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

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It is not known what is required for successive relapses in autoimmune diseases or evolution to a progressive chronic disease. Autoimmune arthritis caused by passive transfer of autoantibodies against glucose 6-phosphate isomerase is transient and therefore lends itself well to test for what might extend the disease. Herpesviruses have long been suspected of contributing to human autoimmune disease. We infected mice with a murine gamma-herpesvirus (MHV-68). In immunodeficient mice, transient arthritis was followed by a relapse. This was due to lytic viral infection of synovial tissues demonstrated by PCR, immunohistochemistry, and electron microscopy. Latent infection could be reactivated in the synovium of normal mice when treated with Cytoxan and this was associated with increased chronic arthritis. We conclude that herpesviruses may play an ancillary pathogenic role in autoimmune arthritis by infection of the inflammatory target tissue. The Journal of Immunology, 2004, 173: 5238–5246.

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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.

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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 KNX mice were purchased (The Jackson Laboratory, Bar Harbor, ME).
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 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 × 10^4 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.
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, K⁎TSINFVKI) (37) (Trudeau Institute, Saranac Lake, NY), with anti-CD8-Cychrome (BD Pharmingen, San Diego, CA) or with anti-Vβ4-FITC 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-solution (Surgipath Medical Industries, Richmond, IL), embedded in paraffin, sectioned (6 μm), and stained with H&E. For immunostaining (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'-AACCTGGAACTCTTTCTGC-3' and 5'-GGGCCGCGACATTTAAAGC-3' (586 bp); inner PCR primers were 5'-CCCCACTTGGTCATATAAGTG-3' and 5'-ATCAC CAGCCATACACATCTG-3' (382bp). Reactions contained 50 mM KCl, 10 mM Tris-Cl (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).

Results

MHV-68 infection alters serum-transferred arthritis

After transfer of serum from arthritic KxN mice into C57BL/6, we observed transient arthritis peaking at days 5–15 and of ~4-wk duration as previously reported (25–27). Severity and duration of disease was directly related to the dose of serum injected (data not shown). To examine the effect of viral infection on serum-transferred transient arthritis, we tested three viruses: MHV-68, HSV-1 (strain 17), and influenza virus (PR-8) (Fig. 1A). Upon infection with MHV-68, mild enhancement of severity and duration of the arthritis was observed in several experiments. However, arthritis remained transient. C57BL/6 mice and RAG1−/− mice infected with MHV-68, but without serum-transferred arthritis showed no joint swelling.

In immune-compromised mice, maintenance of latency for MHV-68 is often deficient. RAG1−/− mice succumbed from poorly controlled viral infection after 4 wk (30). There was no difference between arthritis in wild-type vs RAG1−/− mice (Fig. 1, B and C) (41). However, a second peak of arthritis was observed in infected RAG1−/− mice (Fig. 1C). In four experiments (21 mice), the second peak was always observed (Fig. 2). Not every limb of an affected mouse becomes fully arthritic. The course of arthritis was followed in 84 limbs. Affected joints were grouped according to the severity of the first peak of arthritis. There was a correlation between severity of the initial peak of arthritis and the severity of the second peak (Fig. 2), suggesting perhaps that initial influx of inflammatory cells might import rare virus-infected cells to the synovium.

Histologically, the joints of the infected RAG1−/− mice were more seriously involved. Compared with the normal synovium in a C57BL/6 mouse (Fig. 3A), uninfected mice with adoptive arthritis showed evidence of synovial thickening, residual inflammatory
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 V\(\beta\)4 CD8 response (43, 44) after day 20 and an earlier CD8 response to a peptide of viral P79 measured with K\(^{b}\) 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 V\(\beta\)4 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 10\(^6\) 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
Representative results are shown with averages of three mice. Average percent Vβ4/CD8 cells of the synovium (Fig. 6, A, B, and E) but adjacent subcutaneous and bone marrow tissues were spared even where bone marrow and inflammatory tissues were separated by a thin section of bone (Fig. 6B). Viral Ags were present both within cells and in the extracellular space (Fig. 6B). Some joints were less extensively involved (Fig. 6C), perhaps representing early stages of infection, with predominantly synovial lining cells staining for viral Ags. Viral Ag-positive synoviocytes invading the joint space may represent an early stage of a pannus (Fig. 6C). Tendon fibroblasts and tendon sheath lining cells were positive for viral Ags (Fig. 6F), which is of interest as tenosynovitis is a noted feature of human RA.

Similar observations were made with joint tissues from all infected RAG1−/− mice. However, tissue samples from immunocompetent mice lacked viral Ags even when synovial infiltrates were present (Fig. 6G). As Fig. 4B indicates, 10,000-fold lesser viral DNA in ankles of immunocompetent vs RAG1−/− mice, it is possible that viral Ags were present in insufficient amounts for detection by immunohistology. Productively infected joint cells would be rapidly cleared by a competent immune system, as suggested by the joint infiltration by virus-specific T cells (Fig. 5). To confirm that viral Ags in synovial tissues were due to productive infection, we used electron transmission microscopy. Intracellular and extracellular herpesvirus particles were abundant in synovial tissues (Fig. 6J) and in the spleen (data not shown). The mature particles measured on average 167 nm in diameter, contained a core capsid, tegument, and envelope glycoprotein spikes typical of herpesviruses. There were immature nuclear and cytoplasmic forms as well as fully mature virions and empty capsids. Some lytically destroyed fibroblasts were present in the synovium and a polymorphonuclear cell phagocytosing viral particles is shown (Fig. 6J). It should be noted that viral Ags were not found in nonarthritis joints from the same animals (Fig. 6, H and I).

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. 7, B 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). Comparing 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 spleen 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).
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, specially with cessation of immunosuppressive therapy. Whether this also happens 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 autoimmune 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, Coxsackie 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 adjuvants, e.g., CpG DNA and IFN-α. Viral Ags along with self-Ags from apoptotic cells might stimulate local immune responses, which have little to do with the original stimulus of the autoimmune response, in our case autoantibodies to GPI. Indeed, autoimmune is often characterized by a bewildering combination of seemingly unrelated autoimmune responses. This is also true for patients with RA (56–62). Thus, it will be of interest to examine the generation of new autoantibody specificities in the model of transferred KxN arthritis with or without MHV-68 infection. Indeed, this model is known to lack some autoantibodies typical of human RA, such as rheumatoid factors (25).

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 recurrence or maintenance of inflammation in target organs of the autoimmune disease.

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