Chapter 21
XMRV: Emerging Human Infection or False Alarm

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1 Background

XMRV is closely genetically related to xenotropic murine leukemia virus (MLV or MuLV), a ubiquitous rodent gamma-retrovirus. XMRV was discovered in 2006 during analysis of the association of gene single nucleotide polymorphisms (SNPs) in the 2′,5′-A-dependent Ribonuclease L gene (RNaseL) with prostate cancer [1]. It was proposed that XMRV was the first gamma-retrovirus that might be related to human disease. Later, it was claimed that XMRV might be associated with chronic fatigue syndrome (CFS) as well. The prevalence of XMRV in the general population was unknown; however, its putative prevalence was reported in specific disease populations [2]. Research on the epidemiology, risk, and pathogenesis of some prostate cancers supported the involvement of XMRV in association with allelic variants of the RNaseL locus [3]. These early reputed discoveries of XMRV were from prostate secretions as well as prostate tissues [1, 4–6]. XMRV infection was linked to high-grade prostate cancer with putative evidence for the presence of XMRV DNA and protein expression. Immunohistochemistry (IHC) showed XMRV

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proteins in malignant prostate epithelial cells but were not correlated with XMRV infection and RNaseL SNPs. It was proposed that there might be a more widespread population at risk for XMRV infection and prostate cancer [7]. In addition, in a serum-based assay to detect neutralizing antibodies to XMRV in prostate cancer patients, neutralizing antibodies against XMRV were found that correlated with PCR and fluorescence in situ virus detection in prostate tissue [8].

XMRV was also implicated in chronic fatigue syndrome (CFS). Many studies had searched for viral causes of CFS that had remained unconfirmed and the supposed linkage of CFS with XMRV took the field by surprise [9]. In the new studies, XMRV was putatively detected by culture from blood and blood component of patients with CFS and in their respiratory secretions [10, 11]. In addition, MLV-like *gag* sequences were detected in 86.5 % of PBMCs from CFS patients vs. 6.8 % from healthy volunteers [12]. These studies also suggested XMRV infections could be transmitted to permissive cell lines from CFS patient plasma [13]. However, additional studies failed to show any correlation of XMRV and CFS using quantitative PCR and neutralizing antibodies [14–17]. Indeed, many XMRV studies showed cumulative evidence for laboratory contamination as discussed later in this chapter [18]. In this regard, Table 21.1 summarizes literature that dealt with XMRV detection and Table 21.2 summarizes literature indicating that purported detection of XMRV was due to laboratory contamination.

Table 21.1 XMRV association with disease

Disease	XMRV associated	XMRV unassociated
Prostate cancer	Urisman (2006) [5] Dong (2007) [4] Kim (2008) [71] Fischer (2008) [32] Hong (2009) [6] Knouf (2009) [69] Arnold (2010) [8] Schlaberg (2009) [7] Bhosle (2010) [72]	Sfanos (2008) [73] D'Arcy (2008) [74] Hohn (2009) [36] Martinez-Fierro (2010) [75] Cornelissen (2010) [76] Verhaegh (2010) [77] Switzer (2011) [39] Sakuma (2011) [40] Khodabendehloo (2013) [43] Rezai (2013) [44]
Chronic fatigue syndrome	Lombardi (2009) [10]	Erlwein (2010) [47] Groom (2010) [17] Van Kuppeveld (2010) [49] Hong (2010) [78] Switzer (2010) [16] Henrich (2010) [48] Hohn (2011) [15] Shin (2011) [27] Satterfield (2011) [50] Schutzer (2011) [68]
ALS		McCormick (2008) [62]
Lymphoid malignancies		Waugh (2011) [63]
SLE		Balada (2011) [64]
Autistic disorders		Lintas (2011) [65]
Fibromyalgia		Luczkowiak (2011) [67]

Table 21.2 XMRV laboratory contamination

Study	Technique
Hue (2010) [36]	Taqman qPCR for XMRV *pol* sequences
Oakes (2010) [46]	Taqman qPCR for XMRV *pol* sequences
Robinson (2010) [41]	PCR assays for mitochondrial DNA (mtDNA) or intracisternal A particle (IAP) long terminal repeat DNA
Sato (2010) [15]	One-step RT-PCR kits amplifying the partial gag of XMRV or other MLV-related viruses(primer sets are 419F and 1154R, and GAG-I-F and GAG-I-R)
Sakuma (2011) [40]	Real-time PCR of XMRV gag sequences and nested PCR for XMRV/MLV gag sequences
Yang (2011) [37]	PCR with XMRV specific primers
Garson (2011) [42]	Nucleotide BLAST searches using each of the 14 integration site sequences against the GenBank nr database
Sfanos (2011) [79]	XMRV/MLV IHC; PCR using genomic GAPDH PCR primers; full-length or near full-length viral genomes prepared from the LAPC4, VCaP and EKVX cell lines; Vectorette PCR using a virus-specific forward primer and a vectorette-specific reverse primer; phylogenetic analysis and infectivity assay
Tuke (2011) [45]	Lo et al.'s modified XMRV gag TaqMan assay, using probe P2 and primers F3 and R4, which is able to co-detect the pMLVs [12]
Knox (2011) [52]	PCR and RT-PCR in specimens from CFS patients and those previously positive for XMRV
Katzourakis (2011) [80]	PCR using MLV-like sequences in longitudinal specimens from patients 15 years apart

2 General Virology

XMRV is a gamma-retrovirus with a positive single-stranded RNA genome, 95 % sequence similarity to endogenous MLV, and 93–94 % similarity to several exogenous murine viruses [2, 5, 19]. Using RNA from prostate tissues, an XMRV molecular clone was constructed that was replication-competent in a few PC cell lines, e.g. DU145 and LNCaP. The XMRV-long terminal repeat (LTR) was expressed as well [4, 20]. Purported XMRV infection of primary prostatic epithelial and stromal cells was enhanced by acid phosphatase semen-derived enhancer of virus infection (SEVI). In addition, XMRV RNA was detected in prostatic secretions of some men with PC. It was thus proposed that XMRV might be spreading in the human population [6]. However, many subsequent studies failed to detect XMRV and to verify these disease associations. Moreover, evidence of contamination of specimens, which will be discussed later in this chapter, led Stürzel et al. to study further. They generated replication competent XMRV reporter viruses encoding a green fluorescent protein or a secretable luciferase as tools to analyze virus infection of human cell lines or primary human cells. Transfection of proviral DNAs into LNCaP prostate cancer cells resulted in readily detectably reporter gene expression and production

of progeny virus. Inoculation of known XMRV susceptible target cells revealed that these virions were infectious and expressed the reporter gene, allowing for a fast and highly sensitive quantification of XMRV infection. This technique showed that both reporter viruses were capable of establishing a spreading infection in LNCaP and Raji B cells and could be easily transmitted. However, after inoculation of primary human blood cells such as CD4 T cells, macrophages or dendritic cells, infection rates were very low, and a spreading infection was never established. Moreover, XMRV-infected primary cells-derived supernatants did not contain infectious virus. This evidence suggested that even though XMRV can replicate in some human cell lines, all tested primary cells were largely refractory to XMRV infection and did not support viral spread. Therefore, these results indicated that XMRV is not a human pathogen [21]. The concern became uppermost, that there has been widespread laboratory viral and reagent contamination with murine retroviruses.

3 Antiretroviral Inhibitors

Antiretroviral therapeutic (ART) drugs marketed for treatment of HIV infection had activity against XMRV, in vitro. The retroviral integrase inhibitors, raltegravir and L-000870812, two nucleoside reverse transcriptase inhibitors, zidovudine (ZDV), and tenofovir disoproxil fumarate (TDF) inhibited XMRV replication. It was proposed that using these drugs in combination would delay or prevent the selection of drug-resistant viruses that occurs with HIV treatment [22, 23]. However, XMRV is highly resistant to nonnucleoside reverse transcriptase inhibitors [24]. Additional studies were done advocating the use of ARTs to inhibit the replication of XMRV in PC and CFS. This was, however, at a point in time when there was little information yet about contamination [25]. Nonetheless, APOBEC3 as well as ARTs inhibited XMRV in another study [26]. However, antiretroviral off-label treatment of CFS was proposed as unjustified [27]. This caveat definitely should be applied to possible ART treatments of any postulated XMRV-associated disease.

4 Molecular Biology

The PC 1 (HPC-1) gene is also known as RNaseL (2′,5′-oligoisoadenylate synthetase dependent) and is located at 1q24-q25 in the human genome [28, 29]. Patients with prostate cancer carrying a mutation in the HPC-1 gene locus had stromal cells surrounding prostate tumors that purportedly contained XMRV [30]. The RNaseL innate immunity pathway has been studied since its discovery in 1978 and shown to be a primary line of defense against viruses by cleaving viral RNA [31]. Some RNaseL SNP variants with lower activity were considered permissive for XMRV growth [1]. Specifically, the RNaseL variant SNP, R462Q is associated with prostate cancer and is a prostate cancer susceptibility factor. The homozygous state of

RNaseL allele R462Q (QQ) is underrepresented and XMRV was rarely detected in nonfamilial prostate in Northern European cancer patients. In those patients with the RNaseL allele R462Q (QQ) genotype, 40 % was XMRV positive. In familial prostate cancer patients, XMRV was detected rarely (less than 1 %) [32]. Coevolution of XMRV and RNaseL was hypothesized to result in the spread and pathogenesis due to XMRV [3]. Questions of laboratory viral and reagent contamination have underscored the more recent interpretation of the results. Be that as it may, many additional genes have been implicated in prostate cancer including the following genes (with abbreviations): CTBP2 (C-terminal binding protein 2, active gene expression in prostate tissue), MSMB (microseminoprotein, beta, produces a semen protein, decreased in prostate cancer), LMTK2 (lemur tyrosine kinase 2, cyclin-dependent kinase 5/p35-regulated kinase involved in spermatogenesis), KLK3 (kallikrein-related peptidase 3, prostatic specific antigen [PSA], serum protease, elevated in prostate cancer), JAZF1 (JAZF zinc finger1, normally transcription repressor), CPNE3 (copine 3, mediate membrane–cytoplasm interaction), IL-16 (interleukin-16, lymphocyte chemoattractant factor, anchors ion channels), CDH13 (cadherin 13 (truncated), calcium-dependent cell–cell adhesion glycoprotein), EHBP1 (EH domain-binding protein 1, Links clathrin-mediated endocytosis to the actin cytoskeleton), NUDT10 (nudix-type motif 10, nucleoside diphosphate linked moiety X, inositol phosphatase, signal transduction), and NUDT11 (nudix-type motif 11, nucleoside diphosphate linked moiety X, inositol phosphatase, signal transduction) [28].

5 Detection Methods

As described in the previous sections, detection of XMRV was done in several methods. In the research setting, XMRV was detected using nested and real-time PCR and immune assays included serologic assays, flow cytometry, Western blot, and enzyme-linked immunosorbent assay (ELISA). Initially, SNPs in the RNaseL were used during the initial discovery of XMRV-associated diseases by being identified as the hereditary prostate cancer 1 gene [1]. Further investigations of XMRV in prostate tissues, Dong et al. constructed a Full-Length, Replication-Competent XMRV Clone by using two overlapping partial cDNAs of XMRV strain VP62 and validated by the complete sequencing of the full-length XMRV VP62 (GenBank accession no. EF185282) [4]. Other methods in the early XMRV discoveries utilized microarray-based screening. This method was designed to screen for viruses from all known viral families. The amplified and labeled fragments contained amplified and labeled host and potential viral sequences, then hybridized to a DNA microarray (Virochip, University of California San Francisco, San Francisco, United States). The researchers recovered the entire XMRV genome from the tumor and further examined the association of the virus and the RNASEL genotype by using nested RT-PCR [5]. Moreover, quantitative PCR was used to amplify XMRV proviral DNA from formalin-fixed, paraffin-embedded tissues; and IHC using XMRV-specific antibody detecting XMRV in prostate tissues [7]. In one study, several methods were

used in different type of specimens. Genotyping of RNASEL variant using TaqMan genotyping assay (Applied Biosystems, Foster City, CA). Nested PCR analysis byAmpliTaq gold Kit (Applied Biosystems) is used for detection of XMRV in tissues; and Qiagen QIAquick gel Extraction Kit was used in sequencing DNA bands. Fluorescence in situ hybridization (FISH) assay, which was generated by excision of the full-length XMRV cDNA from the pXMRV plasmid1 using NotI and HindIII restriction enzymes (New England Biolabs, Ipswich, MA) has also been used [8]. Serum-based assay was used to detect neutralizing antibodies against XMRV proteins [7, 8]. Nested PCR or real-time PCR in blood samples or PBMCs were also used in early investigation of XMRV in correlation with CFS [10, 11]. The U.S. Food and Drug Administration (FDA) has not approved any of these methods for testing in the clinic. Moreover, donated blood is not screened for XMRV [33–35].

6 Evidence for Contamination

Many publications confirm continued lack of detection of XMRV. Contradictory and irreproducible results of recent research on the possibility that XMRV may be a human pathogen and a cause of prostate cancer and CFS support questions of contamination. The detection of laboratory reagent and tissue viral contamination is of central concern and vitiated the basis for the original findings related to XMRV. XMRV had been found in healthy controls and the XMRV-specific PCR primers later were found to amplify common murine endogenous viral sequences. Mouse DNA-contaminated patient specimens and nonspecific PCR reactions confounded XMRV detection. XMRV that was isolated from the tumor cell line 22Rv1 was similar to unlinked patient-derived XMRV. The *pol* sequences from these PC patients were possibly derived from XMRV and Maloney MLV. The original findings were made further questionable because the Maloney MLV envelope showed a lack of tropism for human cells [36]. Based on the analysis of the DNA from CWR22 and 22Rv1, the presence of XMRV in 22Rv1 was likely an artifact [37].

Many studies did not support the correlation between XMRV and prostate cancer. Studies in Germany showed a prevalence of 12.9 % for the homozygous SNP R462Q mutation in prostate tumor specimens but failed to show either antibodies for the XMRV gag and envelope proteins or XMRV-specific RNA or DNA in these tissues [38]. XMRV was rarely detected in nonfamilial prostate cancer specimens with homozygous mutation R462Q (QQ) [32]. In addition, detection of XMRV DNA by PCR in PC patient tissue revealed no correlation between XMRV serology and had very low (1.9 %) detectable XMRV DNA, undetectable mouse DNA, and was negative for viral RNA [39]. More recently, the lack of XMRV sequences and of strong anti-XMRV neutralizing antibodies indicated no or very low prevalence of XMRV in a cohort of 110 PC patients and 40 benign prostrate specimens. The prior positive real-time PCR results were due to laboratory and reagent contamination and positive IHC-specimens were due to nonspecific immune reactions [40].

Robinson et al. examined XMRV sequences in DNA purified from prostate cancer tissues. There were only 4.8 % positive for XMRV-like sequences whereas 21.5 % positive for XMRV-negative cases. These findings supported the interpretation that there were mouse DNA contaminants; in addition, intracisternal A particle (IAP) long terminal repeat DNA sequences were detected as well further supporting contamination [41].

Another study using BLAST searches for XMRV integration site sequences in prostatic tissues demonstrated that two of 14 integration sites were identical to sites that had been cloned in the same laboratory using the human prostate DU145 cell line that had been experimentally infected. Retrovirus infections had not previously exhibited identical integration sites. Therefore, it was suggested that PCR contamination had occurred and further weakened the view that XMRV was a human pathogen [42].

Another study of correlation between XMRV and prostate cancers was conducted in Iran. The investigator performed a case–control study with genomic DNA extracted from formalin-fixed and paraffin-embedded prostate tissues of 163 Iranian patients (63 prostate cancers and 100 benign prostate hyperplasias). They used a conventional and a nested PCR assay using primers targeting to an env specific sequence of XMRV. They did not detect XMRV in samples either from prostate cancers or benign prostate hyperplasias using XMRV specific primers [43].

A study failed to illustrate association between prostate cancer and XMRV in matched prostate and normal tissue from Australian patients. Purified genomic DNA (gDNA) matched from normal and cancer formalin-fixed paraffin-embedded (FFPE) prostate tissue from 35 Australian prostate cancer patients. RNase L polymorphism R462Q was determined by allele-specific PCR and contaminating mouse DNA was detected using qPCR targeting mouse intracisternal A particle long terminal repeat DNA. The gDNA was successfully purified from 94 % (66/70) of normal and cancer FFPE prostate tissues. RNase L typing revealed 8 % was homozygous (QQ), 60 % was heterozygous (RQ) and 32 % was wild type (RR) for the RNase L mutation. None of the 66 samples tested were positive for XMRV. The findings were consistent with other studies demonstrating that XMRV is a laboratory contaminant that has no role in the etiology of prostate cancer [44].

To support the evidence of contamination, cDNA from the whole blood of patients with CFS were tested using Invitrogen Platinum Taq (IPT) and Applied Biosystems Taq Gold LD (ABTG) with four gag sequences, followed by further sequencing by ABTG reamplification. Sequence comparisons showed similarity among these sequences, endogenous MLVs, and pMLV. Reagents were contaminated with pMLV sequences [45]. Furthermore, a study using Taqman qPCR failed to detect XMRV pol sequences in any of 112 peripheral blood specimens from CFS patients or 36 healthy controls. Moreover, there were specimens positive for XMRV DNA by a less sensitive PCR assay detecting a different portion of the XMRV genome, and were positive for highly abundant intracisternal A-type particle (IAP) long terminal repeat and murine mitochondrial cytochrome oxidase sequences. This study indicated extensive contamination of human specimens with murine sequences [46].

Several studies that had negative outcomes were originally conducted to demonstrate an XMRV association with disease. A large US study to elucidate the relationship between XMRV and CFS was done in blood specimens from 200 self-reported healthy volunteers and 100 CFS patients included patient specimens from the original study that had reported XMRV in CFS patients. XMRV and related MLVs viral sequences, virus growth, and antibodies to these viruses were not detected in any of the patient specimens, including those from the original study. The authors report that at least some of the discrepancies in previous studies were most likely due to the presence of trace amounts of mouse DNA in the Taq polymerase enzymes used in earlier studies. Virus growth in cell culture in prior studies was considered to be due to contamination as well [21]. The US studies on XMRV and CFS including a study in Kansas and Georgia using multiple molecular and serologic assays showed no evidence of XMRV infection [16]. Moreover, another study failed to show correlation of XMRV in various diseases such as CFS, HIV infection, rheumatoid arthritis and patients who received either organ or hematopoietic stem cell transplants using Lombardi et al. (PCR outer) primer set [10], Urisman et al. (PCR inner) primer set [5], and the Erlwein et al. primer set [14, 47, 48]. There were similar findings for a Dutch cohort between December 1991 and April 1992, using PCR targeting XMRV integrase and *gag* genes. XMRV sequences were not detected in specimens from any of the patients or controls. This study also demonstrated that it is possible to obtain and utilize uncontaminated reagents [49].

A study from 20 states in the US used blood from 45 CFS patients and 42 controls for both XMRV and MLV. Using the same CFS key clinical characteristics as in the Lombardi et al. study [10] highly sensitive and generic DNA and RNA PCR, as well as a new Western blot assay employing purified whole XMRV as antigen, there was no evidence of XMRV or MLV in the CFS patients or controls [50].

A Japanese study used one-step commercial RT-PCR kits, which detected XMRV gag sequences in CSF patient sera. The PCR primer sets were 419F, 1154R, GAG-I-R, and GAG-I-F. The sequences detected were compared with sequences of polytrophic endogenous MLV, XMRV, and endogenous MLV-related viruses derived from CFS patients. The result showed that the *gag*-related sequences were identical (99.4 %) and the *env*-related sequences were identical (99.6 %) to the polytrophic endogenous MLV. The kits were concluded to have been contaminated with MLV genome sequences [51].

Another study of CSF blood specimens from 43 of 61 patients that had previously been identified as XMRV-positive used PCR and reverse transcription-PCR to detect viral DNA and RNA. ELISA was used to detect virus-specific antibodies. There was no evidence of XMRV or other MLVs in any of these specimens. Further analysis in this study of commercial laboratory reagents detected MLV sequences. Thus, previous evidence linking XMRV and/or MLVs to CFS was likely due to laboratory specimen and/or reagent contamination [52].

There was no detection of XMRV DNA by PCR in PBMCs or RNA in plasma from discordant twins for CFS [53]. In addition, there was no detection of XMRV in HIV+ patients with immunosuppression [54]. Another serological studies in Japan found no association between XMRV infection of patients with prostate

cancer or with CFS [55]. Use of combined PCR and immunological techniques indicated and confirmed no role for XMRV in human disease [56].

XMRV-derived plasmids continue to be developed for in vitro and in vivo use for gene transfer. Thus, the danger of contamination is current and continues [57]. In vitro XMRV *env* vaccine studies in mice elicited immunity. Antibodies were detected by ELISA and by virus neutralization. However, immunity only lasted for 3 weeks [58]. Interestingly, Miyazawa pointed out that vaccines themselves are often produced using rodent (xenospecies) retroviral plasmid systems, and may transmit endogenous retroviruses (ERVs) to humans. This is an additional potential source of contamination [59]. In addition, the spectrum of tissue, cell, and nucleic acid preparations as well as patients potentially contaminated by murine viruses may be widening. A related caution is that since porcine tissue is often used for xeno-transplantation in humans, it was proposed that testing should be done for XMRV prior to transplantation. Retroviruses can recombine to produce new strains of virus, and porcine endogenous retroviruses (PERVs) are present in porcine tissues [60]. All these results point to caveats to guard against virus and nucleic acid potential contamination and escape from the laboratory, the dangers of their use in the clinic, as well as contamination of laboratories and reagents. Due to the evidence of contaminations, previously published literature were retracted by the authors including Lombardi et al. (Science 2009), Lo et al. (Science 2010), and Urisman et al. (PLoS One 2006) [61].

7 Clinical Studies

The involvement of XMRV in diseases in addition to prostate cancer and CFS has been investigated. These analyses did not demonstrate any association of XMRV with diseases including amyotrophic lateral sclerosis (ALS) [62], lymphoid malignancies [63], systemic lupus erythematosus (SLE) [64], and autism [65].

Since XMRV is closely related to murine leukemia viruses, the possible association to human lymphoid malignancies was analyzed. Waugh et al. studied DNA specimens from patients in the UK with lymphoid malignancies and benign lymphadenopathy quantitative PCR assays for XMRV. XMRV was not detected in any of the specimens [63]. However, XMRV had been suspected to circulate in the general population. Using TaqMan PCR in peripheral blood DNA, a London cohort of 540 HIV-1-positive patients was analyzed for the presence of XMRV and related viruses. There were no positive specimens in this patient cohort; it was concluded that XMRV or related viruses were not circulating at a detectable level in HIV-1-positive patients in London or in the general population [66]. Blood specimens from 95 SLE patients and 50 healthy controls were also analyzed by PCR. No XMRV was detected by PCR [64]. Moreover, using five sets of nested PCR primer of XMRV *gag* and *env* regions, with confirmation by using full-length molecular viral clone VP62, there was no evidence of MLV-related sequences in the specimens from 15 patients diagnosed with fibromyalgia [67].

In addition, an investigation of XMRV in cerebrospinal fluid did not find any correlation of CFS and XMRV or other common viruses including human adenoviruses, alpha-viruses, herpes viruses (HHV 1, 2, 3, 4, 5, 8), human parvovirus B19, dengue viruses 1, 2, 3, and 4, West Nile Virus, Japanese Encephalitis Virus, St. Louis Encephalitis Virus, enteroviruses A-D, and any coxsackieviruses [68].

In the controversial area of autistic disorder, correlation with XMRV or MLV-related viruses was studied using nested PCR targeted to gag DNA in specimens from blood, postmortem brain tissue, and semen. Once again, no XMRV gag DNA sequences were detected [65].

8 Conclusions

Initial research proposed a causal link for XMRV infection with prostate cancer and CFS. Issues addressed included the origin of the virus, its mode of transmission, its role in disease pathogenesis, and the possible use of chemotherapy and vaccines [1, 31]. However, subsequent work revealed that research laboratory and clinical laboratory contamination were central issues and the data no longer supported the initial claims. Nonetheless, it is important to note the issues raised by the initial findings and the careful and detailed laboratory follow-up. Contamination of tissue and reagents are key issues [69]. It is not the first time that rodents were putatively associated with a human disease. Indeed, rodents have been proposed as the cause of at least 35 human diseases. Many of these findings are now in question because of the potential for rodent contaminants in the reagents and tissues and this needs further follow-up [70]. The initial detection of XMRV and its putative association with human diseases was vitiated by later findings of contamination. This demonstrates that clean reagents and quality control are of crucial importance as well as proper design for controls.

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