shown with averages of three mice. B. Mice with arthritis were sacrificed on day 30. Average percent Vβ4/CD8 was calculated from five mice, mean ± SEM.

JOINTS. Three-color staining was performed with Vβ4-FITC/CD8-Cy5/p79-Kb-PE tetramer. A. Tissues were harvested on day 29 after infection from infected mice without arthritis. Representative results are shown with averages of three mice. B. Mice with arthritis were sacrificed on day 30. Average percent Vβ4/CD8 was calculated from five mice, mean ± SEM.

Role of immunodeficiency in virus reactivation

The effect of MHV-68 infection was most readily detectable in immunodeficient mice. Patients with severe autoimmunity are often treated with drugs like methotrexate or Cytoxan, which have the potential to induce transient immunodeficiency. Therefore, we asked whether Cytoxan might reactivate latent MHV-68 in immunocompetent mice. Mice infected several months earlier with MHV-68 were compared with uninfected mice. After successive episodes of induced arthritis, both groups received Cytoxan for 1 wk (Fig. 7A). This caused a transient decrease in T cell subsets, including Vβ4 CD8 T cells, in PBL (inset, Fig. 7A). In the uninfected mice, Cytoxan significantly diminished serum-transferred arthritis initiated on days 62 and 64 (blue arrow). In the mice latently infected with MHV-68, the arthritis was of much greater magnitude (red arrow) and the therapeutic effect of Cytoxan was lacking. Both groups of mice had previously responded similarly to arthritis induction. All mice were sacrificed on day 89 and joint tissues were stained for viral Ags: synovial tissues were positive in all infected mice after Cytoxan treatment (Fig. 7E), but infected mice examined earlier at day 30 (before Cytoxan) showed no viral Ags in joint tissues (Fig. 7D). Thus, synovial reactivation of latent MHV-68 may have occurred in Cytoxan-treated mice. This correlated with lack of suppression of arthritis by Cytoxan.

To examine whether preexisting arthritis was required for virus reactivation in the joints, naive B6 mice latently infected with MHV-68 were given Cytoxan followed by arthritis induction (Fig. 7B and C). As expected Cytoxan-treated mice had less severe arthritis (solid symbols) than untreated mice (open symbols).
There was no difference between latently infected mice (red symbols) and uninfected mice (blue symbols). In a crossover design, the groups that had not received Cytoxan were then given the drug (Cytoxan #2) and the previously treated groups were not given a repeat course of Cytoxan. Cytoxan diminished the second episode of arthritis compared with that of controls. However, this time the latently infected animals (open red symbols) had a lesser therapeutic effect from the Cytoxan (Fig. 7, B and C). Some authors have expressed their data using this model as millimeters of swelling over baseline (31). The baseline on day 26 was 3 mm for the ankles and 2.3 mm for the wrists (Fig. 7, B and C). The induced swelling after Cytoxan treatment on day 35 was 0.3 vs 0.7 mm (2.3-fold more in the ankles of virus-infected mice) and 0.2 vs 0.7 mm (3.5-fold more in the wrists of infected mice).

These data suggest that MHV-68 reactivation occurs in the affected joints after arthritis has been established earlier. Alternatively, reactivation of MHV-68 may require more severe arthritis, since the second episode of arthritis was more severe than the first episode (Fig. 7, B and C).

**Discussion**

Animal models are required to establish the role of an infectious agent in autoimmunity (1). For example, studies on Theiler’s murine encephalomyelitis virus, which causes a demyelinating disease in mice, have led to understanding on how an antiviral response evolves into an autoimmune response, with direct implications for understanding multiple sclerosis (MS) and human T cell leukemia virus I-associated myelopathy/tropical spastic paraparesis (2).

In this study, we develop a new mouse model to test the effects of a herpesvirus on autoimmune arthritis. Lytic viral infection in the setting of an active, organ-specific autoimmune process changed a self-limited process and caused a relapse of disease. Although this was evident in severely immunodeficient RAG1−/− mice, several clues indicate that importation of virus to the joint tissues also occurs in immunocompetent mice. First, the severity and duration of arthritis was slightly increased in C57BL/6 mice infected with MHV-68. Second, virus-specific CD8 T cells were enriched in the joint tissues of infected C57BL/6 mice (Fig. 5).
Third, viral DNA was present in the joint tissue at a level similar to that in the spleen and was ~100-fold enriched compared with that of the kidney (Fig. 4B) in immunocompetent mice. Fourth, transient immunodeficiency caused by Cytoxan reactivated viral infection in arthritic joints of normal C57BL/6 mice.

It is possible that a similar scenario may apply to some human patients with autoimmune diseases. RA patients undergoing treatment with methotrexate usually respond with clinical improvement. However, treatment failures do occur in ~30% of patients (45, 46). Perhaps herpesviruses are reactivated in situations where transient immunodeficiency occurs, as commonly observed in posttransplant patients. Indeed, there is evidence that some humans with RA may have active herpesvirus infection within the joint (16, 23, 24), although it has not been possible to ascertain whether these viruses contributed to clinical arthritis. Our data suggest that herpesviruses, latent in hemopoietic cells, can be imported into tissue sites of autoimmunity in the setting of an inflammatory infiltrate, and that this is accompanied by reactivation of viral infection. Most importantly, the superimposed infection is clinically significant. This may be a general mechanism that applies to different autoimmune diseases. Indeed, EBV- and CMV-specific T cells may be enriched in the joints of patients with various types of arthritis including RA, psoriatic arthritis, ankylosing spondylitis, and Reiter’s syndrome (18). Compared with PBL, samples from inflammatory target tissues of patients with uveitis or MS were also enriched in herpesvirus-specific T cells (18). Consistent with these findings, there are numerous, often controversial, studies on associations between various autoimmune diseases and several herpesviruses (47–49).

A recent study in mice demonstrated that MHV-68 infection worsened the course of experimental allergic encephalomyelitis, an animal model for MS (50). However, in this study MHV-68 could not be detected by PCR in the target tissue, e.g., the spinal cord. Not only herpesviruses, but also other organisms such as Chlamydiae, may affect autoimmune disease progression in a similar manner (51, 52). Chlamydia pneumoniae given on day 7 of experimental allergic encephalomyelitis induction, but not Chlamydia trachomatis, resulted in accentuated neurological disease. C. pneumoniae, usually present only in splen and lungs, was found in the CNS by RT-PCR and immunohistochemical staining (51).

The mechanism of the virus-related arthritis in RAG1−/− mice (second peak in Fig. 1C) was likely extensive lytic viral replication in the synovia (Fig. 6). The immunostaining clearly showed extracellular (and intracellular) location of Ag. The Ags recognized by the MHV-68-specific Ab included lytic viral Ags (40). Electron microscopy showed fibroblast cell death (Fig. 6J) and many extracellular viral particles. Finally, cidofovir, a drug that inhibits viral replication, prevented second peak arthritis.

Importation of the virus was specific to the target organ. Unaffected joints (Fig. 6, H and I) and other tissues (Fig. 4) contain much less virus. Importation of the virus had to occur during a window in time when inflammatory cells were being recruited to the target synovial tissue. Presumably inflammatory cells such as monocytes or dendritic cells, known to harbor MHV-68, import the virus, and lytic infection then occurs locally involving resident cell types such as fibroblasts and synoviocytes, which are among the first cells to appear positive for viral Ags (Fig. 6).

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FIGURE 7. Cytoxan can reactivate virus in the joint. A, C57BL/6 mice, MHV-68 infected or not, were repetitively injected with arthritogenic serum (five mice per group). Arthritis induction is indicated by the open arrows (days −2/0, days 16/18, days 40/42, and days 60/62). Both groups of mice were treated with Cytoxan i.p., 200 mg/kg, three times over 1 wk. Flow cytometry was performed before Cytoxan treatment, 2 wk and 4 wk later (green, red, and blue arrowheads, respectively) and the results are shown (inset) for the group of infected mice. In uninfected mice (blue symbols), Cytoxan ablated the clinical arthritis (blue upward arrow), but virus-infected mice (red symbols) had undiminished arthritis (red upward arrow). Ankle (B) and wrist (C) thickness in mice with latent MHV-68 infection (red) or uninfected mice (blue) that received either an early course of Cytoxan prearthritic induction (Cytoxan #1, solid symbols) or a late course (Cytoxan #2, open symbols) initiated after the first episode of arthritis in a crossover design. The open arrows indicate arthritis induction (days −2/0; days 26/28); the downward red arrow indicates MHV-68 infection on day −21. D, Ankle of a MHV-68-infected C57BL/6 mouse on day 30 after arthritis induction without Cytoxan-induced reactivation. E, Ankle of a MHV-68-infected C57BL/6 mouse sacrificed on day 89 after Cytoxan treatment (see A).
There are obvious therapeutic implications from the ability to inhibit arthritis with an antiviral drug. Might a similar antiviral drug have a beneficial effect in patients with active herpesvirus infection within their joint tissues? CMV and EBV reactivation in humans given immunosuppressive drugs is common in the transplant setting. Reactivation can be curtailed or prevented by antiviral drugs such as acyclovir, especially with cessation of immunosuppressive therapy. Whether this also occurs in patients with autoimmune diseases, such as RA patients on methotrexate, is not yet clear, but the occurrence of EBV-related lymphomas in methotrexate-treated RA patients (53, 54) suggests that EBV-specific immunosurveillance is deficient.

There are also implications for possible mechanisms by which autoimmunity progresses to a chronic disease. In humans with RA or other autoimmune disorders, it seems unlikely that a lytic herpesvirus infection would advance to the same extent seen in RAG1−/− mice. However, intermittent lytic and productive infection, contained by a competent immune system, could have deleterious effects in several ways. For example, Coxackie virus is known to infect the islets of the pancreas in NOD mice, which can result in release of sequestered islet Ags and restimulation of autoreactive T cells (55). Lytic infection is expected to cause cell death and thus exposure to neo-Ags in the presence of strong viral toreactive T cells. Lytic Epstein-Barr virus infection would advance to the same extent seen in RAG1−/− mice. However, intermittent lytic and productive infection, contained by a competent immune system, could have deleterious effects in several ways. For example, Coxackie virus is known to infect the islets of the pancreas in NOD mice, which can result in release of sequestered islet Ags and restimulation of autoreactive T cells (55). Lytic infection is expected to cause cell death and thus exposure to neo-Ags in the presence of strong viral toreactive T cells. Lytic Epstein-Barr virus infection would advance to the same extent seen in RAG1−/− mice.

Although antigenic mimicry and bystander activation are often cited as the leading theories on the origins of autoimmunity, we propose a contributing pathogenic mechanism, imported infection. This may occur after disease initiation and may contribute to reoccurrence or maintenance of inflammation in target organs of the autoimmune disease.

Acknowledgments

We thank Drs. Skip Virgin and Felipe Suarez, who provided invaluable technical assistance and the rabbit antiserum to MHV-68. The pathologists Dr. Nguyen Hai and Dr. Edward DiCarlo helped with the interpretation of the histology. We are indebted to Leona Cohen-Gould for electron microscopy. Drs. Lionel Iavashik, Mary Crow, William Muller, and Ralph Steinman provided valuable comments.

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