EXACERBATION OF COLLAGEN-INDUCED ARTHRITIS IN RATS BY RAT CYTOMEGALOVIRUS IS ANTIGEN-SPECIFIC

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Collagen-Induced Arthritis (CIA) is an experimentally induced and genetically controlled animal model of chronic joint inflammation. In rats, there are informative strain differences in susceptibility to CIA. DA rats (RT1av) develop severe CIA after immunization with bovine (BII), chick (CII), or homologous rat (RII) type II collagens. In contrast, the MHC-congenic DA.IN(BN) and WF.IN(BN) rats (RT1n) are relatively resistant to CIA and develop moderate CIA in response to immunization with CII but not BII or RII. We previously found that simultaneous infection with rat cytomegalovirus (RCMV) greatly exacerbates the severity of arthritis that develops in BII-immunized DA rats. To examine the mechanism of RCMV amplification of CIA, the effect of simultaneous infection with RCMV on arthritis and autoimmunity to type II collagens was determined in WF.IN and DA.IN rats after immunization with BII, CII and RII. RCMV increased the incidence of CIA and the level of autoimmunity to type II collagen (skin-testing and IgG antibody titer) selectively in DA.IN and WF.IN rats immunized with CII, but not in littermates immunized with BII, although the transient reversal of CD4+/CD8+ mononuclear cell ratios in peripheral blood that is associated with RCMV infection occurred equally in both BII- and CII-immunized DA.IN rats. Likewise, RCMV infection moderately increased the levels of anti-RII autoimmunity and arthritis in DA rats sub-optimally immunized with RII but had no consistent effect on either anti-RHI immunity or arthritis in RII-immunized DA.IN and WF.IN rats. The data show that RCMV augments arthritis only in rats that are genetically susceptible to CIA and that are appropriately immunized with a species of type II collagen that is arthritogenic for the MHC-haplotype being tested. Two possible mechanisms are suggested by these data: RCMV-associated increases in anti-RII autoimmunity in rats with CIA may result from amino acid sequence homologies between RCMV and type II collagen; alternatively, virus-induced pro-inflammatory cytokines may activate RII-reactive lymphocytes thereby potentiating autoimmunity and arthritis.

KEY WORDS: Collagen, Arthritis, Rats, Cytomegalovirus.

INTRODUCTION

Immunization of rats with native type II collagen derived from sternal or articular cartilage induces an autoimmune arthritis, Collagen-Induced Arthritis (CIA), that is controlled by genes both linked and non-linked to RT1. The rat MHC, RT1, is crucial for the development of CIA. CIA requires the development of complement-fixing, anti-type II collagen antibodies and collagen-reactive CD4+ T cells1-3. The pro-inflammatory cytokines such as IFNγ, TNFα, and IL-1 potentiate arthritis in this model10-12. Thus, clinical expression of CIA can be modulated by in vivo manipulation of several immune parameters including changes in the levels or specificities of certain cytokines, T cell subsets or anti-collagen autoantibodies. The type II collagen molecule is a rod-shaped, helical, multi-determinant antigen. There are minor differences in amino acid sequence among the type II collagens of different species. These sequence polymorphisms are recognized as foreign with varying degrees of intensity by the immune response systems of different, genetically inbred rat strains14-16. In this way, they provide an immunogenic stimulus for the production of cross-reactive antibody and the expansion of autoantibodies. In this way, they provide an immunogenic stimulus for the production of cross-reactive antibody and the expansion of autoantibodies. In this way, they provide an immunogenic stimulus for the production of cross-reactive antibody and the expansion of autoantibodies.
Herpesviruses17-19. These common epitopes between a virus and type II collagen, provide a potential stimulator to the development of collagen autoimmunity and arthritis in the infected host due to molecular mimicry, a mechanism that can be viewed as analogous to immunization with heterologous collagens.

With the long term goal of elucidating the potential roles of viruses in human arthritis, we are investigating the effect of a known, exogenous viral infection, rat cytomegalovirus (RCMV), on the rat model of CIA. This system was chosen for several reasons. The host defense against viral infection, and particularly CMV infections, involves an increased production of several pro-inflammatory cytokines including IFNγ, IL-1 and TNFα.20-24 Murine CMV (MCMV) infections are known to elicit NK cells and also both CD4+ and CD8+ T cells with specificity for virus proteins25-27. Also, CMV has sequence homology with class I molecules, thereby potentially modulating antigen presentation to CD8+ T cells by virus infected macrophages and B-cells.28,29. Thus, CIA is an ideal model for evaluating the impact of acute and chronic viral infections on a genetically-controlled, chronic inflammatory process that is targeted to a biochemically well defined and major component of joint tissues.

We previously found that RCMV infection significantly increases the severity of CIA induced in DA rats by immunization with BII.21. RCMV augmentation of CIA was associated with elevated numbers of circulating B cells, a temporary reversal of the CD4+/CD8+ T cell ratio in peripheral blood, a transient increase in circulating NK cells and significant increases in anti-RII autoimmunities.22 However, because of the hyper-reactivity of the DA rat to any of several different arthritogenic compounds and because of the predictability of RCMV to establish latent infections, we could not determine conclusively whether RCMV augmentation of arthritis was due to the enhancement of anti-RII autoimmune reactivity per se or was due to the local production of cytokines by viral infected joint tissues.

RTI-linked genes exert primary control over the level and specificity of anti-collagen autoimmunity that develops in collagen-immunized rats. For example, WF (RTIu) and DA (RTIvl) rats are very highly susceptible to both BII- and CII-induced CIA while the RII isotype is resistant to BII22,23. Thus, WF.IN and DA.IN rats developed anti-collagen antibodies but not arthritis after immunization with BII. In contrast, both anti-collagen autoimmunity and arthritis occur in these two strains after immunization with CII. The data presented in this report show that RCMV augmentation of arthritis and autoimmunity to RII occur in DA.IN and WF.IN rats only when they are immunized with chick type II collagen. The data support the hypothesis that RCMV enhances CIA by promoting the reactivity of cells involved in the development of anti-RII autoimmunity and not by antigen non-specific mechanisms such as viral infection of joint tissues.

**METHODS**

**Rats**

DA (RTIu) and DA.IN rats were from BioLabs and Kingman (Freemont, CA). WF,1N(BN) and DA.1N(BN) are maintained by sib-sib matings in our colony at the University of Utah and their origins have been detailed.1 These are inbred, MHC-congenic strains that express the RTI+ genes of BN on the non-MHC genomes of WF and DA parental strain rats and were developed by classic backcrossing protocols. Experimental rats were housed in our animal resources facility at the Veterans Affairs Medical Center in pann provided with shavings to prevent trauma to arthritic paws.

**Collagens**

Chick (CII) and bovine (BII) type II collagens were purified from sternal and articular cartilages. Rat type II collagen (RII) was purified from a rat chondrosarcoma established in DA rats. Rat type II collagen (RII) was prepared from a rat chondrosarcoma carried on DA, WF.IN and DA.IN rats.24 A rabbit antiserum to rat cartilage was a kind gift of Dr. Michael Cremer, Veteran's Affairs Medical Center, Memphis, TN. Protease inhibitors (0.01 M EDTA, 0.001 M Benzamidine, HCl, 0.01 M N-ethylmaleimide) were included in the early stages of purification and all procedures were performed at 4°C. Collagens are stored lyophilized under desiccation at −20°C.

**RCMV, Propagation and Detection**

Our rat cytomegalovirus was originally obtained from Priscott25-27 and has been passaged 8 times through specific pathogen free DA rats. Virus is inoculated i.p. into 4-6 week old rats and salivary glands are harvested and pooled at three weeks. The salivary glands are homogenized (10%) in Eagles Minimum Essential Medium (MEM) with 10% fetal bovine serum as a stabilizer and stored in 1-2 ml volumes at −70°C. Concentrations of RCMV in inoculum pools and in salivary gland extracts are quantitated by titration assays (16-fold dilutions; 1:10 to 1:10⁶ for...
plaques-forming units (PFU) on monolayers of rat embryo fibroblasts grown in 96 well, flat-bottom, microtiter plates as previously described19.

**Collagen Induced Arthritis: Induction and Assessment**

Our standard protocol for induction of CIA15 was followed. Collagen was dissolved in cold 0.1 N acetic acid at 1 mg per ml and emulsified 1:1 (vol/vol) with IFA (Incomplete Freund's adjuvant; Difco). Rats at 8 to 12 weeks of age are injected intradermally at multiple sites on the back to obtain a final collagen dose of 2 mg/kg rat weight (100%). Rats are scored twice weekly for arthritis onset and severity. Arthritis is evaluated on an expanded scale such that the maximum possible score per animal is 2030. This scoring system includes an evaluation of each joint individually, thus providing a total score that is effectively weighted for articular surface areas. Rats were observed for 60–90 days after collagen immunization unless otherwise stated. Variations on this standard protocol are described in the text where appropriate.

**RCMV Augmentation of CIA**

Equal numbers of male and female rats were immunized with IFA emulsions of CII, BII, RII (dose: 0.5, 1.0 or 2.0 mg/kg) or 0.1 N acetic acid, the collagen solvent. Sub-optimum immunization schedules were used in some experiments so as to maximize the potential degree of RCMV augmentation of arthritis. The collagen concentrations were adjusted such that all rats received the same total amount of IFA relative to their body weight. Collagen emulsions were injected, intradermally on the back; test rats were simultaneously administered 5 × 10^5 to 1 × 10^6 PFU of a stock RCMV pool or PBS in 0.1 ml volumes by intraperitoneal injection. We have previously shown that RCMV alone does not induce arthritis in immununized nor in acetic acid: IFA sham immunized with RCMV or placebo (PBS) was administered by i.p.

**Measurement of Anti-Collagen Immunity**

IgG antibody titers to RII were determined by an ELISA assay using 96 well microtiter plates coated with native RII, blocked with bovine serum albumin and developed with a goat anti-rat IgG (heavy and light chain specific) second antibody conjugated with horse-radish peroxidase (Cooper Biomedical, Inc.) as previously detailed7. Sera are tested at 4-fold dilutions (initial dilution of 1:25) and plates are exposed at 4°C overnight. Positive and negative control sera are run on each plate in duplicate. Data are presented as antibody units (Ab U/ml) calculated by comparison with the linear portion of a standard curve established using a positive control serum pool arbitrarily assigned a value of 100,000 Ab U/ml with RII as the antigen. Sera were obtained from all rats at 28 days. Measurement of skin-test reactivity to native type II collagens has been previously described8. Rats are injected intradermally on the shaved abdomen with 50 μg test collagen in 0.05 ml volumes of 0.15M NaCl in 0.05 M Tris-HCl buffer, pH 7.4 and the diameters of induration and redness are measured at 24, 48, and 72 hours intervals. Positive skin test responses are those with mean diameters of ≥ 4 mm at 48 to 72 hours as calculated from 3 repetitive measurements by the same technician. Skin testing was performed at 2 or 8 weeks. None of the strains tested, including DA males and females, have shown any skin test reactivity nor pre-existing IgG antibody to native or denatured rat type II collagen under these conditions in naive rats.

**FACS Analysis of Circulating T Cell Subsets**

The ratio of CD4+ /CD8+ mononuclear cells in peripheral blood was determined by a modification of previously described techniques5 using a Model 151 Ortho Cytofluorograph outfitted with an Omnichrome argon laser and calculation of data with a Cytomation, Inc personal computer by Cytomation. Single color analysis of ficoll-hypaque purified rat mononuclear cells employed the following reagents obtained from Accurate Chemical & Scientific Corp (San Diego, CA): Primary (mouse IgG) monoclonal antibodies were: Clone W3/13 HLK to detect T lymphocytes — W3/13 reacts with B-cells and was used as a technical control stain for ficoll-hypaque centrifugation, brain and plasma cells. It does not react with B-cells and was used as a technical control stain in the current study. Clone W3/25 was used to detect CD4+ T cells — W3/25 recognizes the rat CD4 glycoprotein expressed on the T helper subset of T cells and on peritoneal macrophages which would be absent in the test lymphocyte suspension; Clone MRC OX-8 was used to detect CD8+ T cells and Natural Killer cells (NK cells). The second antibody was a fluorescein-conjugated, affinity purified F(ab')2, fragment of a donkey anti-mouse IgG (heavy and light chain specific) antisera obtained from Jackson Immuno-Research Laboratories Inc (West Grove, PA). For this analysis, test rats were immunized with BII or CII at 1 mg/kg (50% standard dose) and not boosted. RCMV or placebo (PBS) was administered by i.p.
injections as described. Peripheral blood was obtained from randomly selected rats of each group by cardiac puncture under ether anesthesia. Rats were bled only once. Simultaneous analyses of test rats were usually in the ratios of, CII + PBS (1); CII + RCMV (3); BI + PBS (1); BI + RCMV (3). In addition, data on naive DA.IN and DA rats were obtained for comparison as part of each sequential analysis of the test rats. Data are presented as the ratio of CD4+ to CD8+ periphera blood mononuclear cells at each time point and as group means ± S.D.

**Statistical Analysis**

One-tailed student's t-test was used to analyze serum titers of IgG anti-RII antibody for significance of RCMV-associated increases of this autoantibody in CII- versus BI-immunized WF.IN and DA.IN rats. Paired data comprised mean group responses of RCMV-infected versus RCMV-non-infected rats of the same strain and sex tested in the same experiment and under identical conditions of immunization but with or without virus infections.

## RESULTS

**RCMV Augmentation of Arthritis in CII-Immunized Versus BI-Immunized Rats**

The effects of RCMV infection on the incidence of CIA in DA, DA.IN and WF.IN rats is shown in Figure 1. These data combine the results of 6 different experiments in which male and female rats of each strain were immunized with collagen at dosages that are equivalent to 25%, 50%, or 100% of the standard immunization dose of 2 mg/kg. Rats were considered positive for arthritis if they developed severity scores of 4+ or greater and showed sustained arthritis for a minimum of 10 days. RCMV-infected and non-infected rats were included in each experiment. RCMV infection caused, respectively, 2 and 3.5 fold increases in the incidences of arthritis in the WF.IN and DA.IN rats that had been immunized with CII. There were no significant differences in RCMV effects on CIA among the three CII dosage groups. In contrast, RCMV did not increase CIA in WF.IN or DA.IN rats that were immunized with BI (100% dose) or that were injected with an acetic acid/IFA control emulsion.

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>ARTHRITIS INCIDENCE (%)</th>
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<tbody>
<tr>
<td>STRAIN NUMBER COLLAGEN</td>
<td>20  40  60  80  100</td>
</tr>
<tr>
<td>DA 6 6 CHICK 0</td>
<td></td>
</tr>
<tr>
<td>DA 6 8 CHICK +</td>
<td></td>
</tr>
<tr>
<td>DA.IN 40 6 CHICK 0</td>
<td></td>
</tr>
<tr>
<td>DA.IN 15 6 BOVINE 0</td>
<td></td>
</tr>
<tr>
<td>DA.IN 16 6 BOVINE +</td>
<td></td>
</tr>
<tr>
<td>DA.IN 14 6 NONE 0</td>
<td></td>
</tr>
<tr>
<td>DA.IN 13 6 NONE +</td>
<td></td>
</tr>
<tr>
<td>WF.IN 23 6 CHICK 0</td>
<td></td>
</tr>
<tr>
<td>WF.IN 22 6 CHICK +</td>
<td></td>
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<tr>
<td>WF.IN 11 6 BOVINE 0</td>
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<td></td>
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<tr>
<td>WF.IN 10 6 NONE +</td>
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**Figure 1** RCMV infection increases the incidence of CIA in CII-immunized but not BI-immunized RII+ rats. Equal numbers of male and female WF.IN and DA.IN were immunized with IFA emulsions of BI (2 mg/kg) or CII (2, 3, or 0.5 mg/kg) rat weight and were (+) or were not (0) infected with RCMV (1 × 10⁶ PFU/ml) and subsequently observed for CIA for 60 days. None: Control rats were immunized with IFA emulsions with 0.1% acetic acid, the solvent for CII or BI. Data represent combined results of 6 different experiments.
Although DA.IN rats are resistant to chronic BII-induced CIA, DA.IN males occasionally and sporadically develop a low incidence of mild erythema that is associated with a slight swelling of one or two distal joints which are tender to touch and manipulation. The reaction onsets at 23 to 28 days after immunization with BII and is transient, resolving within 72 to 96 hours. This very weak response occurred in 2/15 BII-immunized (male) DA.IN rats without RCMV infection and in 4/16 BII-immunized and RCMV infected DA.IN rats (3 males and 1 female). The severity score for this BII induced response was 1.7 ± 0.4 (mean ± S.D.) compared with severity scores ranging from 20 to 50 in CIA-immunized DA.IN rats. This transient, mild joint inflammation was not observed in any of 23 BII-immunized WF.IN rats in either the presence or absence of RCMV. These combined results show that RCMV augmentation of CIA occurs only in RTI15 rats that are immunized with a species source of type II collagen (chick) that is fully arthritogenic for rats with this MHC haplotype.

**RCMV Augmentation of CIA Is Not Overtly Influenced by Gender**

The data in Table 1 compare CIA in a subpopulation of the RCMV-infected and RCMV-non-infected, DA.IN and WF.IN, male and female rats that were immunized with CII at 50% and 100% dose ratios and presented in Figure 1. RCMV infection primarily affected the incidence of clinically detectable CIA rather than the rate of onset or the severity of joint inflammation. Control DA rats simultaneously immunized with the same CII emulsions developed CIA in 100% incidence by 29 days and with a mean arthritis score of 63 ± 2. Sex-associated differences in susceptibility to CII-induced CIA were seen in DA.IN rats but not in WF.IN rats. WF.IN male and female rats showed similar responses to CII-immunization (37%; 23% respectively) and reached equivalent incidences of CIA when infected with RCMV (58% ± 60%). In contrast, the incidence of CIA in CII-immunized DA.IN male rats (6.7%) was much lower than that of CIA-immune female DA.IN rats (24%). When infected with RCMV, CII-immunized male and female DA.IN rats showed the same magnitude of increase in the incidence of CIA (2-3 fold) and therefore CIA-immunized and RCMV-infected DA.IN females continued to show a higher incidence of arthritis (50%) than similarly immunized and RCMV-infected DA.IN males (20%).

**Effects RCMV on Anti-Type II Collagen Autoimmunity in CIA Immunized WF.IN and DA.IN Rats**

In association with the higher incidence of clinical arthritis, RCMV increased the incidence of positive skin test responses to CII in DA.IN rats as measured 8 weeks after immunization with a single injection of a CII-IFA emulsion at 25% the optimum dose (Table II). In prior studies with BII-immunized DA rats, we found the greatest enhancement of skin-test reactivity to RII by RCMV was evidence at 14 days after the primary immunization. Thus in a second study, female DA.IN rats were immunized with CII at 50% dose (1 mg/kg), injected with RCMV (10^6 PFU) or PBS, and skin tested at day 14 with CII and RII. In these rats, RCMV infection increased the incidence and diameter of skin test reactivity to 50 μg CII from 3/6 and 4.3 ± 4.1 mm (means ± S.D.) respectively, in the absence of RCMV to 6/6 and 8.5 ± 0.6 mm in the presence of RCMV (p < 0.05). Likewise, skin test reactivity to RII in the day 14 analysis of CIA-immunized DA.IN rats were elevated from 2/6 positive responses (2.7 ± 1.8 mm) in RCMV-negative rats to 5/6 positive responses (4.8 ± 1.7 mm) in the RCMV-infected rats. RCMV infection was also associated with a two to three-fold increase in serum IgG anti-RII antibody.

**Table I Effect of RCMV on Incidence of CIA in CII-immunized male and female RTI15 rats**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>RCMV</th>
<th>Arthritis</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incidence</td>
</tr>
<tr>
<td>WF.IN</td>
<td>M</td>
<td>0</td>
<td>4/11</td>
</tr>
<tr>
<td>WF.IN</td>
<td>M</td>
<td>*</td>
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<td>1/15</td>
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<td>M</td>
<td>*</td>
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<tr>
<td>DA.IN</td>
<td>F</td>
<td>*</td>
<td>9/18</td>
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</table>

Rats were immunized with chick type II collagen at 5 mg/kg of CII emulsions at 1 mg/kg or 3 mg/kg rat subcutaneously injected with 0.1 ml PBS (0) or RCMV (+). Data are group means ± S.D. for arthritis severity (maximum possible score of 80) and onset of arthritis (days) within a 90 day observation period.
TABLE II  

RCMV infection increases the incidence of arthritis and positive skin test responses to type II collagen in CII-immunized DA.IN rats.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RCMV</th>
<th>ARTHRITIS* (Incidence)</th>
<th>SKIN TEST† (Incidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA.IN</td>
<td>0</td>
<td>1/8</td>
<td>0/8</td>
</tr>
<tr>
<td>DA.IN</td>
<td>+</td>
<td>5/8</td>
<td>6/8</td>
</tr>
<tr>
<td>DA</td>
<td>0</td>
<td>4/5</td>
<td>2/2</td>
</tr>
<tr>
<td>DA</td>
<td>+</td>
<td>6/6</td>
<td>2/2</td>
</tr>
</tbody>
</table>

*Rats were immunized with CII in IFA emulsions at 0.5 mg/kg. Skin tests were with 50 μg CII in 0.1 ml PBS at 8 wks. Positive DTH: ≥4 mm diameter at 48-72 hours. Incidence: No. positive/No. tested.

†Titer in both male and female DA.IN and WF.IN rats that were immunized with CII at each of the three doses (Figure 2). This increase was statistically significant for both the WF.IN (p < 0.05) and the DA.IN (p < 0.005) rats. In both strains, the highest levels of anti-RII antibody developed in RCMV-infected rats that were immunized with CII at 2 mg/kg and not boosted. The approximately 10-fold difference in anti-RII titers between comparable groups of WF.IN and DA.IN rats were expected; this large interstrain difference in serum titers of anti-RII antibody activity is characteristic of WF versus DA non-MHC genomes. In contrast, RCMV did not significantly alter the levels of IgG anti-RII antibody in BII-immunized WF.IN rats. A small increase in anti-RII antibody was seen in BII-immunized male DA.IN rats which was consistent with the mild transitory joint inflammation that occurs, albeit infrequently, in BII-immune DA.IN rats. Statistical analysis confirmed that IgG anti-RII antibody levels in BII-immunized rats were not significantly increased by RCMV in either WF.IN (t = 1.08) or DA.IN (t = 0.53) rats. Two of 3 BII-immunized, DA, emulsion-control rats developed arthritis and produced antibody levels of 5,000 ± 1,200 units/ml showing that the BII:IFA emulsion used in generating this data was active in produc-
ing arthritis and pathogenic anti-RII autoimmunity in CIA-susceptible DA rats.

The effect of RCMV on arthritis and collagen autoimmunity in DA.IN and WF.IN rats after immunization with autologous RII was also examined (Table III). Kyoto strain DA, DA.IN and WF.IN rats were responders to RII, showing negative skin test reactivity to RII at 56 days and mild to no arthritis. There was no consistent effect of RCMV infection on 28 day anti-RII antibody levels nor on 42 or 56 day skin test reactivity to RII in RII-immunized DA.IN rats. As previously observed in DA.IN rats immunized with heterologous type II collagens, RII-immunized DA.IN rats showed a very low incidence of a mild and transitory joint inflammation which was not substantially increased by RCMV infection. Among RII-immune WF.IN rats, RCMV infection boosted the level of IgG anti-RII antibody by approximately 50% but skin test reactivity to RII was negative and none of the RII-immune WF.IN rats developed joint inflammation, whether or not they were RCMV-infected.

Among DA rats, which are very susceptible to RII-induced CIA, RCMV caused a modest increase in the severity of arthritis (score of 49 ± 8 vs 64 ± 8) and tripled the level of IgG anti-RII antibody. In DA rats immunized with this dose of RII (2 mg/kg), skin test reactivity to RII at 56 days was high in the absence of RCMV and was not increased further by RCMV infection. The effect of RCMV on skin test reactivity to RII in RII-immunized DA, DA.IN and WF.IN rats was also measured at 14 days. For this analysis, rats were immunized at the standard dosage of RII emulsified with IFA but were not boosted at 7 days. Under these conditions, RCMV infection increased the rate of onset of arthritis and the level of skin test reactivity to RII at 14 days among RII-immunized DA rats but not among DA.IN nor WF.IN rats (Table III).

Titters of IgM anti-RII antibody in 21 day serum samples were determined (data not shown) and were not consistently altered in RII-immunized DA.IN or WF.IN rats. Neither IgM anti-RII nor IgG anti-RII antibodies were detected in 21 day serum from any of 6–8 control rats of all three strains that were infected with RCMV but not immunized with collagen.

CD4+ and CD8+ Peripheral Blood Mononuclear Cells in RCMV Infected Rats

RCMV infection causes a temporary reversal of the CD4+/CD8+ T cell ratios in naive and BII-immunized DA rats. This response peaks at day 8 and parallels the induction of increased NK cell activity in peripheral blood. To question the role of CD8+ cytotoxic lymphocytes and/or associated cytokines in the RCMV augmentation of CIA, we determined the circulating CD4+/CD8+ mononuclear cell ratios in DA.IN rats at weekly intervals after they were infected with RCMV and simultaneously immunized with either BII or CII (Table III). Immunization with collagen alone did not significantly change the CD4+/CD8+ mononuclear cell ratios in RCMV-infected rats over time. In contrast, RCMV infected DA.IN rats showed a temporary reversal of CD4+/CD8+ mononuclear cell ratios that was evident at day 7, reached a nadir at day 14 and gradually returned to normal by day 28. Importantly, this change in the subset composition of the circulating mononuclear cell populations was seen in both CIA-immune and BII-immune DA.IN rats although RCMV augments arthritis only in CIA-immunized DA.IN rats. Thus, the lack of augmentation of CIA in the BII-immune RT1n rats with RCMV is not due to an ineffective, aberrant or suboptimal mononuclear cell response to RCMV in the BII-immunized rats.

Verification of RCMV Infections

In all experiments, salivary glands were obtained from experimental and control rats at 60 days, homogenized and analyzed for RCMV infectious particles by a standard plaque-forming assay as previously described. Equivalent titers of RCMV plaque forming units (10^2–10^1 PFU/ml of 10% homogenate) were recovered at 56 days from the salivary glands of RCMV-infected DA, DA.IN and WF.IN rats regardless of the collagen immunization or the presence or absence of arthritis. Thus, comparable levels of viremia likely developed in all three strains although exacerbation of arthritis was strain and collagen specific. All control rats (collagen-injected, not RCMV inoculated) were negative for RCMV in salivary gland extracts (at ≥1:100 dilutions) showing that there was no horizontal spread of RCMV during the experimental observation times.

DISCUSSION

There are several mechanisms by which RCMV could exacerbate arthritis and autoimmunity in the rat model of CIA: 1) molecular mimicry; 2) induction of pro-inflammatory cytokines; 3) latent infection of joint tissues. All of these hypothetical mechanisms are compatible with our current understanding of the immunopathology of CIA. For example, the severity of arthritis in the CIA model generally reflects the level of anti-RII autoimmunity. A very sensitive arthritogenic response to RII-immunization is associated with the RTI^6 haplotype of DA rats. By introducing viral proteins having amino acid sequence homologies with type II collagen, RCMV could augment CIA in DA rats by providing an antigenic stimulus to type II...
TABLE III RCMV amplifies CIA in RII-immunized DA rats but not in RII-immunized WF.1N nor DA.IN rats.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group I</th>
<th>Group II</th>
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<tbody>
<tr>
<td></td>
<td>Skin Test (14 days)</td>
<td>Antibody (28 days)</td>
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<tr>
<td>DA.IN</td>
<td>3.5 ± 2.4</td>
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<tr>
<td>WF.IN</td>
<td>0.0</td>
<td>0/5</td>
</tr>
<tr>
<td>DA.IN</td>
<td>0.0</td>
<td>0/5</td>
</tr>
<tr>
<td>DA</td>
<td>5.5 ± 0.5</td>
<td>0/2</td>
</tr>
<tr>
<td>DA</td>
<td>9.0 ± 1.0</td>
<td>3/3</td>
</tr>
</tbody>
</table>

**Skin testing with 50 RII in PBS; group near diameters ±S.D. in mm.
***Mild transitory arthritis of < 5 severity scores compared to mean score of 50-60 in RII immune DA rats.

Collagen auto-reactive T and B lymphocytes. Alternatively, Il-1 and IlFN are transiently elevated during acute CMV infections. Il-1, IlFN and Tfnx exacer­bate CIA, presumably by activating synovial cells and chondrocytes to release cartilage destroying enzymes. Finally, Cytomegaloviruses establish latent infections in several cells and tissues important to CIA such as macrophages and the bone marrow. CD4+ and CD8+ T cells specific for virus proteins are recruited during CMV infections22. RCMV infection of DA joint cells and local activation of anti-viral immune reactivities in a tissue that is already compromised by the presence of complement-activating, anti-collagen antibodies on nearby cartilage surfaces could present clinically as an augmented arthritis. In this situation, increased anti-RII antibody levels might actually derive from cartilage breakdown products and RCMV amplification of anti-RII immunity should be most evident late and after arthritis onset rather than during the early stages of arthritis and acute infection.

One approach to differentiating among these mech­anisms, is to analyze the antigen-specificity of RCMV amplification of CIA. Because DA rats are highly susceptible to CIA induction by diverse species of heterologous and homologous type II collagen including bovine, porcine, human, deer, rat and chick6, it was not possible to test for antigen-specificity of RCMV augmentation of CIA using this strain. WF.1N and DA.IN rats were selected for study because they develop CIA only after immunization with CII and are very resistant to CIA induction by immunization with mammalian type II collagen5. Also, past analyses of antisera raised in DA.IN and WF.1N rats, have shown that these rats develop significantly different profiles of immune reactivity towards a panel of renatured, cyanogen-bromide peptides derived from RII after immunization with BII as compared to CII15. Thus, there are important differences in epitope recognition between RT1+ and RT1− rats in the CIA model. By using these two strains under conditions of sub­optimum immunization, we were able to demonstrate direct correlations between RCMV infection, higher incidences of arthritis, higher, IgG anti-RII serum antibody titers, and increased levels of skin test re­ponses to RII and CII in RII-immunized WF.1N and DA.IN rats. Importantly, RCMV infection precipi­tated arthritis and amplified the immune response to RII only in RT1− rats that were immunized with chick type II collagen. In contrast, RCMV-infected but BII-immunized RT1+ rats did not develop arthritis and did not show increased levels of anti-RII autoimmu­nity at either 14 or 56 days, although these rats experi­enced an RCMV viremia similar to that of their CIA-immune siblings. Likewise, RCMV infection en-

### TABLE IV The transient effect of RCMV infection on peripheral Mononuclear cell subsets is identical in BII- and CII-immunized DA.IN rats

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4*/CD8* Mononuclear Cell Ratios</th>
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<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>CII + PBS</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>CII + RCMV</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>BII + PBS</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>BII + RCMV</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

DA.IN rats were immunized with BII or CII at 1 mg/kg and injected i.p. with RCMV (106 PFU) or PBS. CD4* and CD8* T circulating mononuclear cells were quantitated by FACS analysis using specific monoclonal antibodies and FITC-conjugated secondary antibody. Data are means ±S.D. of 3 rats (2 females, 1 male) at each time point.

CD4*/CD8* ratios for naive DA.lN x FACS control rats (n = 6) were 2.2 ± 0.2.
hanced the anti-RII autoimmune reactivity and arthritis in DA rats that were immunized with rat type II collagen, but had no significant effect on RII-immunized WF.1N and DA.IN rats, both of which are resistant to RII-induced CIA.

Because of the hyper-reactivity of the DA rats to a variety of stimuli, it was of interest to determine if other rat strains were subject to modification of CIA by RCMV infection or if the phenomena was unique to DA rats. DA.IN and WF.1N rats carry the major histocompatibility complex (MHC) alleles of the BN rat strain on the non-MHC genomes of DA and WF rats respectively. The finding that RCMV augments CIA in both DA.IN and WF.1N rats demonstrates that neither RT1avl alleles nor non-MHC genes of DA origin are necessary for viral exacerbation of arthritis and anti-RII immunity. In summary, these data show that RCMV augments CIA only in rats that are genetically susceptible to CIA and that are appropriately immunized with a species of type II collagen that elicits an arthritogenic, autoimmune response to RII in that particular strain of rat. Importantly, this phenomena is not restricted to DA rats.

RCMV-associated reversals of CD4+ /CD8+ mononuclear cell ratios occurred in BII-immunized DA.IN rats without causing an increase in anti-RII immune responses and without precipitating clinical arthritis. Likewise, we found no evidence for differences in the systemic spread of virus particles among BII- and CII-immune DA, DA.IN and WF.IN rats as detected by recovery of infectious (PFU) particles from salivary tissue of test rats. The combined data argue against the selective RCMV augmentation of CIA in CIA-immune RT1avl rats being due to viral infection of joint tissues because all rats apparently experienced similar acute, viremic episodes. These results do not negate the possibility that anti-RCMV CD8+ T cells (NK cells or CTL's) plus virus-elicited IL-1 and INFy contribute synergistically to the severity of arthritis in RCMV-infected RT1avl rats. For example, administration of recombinant IL-1 is reported to precipitate CIA in mice only if they are of a genetically susceptible strain.

DA female rats develop a more severe arthritis than DA males in both the CIA and the adjuvant arthritis models. In this study, DA.IN females suboptimally immunized with CII had a higher incidence of arthritis than DA.IN males. However, RCMV infection augmented CIA in both male and female DA.IN rats to the same extent such that the incidence of CIA in DA.IN females was more than twice that of DA.IN males regardless of the presence or absence of RCMV. Likewise, CII-immunized, WF.1N male and female rats (which do not show gender differences in CIA) were equally affected by RCMV infection. These data argue against RCMV amplification of CIA being due to disruption of the normal levels of corticosteroids, sex hormones or other sex-linked, pro-inflammatory mediators.

Because there is a ten-fold difference in serum anti-RII antibody titers between collagen-immunized WF.1N and DA.IN rats, even though the two strains show equivalent clinical CIA phenotypes, the exacerbation of CIA by RCMV cannot be attributed solely to an overall increase in total anti-RII autoantibodies. An alternative hypothesis, is that RCMV could enhance CIA by causing a shift in the epitope specificity of anti-type II collagen reaction response towards novel or more pathogenic determinants on exposed cartilage collagens. For example, a major arthritogenic determinant of murine CIA is found on the chick cyangogen-bromide peptide CB-11. A short amino acid motif (F-x-y-Q) was identified in the immunodominant region of chick CB-11, in the glycoprotein B of MCMV, and in the mycobacterial heat-shock proteins that is associated with the adjuvant arthritis model of autoimmunity in rats. RT1avl strains develop a higher antibody response to the CB-11 peptide of RII when immunized with CII as compared to RII that directly correlates with the induction of arthritis in RT1avl rats by CII but not BII. Alternatively, rats develop substantial immune reactivity to other regions of RII and this is particularly true of DA rats. There is substantial sequence homology between EBV proteins and the C-terminal one/fifth of the helical portion of human type II collagen. This region is a major antigenic target in humans with HCMV and EBV infections and is analogous to the EBV,7 peptide of RII, an important B cell antigen in the rat model of CIA. Thus, RCMV may increase anti-RII immune reactivity in genetically susceptible rats by antigenic mimicry and much of this autoimmunity may be directed towards arthritogenic B cell and T cell epitopes of RII.

In summary, the data in this paper have shown that viral infection can magnify the severity of ongoing CIA in association with an increase in autoimmune reactiv­ity to cartilage collagen. This suggests that the phenomenon is antigen-specific and occurs only in genetically sus­ceptible rats. Importantly, neither RT1avl genes nor non-RT1 genes of the hyper-reactive DA rats are a pre-requisite for RCMV-augmentation of CIA. Also, the fact that RCMV infection did not precipitate ar­thritis in BII-immunized WF.1N rats, although these rats developed substantial anti-RII antibody, argues strongly against RCMV-amplification of CIA being due to latent joint infections. The two most likely mecha­nisms for amplification of CIA are molecular mimicry and virus-induced, release of immunomodulating and pro-inflammatory cytokines into joint tissues, draining nodes, and adjacent bone marrow. These are not mutu­ally exclusive effects of RCMV infection and both may prove to be important mechanisms of a virus-associated initiation or flaring of arthritis in humans with autoimmune rheumatic disease.
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FOOTNOTES

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3) Abbreviations used in this manuscript are: B1, CII, Ri, Type II collagen prepared from bovine, chick, and rat cartilage, respectively; CIA, collagen-induced arthritis; PFU, plaque forming units; RTI, rat major histocompatibility complex; RCMV, rat CMV.