Effects of Free Immunoglobulin Light Chains on Viral Myocarditis

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Rationale: In recent work, we have demonstrated a crucial role of mast cells in the development of viral myocarditis. Viral infection could lead to increased synthesis of free immunoglobulin light chains (FLC) and our earlier work showed that FLC can trigger mast cell activation.

Objective: We studied the possible involvement of FLC in the pathogenesis of viral myocarditis, and therapeutic effects of FLC using an animal model of viral myocarditis.

Methods and Results: DBA/2 mice were inoculated intraperitoneally with encephalomyocarditis (EMC) virus. Serum levels and concentrations in the heart of κ FLC on day 14 in mice inoculated with EMC virus were significantly increased compared with controls. Myocardial viral concentration was significantly inhibited, the area of myocardial lesions was smaller in mice treated with κ or λ FLC, and survival of mice given FLC significantly improved. In contrast, an FLC antagonist deteriorated myocarditis. κ and λ FLC chains inhibited EMC viral replication in human amnion cells in vitro. λ FLC significantly increased the gene expression of interleukin-10 in the heart which was previously shown to improve viral myocarditis when given exogenously. FLC also tended to increase the gene expressions of interferon-α and -γ in the heart mice.

Conclusions: FLC have antiviral and antiinflammatory effects and improved viral myocarditis in mice. FLC may be promising agents for the treatment of viral myocarditis. (Circ Res. 2010;106:1533-1540.)

Key Words: free immunoglobulin light chains ■ virus ■ myocarditis ■ heart failure ■ cardiomyopathy

Free immunoglobulin light chains (FLC) are produced by plasma cells and can be found in various body fluids. Interestingly, viral infection has been shown to increase the occurrence of immunoglobulin free light chains in various body fluids.1–3 In this study, we show that FLC concentrations are greatly increased during viral myocarditis and that FLC may play a protective role in the pathogenesis of disease.

Immunoglobulin is composed of 2 identical heavy chains and 2 identical light chains and provides defense against all extra-cellular and some intracellular pathogens.4 In mammals immunoglobulin light chain genes generally exist in 2 distinct isotypes called κ and λ. The genes for the 2 light chain isotypes are encoded at separate and unlinked loci, and the organization of κ and λ chain locus differs significantly.5,6

Previous results have suggested various roles for nuclear factor (NF)-κB during B cell development, ranging from involvement in light chain gene rearrangements7 to being dispensable for B cell development.8 Because receptor editing involves a specific subset of light chain gene rearrangements, it has been suggested that NF-κB could be involved in light chain rearrangements without being required for B cell development.9 In additions, the role NF-κB in receptor editing could be either direct or indirect, and its activation in pre-B cells might be via the canonical or noncanonical pathway.9

Recent studies have clarified the roles played by several cytokines in various cardiovascular disorders,10 and have found increased levels of circulating tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and other proinflammatory cytokines in patients with myocarditis, cardiomyopathy, heart failure and acute myocardial infarction.11,12 NF-κB regulates the expression of a wide range of genes involved in immune and inflammatory responses. In our previous study, EMCV activated NF-κB in vitro. In a murine model of EMCV-induced myocarditis, a NF-κB inhibitor lowered the mortality of the animals, as well as attenuated myocardial necrosis and cellular infiltration, and decreased the intracardiac production of IL-1β and tumor necrosis factor-α.13 Therefore, it is possible that production of immunoglobulin free light chains may be modulated in viral myocarditis.
We also studied the gene expressions of interferons and IL-10 which had beneficial effect in our model of viral myocarditis.14–16

Methods

Experimental Myocarditis Model
Stocks of the myocardiotrophic variant of encephalomyocarditis (EMC) virus were prepared as previously described17,18 and stored at −80°C. The 4-week-old male DBA/2 mice used in this study were treated in accordance with local institutional guidelines at all stages of the experiments. Mice were inoculated with 0.2 mL EMC virus (EMCV) in PBS diluted to a concentration of 10 pfu/mL.

Effects of FLC Antagonist on Experimental Myocarditis
Tamm–Horsfall protein (THP), a monomeric glycoprotein produced by cells in the ascending limb of Henle of the kidney, has a specific binding affinity to FLC. F991 is a 9-mer peptide derived from the amino-acid sequence in THP responsible for binding.19 F991, a FLC antagonist, was synthesized as previously described20. To investigate the effect of F991 on viral myocarditis, 50 μg of F991 dissolved in 0.5 mL of PBS was administered intraperitoneally immediately after EMCV inoculation and once daily until day 5. Control mice were given PBS. Mice were euthanized at day 5 for histological studies.

Analysis of FLC Levels in Serum and Heart
Before sample use, serum samples were precipitated to deplete high amounts of albumin using trichloroacetic acid/acetone as previously described.21 Heart tissue samples were crushed in liquid nitrogen using a mortar and pestle and dissolved in 300 μL of PBS containing 0.5% NP-40 (Sigma-Aldrich, Tokyo, Japan) and 1 tablet of Protein Inhibitor Cocktail (Roche, Almere, The Netherlands). Homogenates were centrifuged at 14,000 g of total protein) samples were separated by SDS-PAGE and blotted onto a PVDF membrane (Bio-Rad Laboratories, Veenendaal, The Netherlands). The next day, membranes were incubated with 0.1 μg/mL HRP-labeled goat anti-mouse light chain (SouthernBiotech, Birmingham, Ala) or a light chain (Genway Biotech, San Diego, Calif) diluted in block buffer (2% non fat dry milk in PBS/0.1% Tween-20 for 5 minutes, blocked in block buffer for 2% non fat dry milk in PBS/0.1% Tween-20) for 1 hour, and incubated with 0.1 μg/mL HRP-labeled goat anti-mouse κ light chain (SouthernBiotech, Birmingham, Ala) or a light chain (Genway Biotech, San Diego, Calif) diluted in block buffer for 1 hour. After washing the membranes with PBS/0.1% Tween-20 3×10 minutes, ECL (Thermo Scientific, Ettten-Leur, The Netherlands; for serum) or ECL plus (GE Healthcare, Buckinghamshire, UK; for tissue samples) was used for detection of immobilized antibodies according to the manufacturer’s protocol, followed by film (CL-XPosure film; Thermo Scientific, Ettten-Leur, The Netherlands) exposure and development. Densitometry was performed on a GS-710 Calibrated Imaging Densitometer using software package Quantity One (Bio-Rad Laboratories, Veenendaal, The Netherlands) to analyze the density of the appropriate bands.

Effects of FLC on EMC Virus Myocarditis
Human κ FLC (10 μg/mouse), or a FLC (10 μg/mouse) (Bethyl Laboratory Inc, Montgomery, Tex) were dissolved in 0.2 mL distilled water and given subcutaneously or intraperitoneally daily starting 1 day before the EMCV inoculation (each n=10). Control mice were given 0.2 mL of distilled water. At day 5, we observed that some mice began to die, which was expected, and could have been attributable to viremia and/or encephalitis. Sera were obtained from surviving mice, and were euthanized by cervical dislocation on day 5 for the histopathologic experiments and viral concentration assay. The hearts were dissected, immediately frozen and stored at −80°C, and the section of interest fixed in formalin.

Histopathologic Examination
We examined the histopathologic changes on day 7, the time when they began to appear. The hearts were fixed in 10% formalin and embedded in paraffin. The left ventricles (LV) were sliced horizontally to the long axis, and stained with hematoxylin, eosin and Masson’s trichrome for light microscopic examination. The extent of myocardial lesion in the complete section was evaluated by measuring the ratio (%): myocardial necrosis area/
Inc.) 0.1 g/mL, or F991, 1 µg/mL was added to culture plates, and plaque formation was observed 24 hours after EMCV inoculation. Plaque formation in presence of FLC was calculated as percentage of control plaques. Myocardial viral concentrations were determined only in the euthanized mice, and expressed as plaque-forming units (pfu) per milligram of myocardium.

**Quantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis**

Total RNA was isolated from the heart tissue by the acid guanidinium thiocyanate-phenol-chloroform method and the RNA concentration was measured spectrophotometrically. First-strand cDNA was synthesized using SUPERSCRIPT Preamplification System for FirstStrand cDNA Synthesis (Invitrogen). Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp, Foster City, Calif) was performed according to the manufacturer’s protocol. Interferon-α and IL-10 were performed using SYBR green Master Mix (Life Technologies). We used 2 µL of the first-strand cDNA in the following assay.

Synthesis of cDNAs was controlled and normalized by performing real-time PCR amplification of the housekeeping gene GAPDH for mouse. The following forward and reverse oligonucleotides, and probes were used for the quantification of mouse interleukin-10, interferon (mIFN)-α, mIFN-β, mIFN-γ.

- GAPDH, forward: 5′-TTACCAACACATGGAGAAGGC-3′
- GAPDH, reverse: 5′-GGCATGGACGTGCTAGTA-3′
- GAPDH, probe: 5′-TCTGTAGACGGAGTGGCC-3′
- Interleukin-10, forward: 5′-GGTTGCCAACCACTATGCTTAG-3′
- Interleukin-10, reverse: 5′-ACC TGC TCC ACT GCC TTG CT-3′
- mIFN-α, forward:5′-TCTGTAGACGGAGTGGCC-3′
- mIFN-α, reverse: 5′-AGGGCTCTCCAGACTTCTGCT-3′
- mIFN-β, forward:5′-GACTGTGGCATCACCTGCTATG-3′
- mIFN-β, reverse:5′-AAGCAGAAGGTACCCCAGGTTC-3′
- mIFN-γ, probe: 5′-TACAGTGGCATCACCTGCTATG-3′
- mIFN-γ, reverse: 5′-AGGGCTCTCCAGACTTCTGCT-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′
- mIFN-γ, probe: 5′-AAAGGAAAGCAACAGCGTTGTC-3′

**Figure 2.** Immunoglobulin FLC levels are increased in hearts of mice with viral myocarditis. **Left,** Anti-κ Ig light chain immunoblot of serum proteins separated by nonreducing SDS-PAGE. **Left lanes 1 to 6,** Heart tissue from EMCV-infected animals collected at day 14 after inoculation. **Right lanes 1 to 5,** Heart tissue from control animals. κ FLC monomers and FLC dimers appear at approximated molecular weights of 22 and 44 kDa, respectively. **Right,** Quantification of κ FLC monomers in heart tissue by calibrated densitometry. Significant difference between monomer FLC in control (n=5) vs EMCV-infected heart tissue (day 14 after inoculation) (n=6). *P<0.0001.

**Figure 3.** Immunoglobulin FLC levels are increased at day 14 after viral infection. **A,** Representative anti-κ Ig light chain immunoblot of serum proteins separated by nonreducing SDS-PAGE. **Left 4 lanes,** Serum from control animals collected at day 6 and 14. **Right 6 lanes,** Serum from EMCV-infected animals at day 6 and 14 after inoculation. κ FLC monomers and FLC dimers appear at approximated molecular weights of 22 and 44 kDa, respectively. **B,** Quantification of κ FLC monomers in serum using calibrated densitometry. Serum from control animals (n=10) and from EMCV-infected heart tissue (n=12) were collected at day 6 and 14 after inoculation or mock-treatment. *P<0.01.
The conditions for the TaqMan PCR were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s and 60°C for 1 minute.

Statistical Analysis
Comparisons were made using one-way analysis of variance with multiple comparisons by Fisher’s least significant difference. A probability value <0.05 was considered significant. Results were expressed as means±SD.

The survival rate of mice was analyzed by the Kaplan–Meier method, and survival differences between groups were tested by the log-rank test.

Results
Effects of FLC Antagonist F991 on EMC Virus-Induced Myocarditis in Mice
To investigate whether FLC were involved in the pathogenesis of virus-induced myocarditis, the FLC antagonist F991 was injected daily during 5 days after inoculation. Surprisingly, inhibition of free light chains exacerbated myocarditis as evidenced by an increase in the area of myocardial lesion at day 5 in the hearts of mice treated with F991 (21.5±8.2%, n=10) as compared with controls (9.1±4.8%, n=10, *P*<0.001; Figure 1A and 1B).

FLC Levels in Heart Tissue and Serum of Mice With Viral Myocarditis
Next, we examined if FLC could be detected in the heart tissue of mice inoculated with EMCV. On day 14, monomer κ FLC concentrations were significantly higher in virus infected heart tissue compared to controls (211.6±18.6 OD/ mm² (n=12) versus 29.2±37.6 (n=10); *P*<0.001) (Figure 2). FLC dimers were also increased in EMCV-infected hearts, but were not detectable in control hearts. Similar results were found for λ FLC in heart tissue, but as expected for mice,23...
were monitored subsequently. Various hallmarks of the viral myocarditis were daily injected subcutaneously with 10 g/mouse, SC) (n = 10) or solvent only (n = 10). Significant increased survival was observed in the κ FLC-treated group (80% vs 0% in controls, P < 0.001).

Myocardial Histology

The area of myocardial lesion on day 7 was significantly smaller in the hearts of mice treated with κ FLC (9.3 ± 5%, n = 10, mean ± SD) or λ FLC (9 ± 3.6%, n = 10) compared with controls (16.9 ± 8%, n = 9, P < 0.01) (Figure 4A and 4B). Intraperitoneal treatment with λ FLC improved myocardial damage similarly (7.9 ± 4.1%, n = 10 versus control 13.3 ± 4.8%, n = 10, P < 0.05; Figure 4C).

Survival

All virus-inoculated mice died before day 14. However, 80% of mice treated with κ FLC were still alive at day 14 (n = 10, P < 0.001) (Figure 5).

Myocardial Virus Concentration

Myocardial virus concentration at day 7 was (4 ± 3) × 10^3 pfu/mg in mice treated with κ FLC (n = 10), (6 ± 5.7) × 10^3 pfu/mg in mice treated with λ FLC (n = 10), and (14 ± 9) × 10^3 pfu/mg in control mice (n = 9). The difference between the FLC groups and the controls was statistically significant (P < 0.01 and P < 0.05, respectively) (Figure 6).

FLC Reduce Viral Replication in an In Vitro Plaque Assay

The in vivo experiments indicated that FLC reduced viral load of the animals. To investigate whether FLC may have direct effects of viral replication, an in vitro plaque assay was performed. Interestingly, κ FLC 0.1 μg/mL or 1 μg/mL reduced plaques to 87 ± 15% (61.83 ± 10.53 plaques P < 0.05) or 72 ± 7% (51.33 ± 6.44 plaques P < 0.01) of control 71.33 ± 8.02 plaques, respectively. λ FLC 0.1 μg/mL or 1 μg/mL reduced plaque formation to 68 ± 8% (48.33 ± 3.20 plaques P < 0.01) and 67 ± 4% (48.17 ± 5.46 plaques P < 0.01) of control 71.33 ± 8.02 plaques (Figure 7).

F991 Enhances Viral Replication In Vitro Plaque Assay

F991, 1 μg/mL enhanced plaque formation to 871 ± 42% (37.01 plaques vs 9.33 plaques in control) plaques control (65.40 ± 10.24 plaques vs 15.33 plaques in control) plaques at day 6 (P < 0.01, Figure 7).

Gene Expressions of Mouse Interleukin-10, Interferon-α, β, and γ mRNA

The gene expression of IL-10 in the heart of mice treated with 10 μg of λ FLC significantly increased (0.86 ± 0.84, versus 0.36 ± 0.10, P < 0.05, Figure 8). The gene expressions of
interferon-α, and γ tended to increase in the hearts of mice treated with 10 mg of λ FLC (0.27±0.28 versus 0.14±0.23, 2.28±1.58 versus 1.51±0.9/GAPDH, respectively; Figure 8). However, these differences did not reach statistical significance. There was no significant change in the expression of interferon-β.

Discussion

In this study, we show that FLC production is greatly enhanced during infection with EMC-virus. Furthermore, FLC are shown to have protective effects in viral myocarditis, most likely through direct antiviral activity and antiinflammatory effect by increased expression of IL-10.

It seems to be paradoxical that FLC levels are elevated, but that FLCs are beneficial for viral myocarditis. However, increased circulating levels of certain molecules do not necessarily mean that they have harmful effects in the diseases. For example, circulating brain natriuretic peptide levels are increased in heart failure, but beneficial when it is given heart failure patients. Nesiritide, an human brain natriuretic peptide, has been shown to improve hemodynamics and clinical status.

Figure 7. κ and λ FLC reduce EMCV replication in vitro. Virus replication was measured in a plaque assay in presence of 0.1 and 1.0 μg/mL κ or λ FLC, respectively. Viral replication 24 hours after EMCV inoculation was calculated as number of plaques in the experimental groups vs control (no FLC added). Values represent means±SD (n=6). Significant difference vs control: *P<0.05; **P<0.01.

Figure 8. The gene expressions of IL-10 (A) and interferons α (B), β (C), and γ (D). λ FLC significantly increased the expression of IL-10 and tended to increase the expression of interferon-α and -γ.
In all classes of immunoglobulin, the light chains comprise 1 of 2 subtypes, known as κ and λ. Immunoglobulin light chains are synthesized in excess during the generation and assembly of complete immunoglobulins and can be found in the circulation under normal physiological conditions, whereas during inflammatory conditions, greatly enhanced concentrations are found in various body fluids. Immunoglobulin free light chains exert a range of biological activities including enzymatic activity (proteolysis of diverse substrates), specific binding activity for substrates and antigens and binding to different cells, although its immunologic function remains to be clarified. We found that circulating κ FLC monomers and dimers are slightly increased in mice with EMC viral myocarditis on day 6 when myocardial necrosis becomes apparent, but are significantly increased on day 14 when heart failure developed. Infection with EMCV was also associated with an increased expression of FLC in mouse heart tissue at day 14. We are the first to report increased FLC expression in an animal model of viral infection. Our data are supported by analysis of FLC in virus-infected human subjects, where increased levels of λ and κ FLC in sera and/or cerebrospinal fluid have been found in patients infected with immunodeficiency type-1 virus and hepatitis C virus and in cerebrospinal fluid from patients with viral meningitis. Recently, we found that circulating λ FLC was elevated in patients with myocarditis and heart failure (A Matsumori, T Nakano, T Shimada, T Horii, T Isomura, J Hoshino, H Suma, M Shimada, S Miyazaki, A Nagata, unpublished observation, 2009).

Application of the FLC antagonist F991 during the first period of viral infection worsened pathology of viral myocarditis suggesting a protective role of FLC. The latter was further substantiated by the protective effect of supplementation of FLC at the start of the viral infection leading to reduced necrosis and greatly enhanced survival. Our data indicate that FLC may directly inhibit viral replication as shown in an in vitro plaque assay. Indeed, in FLC-treated animals viral load of the heart tissue was significantly reduced.

Enhancement of viral replication in vitro by F991 supports the concept that deterioration of myocarditis by F991 in vivo may be attributable to the effect of enhancing viral replication.

Intravenous immunoglobulin (IVIG) is produced from pooled plasma of thousands of healthy humans. During the 1940’s-50’s, it was used for its anti-infectious properties, to treat viral diseases and congenital immunodeficiency. Over the years there has been accumulating evidence of IVIG efficiency in various other indications. Currently, there are 6 FDA approved indications for IVIG treatment: primary immunodeficiency, pediatric HIV infections, secondary immunodeficiency in chronic lymphocytic leukemia, idiopathic thrombocytopenic purpura, Kawasaki disease, and allogenic stem cell transplantation. Nevertheless, IVIG is accepted as first line treatment in other indications such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, multifocal motor neuropathy, dermatomyositis, stiff-person syndrome, fetal alloimmune hemolytic disease, and various autoimmune diseases. IVIG has antiinflammatory properties and induces a change of circulating cytokines. Therefore, it was considered as treatment for heart failure patients, induced an antiinflammatory effect, with an associated significantincrease of left ventricular ejection fraction regardless of the cause of the chronic heart failure. IVIG treatment also has been reported in patients with myocarditis. A wide variety of immunoglobulins are present in the IVIG preparation. Therefore extended spectrum of activities may arise including immunomodulation, antinflammatory and anti-infectious properties.

However, IVIG treatment in patients with recent-onset cardiomyopathy did not affect improvement of the left ventricular ejection fraction and functional capacity during follow-up.

In our previous study, exogenously administered IL-10 induced myocardial lesion in our model of viral myocarditis. In this study, gene expression of IL-10 in the heart was increased in mice treated with λ FLC, suggesting that a part of the effect of λ FLC might be attributable to this effect.

In addition, the gene expressions of interferon-α and -γ tended to increase in the heart of mice treated with κ and λ FLC, suggesting that these effects may also contribute to inhibition of viral replication and improvement of myocardial lesions.

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Disclosures
None.

References
Novelty and Significance

What Is Known?
- Immunoglobulin protects against pathogens. It is composed to 2 identical heavy chains and 2 light chains.
- It is well known that free immunoglobulin heavy chains protect against infectious disease, but the function of the free light chains (FLCs) remains unclear.
- Mammalian immunoglobulin light chains exist in 2 distinct isotypes: λ and κ. Although the immunoglobulin free chains display a range of biological activities (eg, catalysis, proteolysis), their immunologic function is not known.

What New Information Does This Article Contribute?
- Circulating κ FLC monomers and dimers are increased in encephalomyocarditis (EMC) virus-induced myocarditis.
- Treatment with an FLC antagonist worsens pathology of viral myocarditis.
- Treatment with the κ FLC increased the gene expression of IL-10 in the heart.

This study demonstrates for the first that circulating FLCs and FLC levels in the heart are increased during viral myocarditis and that measurement of FLCs may be useful for the diagnosis of myocarditis. FLCs inhibit viral replication in vitro and treatment with an FLC antagonist increases replication. Treatment with FLCs inhibits viral replication in the heart and improves myocardial lesions and the survival of mice with viral myocarditis. Measurements of FLCs may be useful for the diagnosis of viral myocarditis and FLCs may be useful therapeutic agents to prevent cardiac injury during viral myocarditis.
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