Association of Measles Virus with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis; while the cause is unknown, it has been speculated that an infectious agent could be the trigger for the disease. Numerous attempts at isolating an agent have been unsuccessful. Our purpose was to identify a virus from diseased tissue from a patient with RA.

Methods. Diseased tissue taken at the time of knee replacement surgery from a patient with RA was inoculated into several cell lines and observed for cytopathic effect. Cells from the tissue were also grown as explants and were examined for viruses. Synovial fluid drawn 4 years prior to the surgery and frozen at –70°C was also inoculated into cell lines. Following the development of a cytopathic effect and identification of the agent, sera from 50 patients with rheumatoid factor (RF)-negative RA were examined for IgM antibodies to the agent.

Results. After many inoculations and numerous subpassages, measles virus was identified in 6 cell lines inoculated with either the minced tissue or synovial fluid. Six cell lines co-cultivated with one or more of 9 explants also showed the presence of measles virus. Measles virus was confirmed by immunofluorescence and by neutralization. Eleven of 50 (22%) sera samples from patients with RF-negative RA had IgM antibodies to measles virus recombinant nucleoprotein.

Conclusion. There is an association between measles virus and RA. (J Rheumatol First Release March 1 2009; doi:10.3899/jrheum.080856)

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects mainly synovial tissues. If left untreated, joint destruction develops, causing considerable disability and reduced quality of life. RA occurs worldwide, affecting approximately 1% of adults[1]. Women are more commonly affected, at a ratio of 3 to 1 and at a younger age than men[1]. In addition to disability, RA is associated with an increased risk for the development of cardiovascular disease, infections, and malignancies, especially lymphoma[2]. Further, the monetary cost of RA in industrialized countries is estimated at $26 to $32 billion per year[3].

The cause of RA is unknown; it has been hypothesized that a microorganism might be the trigger. Our study represents an effort to isolate a virus from diseased tissue from a patient with RA. After isolation and identification as measles virus, sera from 50 patients with rheumatoid factor (RF)-negative RA were tested for IgM antibodies to measles in order to establish the existence of a latent infection.

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MATERIALS AND METHODS

Virus isolation. Synovial tissue was removed from both knees from a patient with RA during knee replacement surgery with an interval of 6 weeks between surgeries. The patient was born in 1893 and had measles as a child. The synovial tissue was placed on wet ice, and brought immediately to the virology laboratory, Massachusetts Department of Health, State Laboratory Institute, Boston, MA. The tissue was finely minced and frozen at –70°C. This material was thawed 1 to 3 months later and inoculated into primary cell cultures and continuous cell lines: RMK, WI-38, HEK, and AGMK (Flow Laboratories, McLean, VA, USA) are all primary cultures; BHK-21, Vero, RK-13, and Hep-2 (American Type Culture Collection, Rockville, MD, USA) are continuous cell lines. These cell lines were used because they were commonly available in the virus isolation laboratory and because all had previously supported growth of a virus. Because the virus sought was unknown, it was hoped that at least one of the lines would be suitable for growth should there be a virus present.

Additional minced tissue was planted directly into flasks containing Medium 199 (Sigma, St. Louis, MO, USA) and 30% fetal bovine serum (FBS; Sigma). After these explanted cells were growing well the maintenance medium was Eagle's minimal essential medium (MEM; Sigma) with 2% FBS. Every 2 to 3 weeks fluid and a few cells from the explanted cultures were inoculated into new tissue cultures and lines (as above). In addition the minced tissue was maintained in culture for 3 years and then cells were scraped off and transferred to cell lines, producing a co-culture.

Synovial fluid obtained from the patient 4 years prior to surgery was stored at –70°C. This fluid was then thawed and inoculated into the same cell lines.

All cultures were examined 2 to 3 times per week for cytopathic effect (CPE), which was customary in this virus isolation laboratory, using standard virologic technique and assessed semiquantitatively as 1–4+. Subpassages were made blindly or to enhance what appeared to be a beginning CPE. When the pH became too acid (e.g., 6.5 from an ideal of 7.2), as demonstrated by a color change (from red to yellow), the MEM with 2% FBS was changed.

Measles virus was identified by indirect immunofluorescence[4]. Smears of infected and uninfected cells were incubated with bovine anti-measles antiserum, and then fluorescein-conjugated rabbit anti-bovine immunoglobulin (both from Burroughs Wellcome Co.; Research Triangle Park, NC, USA). Measles virus [Edmonston strain; US Centers for Disease Control (CDC), Atlanta, GA, USA] was used as a positive control. As a negative control the smears were tested using FBS as a substitute for the bovine anti-measles antiserum.

All smears were examined microscopically for immunofluorescence.

Measles was also identified by performing a neutralization test utilizing pre- and postvaccination guinea pig antisera specific for measles virus (Flow Laboratories). After the virus was showing 4+ CPE in Hep-2 cells, 0.5 ml of fluid from these tubes was mixed with 0.5 ml of either prevaccination or post-vaccination sera. After incubating for 30 min at room temperature, 0.5 ml of each mixture was added to fresh Hep-2 cells. These cells were examined for CPE over 21 days. Cells inoculated with virus plus prevaccination serum demonstrated a 4+ CPE within 19 days, while CPE did not form in cells inoculated with postvaccination sera. These neutralization experiments demonstrated that the virus causing the CPE was measles. A similar experiment was performed utilizing measles virus from the CDC, further confirming the specificity of the antisera.

A hemadsorption test was also performed; test is positive when viral hemagglutinins are incorporated into the cell membrane. Cells infected with the measles virus are detected by adsorption of AGMK erythrocytes (Flow Laboratories)[5]. While this test is not pathognomonic, a positive test does suggest the presence of measles. Uninfected cells were tested as controls; measles virus (CDC) was used as positive control.

Serology. After isolating a virus from diseased tissue we sought to corroborate this finding by serological methods in a larger cohort of patients with RA. Fifty sequential serum samples from patients with RF-negative RA were obtained from discarded samples in the Clinical Immunology Laboratory, Brigham and Women's Hospital (this protocol was approved by the hospital institutional review board). Their electronic medical records were reviewed and their demographic data are given in Table 1. Sixty sera were obtained from normal individuals as controls.

Measles IgM capture-enzyme immunoassay. Kits were obtained from Millipore (Temecula, CA, USA; catalog no. 4050); the assay is approved by the US Food and Drug Administration. The assays were performed using the manufacturer's protocol: 100 µl of each test serum (plus positive and negative controls provided with the kit), diluted 1:200 in the manufacturer's diluent, were added to 2 wells coated with anti-human IgM on plates provided by the manufacturer. The plates were then incubated 30 min at 37°C. After washing, 100 µl of measles virus nucleoprotein (P) was added to one well, and uninfected lysate (N) was added to the second well to control for specificity. The plates were then incubated 30 min at 37°C and then washed. Plates were next incubated for 30 min at 37°C with horseradish peroxidase (HRP)-conjugated monoclonal anti-measles antibody that binds to the (measles/anti-measles) immune complexes immobilized in the wells. Addition of the chromogenic substrate TMB reveals the presence of the immune complexes. The colorless TMB is oxidized by the HRP to a blue product.

This kit was developed for detection of acute-onset measles. As we were trying to detect latent or chronic infection, which is associated with an increase in IgM antibodies (see Discussion), whose titers might be lower than in an acute infection, we modified the procedure as follows: sera were diluted 1:50 instead of 1:200; sera were incubated for 60 instead of 30 min in step 1, allowing more time for IgM capture[6]. In addition, to control for specificity using the different dilution, 60 sera from normal individuals were tested in this modified assay to determine a normal range. The positive to negative ratio (P/N) was ≥ 7.44 for ≥ 3 SD above the normal mean and ≥ 6.32 for ≥ 2 SD above the normal mean.

Using the manufacturer's method a positive finding is defined as a P/N of ≥ 6.5, indeterminate as 4.5 to 6.5, and negative as < 4.5. For our modified method we calculated that positive was ≥ 7.44, indeterminate was 6.32 to 7.44, and negative was < 6.32. In addition, according to the manufacturer, the P − N value had to be ≥ 0.15 to be considered positive; we adopted the same guideline for the modified assay.

Optical densities were read in a plate reader (Titertek Multiskan) at 450 nm with a reference filter at 620 nm.

RESULTS

Virus isolation and identification. Cell cultures that became infected after receiving inoculum from minced diseased tissue began to show a cytopathic effect at a mean of 13 days. In order to see a fully developed CPE, that is, extensive syncytia, it was necessary to subculture at least twice. The virus grew well in HEK, WI-38, PMK, AGMK, Vero, and especially in the Hep-2 cell lines, but not in the BHK-21 or RK-13 cell

Table 1. Group characteristics.

Feature	Total Group	Positive Antibody (manufacturer method; positive and indeterminate)	Negative Antibody (manufacturer method; positive and indeterminate)	Positive Antibody (modified method; positive and indeterminate)	Negative Antibody (modified method; positive and indeterminate)
No.	50	24	26	13	37
Male/female	13/37	7/17	6/20	2/11	9/28
Age, mean yrs	57	57	56	57.5	56
Age at onset of RA, yrs	49	49	48	48	49
History of smoking (+/total)	10/43	5/19	5/24	1/13	9/37
Erosions (+/total)	9/41	3/17	6/24	2/13	7/37
ESR, mean mm/h	22	22	22	23.7	21.5
CRP, mean mg/dl	6.8	6.9	6.8	7.5	6.6
Anti-CCP, mean U	6	7.5	5.3	3.4	5.5
Therapy					
Prednisone	15	9	6	5	10
Plaquenil	12	5	7	4	8
Methotrexate	18	7	11	7	11
Anti-TNF	17	6	11	4	13

None of these differences was statistically significant.

lines. Uninoculated controls and controls containing just media were negative.

The patient's cells, which had been explanted directly after surgery, were maintained only by changes of medium as determined by the acidity. They were never trypsinized. The cells formed large islands but never a complete sheet. There was no CPE in the explanted cells. However, when cells from the explants were inoculated at intervals into any of the 6 cell lines that allowed viral growth, a CPE was obtained usually by 19 days after inoculation. The original explant was maintained for 3 years. After growth for the 3 years in 75-ml flasks, a few cells (approximately 50) from each of the 9 cultures were scraped off and inoculated into Hep-2 cell lines. At 16 days there was a suggestion of CPE. The fluids from these cultures were passed again to fresh Hep-2 cell lines. These cultures showed the complete spectrum of measles cytopathic effects: a few spindle and stellate forms progressing to 100% syncytia. Uninoculated Hep-2 cultures showed no CPE.

Synovial fluid that had been collected from this patient 4 years earlier and stored unopened at –70°C was thawed, and 3 drops were inoculated into each of 4 Hep-2 cell tubes. Syncytia developed by 19 days. Control tubes showed no CPE. A hemadsorption test using AGMK red blood cells was positive with the isolate grown on Hep-2 cells, indicating that the isolate was probably measles.

The indirect fluorescent antibody test using an isolate from one of the explants confirmed the identification of the isolate to be measles virus, as did the neutralization test. Controls utilizing just media were negative.

Subsequent inoculations confirmed that virus from the synovial fluids and 9 explants all grew on the same 6 cell lines, that is, HEK, AGMK, Vero, Hep-2, WI-38, and RMK, as virus that was isolated from the direct inoculation of diseased tissue. Thus the presence of the virus was confirmed in all 3 modes: direct inoculation, explants from both knees, and in synovial fluid that was available from one knee. Both direct inoculation and synovial fluid tests were short-term, that is, the CPE was evident in 2 to 3 weeks following direct inoculation (they were not held longer). Isolation from explants demonstrated the persistence of the virus in longterm cultures.

Three samples each of Vero cells, inoculated directly from diseased tissue, from explants or from synovial fluid, were tested by indirect immunofluorescence for measles virus; all 9 samples were positive. Three positive controls were positive by immunofluorescence; 3 uninoculated cell smears were negative.

Serology. Two of the 50 (4%) patients with RA were positive at the 1:200 dilution, and 9 (18%) at the 1:50 dilution. The 2 sera that were positive at 1:200 dilution were also positive at 1:50 dilution. Further, 22 (44%) additional sera were considered indeterminate in the manufacturer's assay (at the 1/200 dilution), and 4 (8%) in the modified assay at the 1/50 dilution. According to the manufacturer's guidelines these indeterminate values are considered "possible borderline positive." Therefore when the "positives" are added to the "possible pos-

itives," 24 of the 50 (48%) in the manufacturer's method and 13 of the 50 (26%) for the modified could also be considered potentially positive.

There was no statistical difference between those positive and negative in respect to sex, race, age, age of disease onset, history of smoking, erosions, mean anti-cyclic citrullinated peptide antibody, mean erythrocyte sedimentation rate, mean C-reactive protein, and use of anti-RA medication (Table 1).

DISCUSSION

This study demonstrates an association between RA and the measles virus. There are many common viral infections that are associated with arthritic symptoms. These arthritic symptoms may persist for weeks or months, but generally resolve. Viruses implicated in causing arthritis include hepatitis A, B and C[7], parvovirus[8,9], rubella[10,11], rubella vaccine virus[12], mumps[13], enteroviruses[14], adenoviruses[15], and herpes viruses[16-19]. Patients infected with human immunodeficiency virus can develop a self-limited arthritis usually lasting less than 6 weeks[20]. Alpha-virus infections are also associated with musculoskeletal symptoms. Infection with the Chikungunya virus was reported to cause chronic arthralgia for more than 3 years in 12% of patients[21].

There are no reports that measles is associated with arthritis[22]. However, measles virus can cause a subacute inclusion body encephalitis[23,24], as well as subacute sclerosing panencephalitis (SSPE), in which neurological symptoms develop years after the initial viral illness[25]. The patient had no symptoms or signs of SSPE.

In addition to neurological disease, measles has been thought to be involved in Paget's disease, in which measles antigens and nucleic acids have been observed in affected tissue, but the virus has not been isolated[26].

Measles is an extremely contagious virus. According to the CDC, in the prevaccine era, 3 to 4 million cases of measles occurred each year[27]. Today in the United States, because of the vaccination program that began in 1963, fewer than 100 cases are reported annually[27]. Persons born prior to 1963 are considered to be immune from wild-type measles infection[27]. Vaccination is typically given at about age 12 months. In our cohort, 40 of the patients were born prior to 1963, including all who were seropositive, except for one patient born in 1968. These observations suggest that most of the serological response reflected wild-type virus infection.

The mechanism by which measles virus can become latent is not known. One theory suggests that exposure to measles virus early in life, before the immune system can mount an adequate immune response, may contribute to viral latency[28]. The fact that measles vaccine virus is attenuated, but still viable, raises some possibilities. Some children may be protected enough by the vaccine to avoid overt "measles" disease, but may harbor the vaccine virus in their tissue. In addition, the vaccine virus may allow less virulent wild-type virus to become latent.

It is known that, *in vitro*, cells can become persistently infected with measles virus[29]. Continued subculture results in the disappearance of the cytopathic effect[29]. We noted the same phenomenon in our explants. These cells were allowed to grow for 3 years without antibody. There was never a CPE, but when cells were co-cultured at varying intervals during these 3 years with Hep-2 cell lines, marked syncytia developed, indicating the presence of viable virus. It seems likely/possible that *in vivo* the relentless persistence of virus leads to overwhelming production of antigen-antibody immune complexes, which in turn produce inflammatory pathology.

Our rationale for studying IgM antibodies is as follows: IgM antibodies are well known to be part of the early acute immune response to infection. However, IgM antibodies may also be elevated in the serum of patients with reactivation of a persistent viral infection[30-40] and chronic spirochete infection[41]. For our study we chose to test only RF-negative sera in order to exclude possible interference by IgM RF in the serological assay[42]. It is possible that RF-positive sera would react differently. It has been postulated that RF-positive and RF-negative RA might have different etiologies.

A possibility that must be considered is that the patient whose cells were cultured did not have RA but had a chronic measles infection. This seems unlikely, since several competent rheumatologists who evaluated the patient over 20 years each made a diagnosis of RA. Even if the measles infection had become latent or coexisted with RA, one could also assume that the measles virus was somehow involved in the RA process because it was found in diseased tissue.

There is also the possibility that the cell lines were contaminated with measles virus; this is unlikely, as so many different cell lines would all have to have been contaminated. Further, uninoculated cultures were negative for measles virus. As a precaution to prevent contamination, all of the isolation work was performed first in the morning before any other procedures were performed, and after the safety cabinet had been flooded with ultraviolet light overnight.

The finding of IgM antibody to measles virus in up to 56% of patients with RA also tends to corroborate an association between chronic measles by latency and/or infection in at least some patients with seronegative RA.

To our knowledge this is the first autoimmune disease in which a virus has been isolated and IgM antibody identified in patients with long-standing disease. It will be interesting to see whether other autoimmune diseases are also viral-associated.

An interesting hypothesis regarding the cause of RA has been set forth by Ford[11,43], that one or more antigens of an infectious agent react specifically with intrasynovial but not synovial blood lymphocytes. With prolonged antigenic stimulation autoimmune phenomena develop. The unsuccessful attempts to detect genomic measles RNA in the synovial fluid and peripheral blood of patients with RA are also worth pondering[22]. Patients selected for that study were required to have been diagnosed with RA within 1 year. The authors thought

they were less likely to detect measles RNA as the disease progressed. However, if a persistent virus such as measles attaches to cells and unceasingly churns out virus, lymphocytes will surely increase and combine with the agent in an attempt to vanquish the invader. The longer this goes on the more likely one is to isolate the inciting agent. The patient in our study had RA for 20 years.

Finally, antiviral drugs will need to be evaluated for their efficacy in the treatment of RA and other autoimmune diseases.

In summary, measles virus is associated with some cases of rheumatoid factor-negative rheumatoid arthritis; several avenues for future research are suggested.

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