Rheumatoid Arthritis is Associated with IgG Antibodies to Human Endogenous Retrovirus Gag Matrix: A Potential Pathogenic Mechanism of Disease?

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ABSTRACT. ***Objective.*** Human endogenous retrovirus (HERV)-K10 has been implicated in the etiology and pathogenesis of rheumatoid arthritis (RA). A secondary immune response to this virus might suggest an antigen-driven response in patients. The Gag region of HERV-K10 could provide a key epitope important for immunological reactivity. We investigated the presence of IgG antibodies to this region and assessed its significance in RA.

Methods. We determined an antigenic peptide on the matrix segment of HERV-K10 and developed an ELISA system to detect IgG antibodies in patients with RA and controls. The presence of antibodies to the matrix peptide (denoted as MAG1: RIGKELKQAGRKGNI) was correlated with patient details.

Results. On screening patients' serum, we found a significantly higher mean IgG antibody response to MAG1 in 30 patients with RA as compared to 23 patients with inflammatory bowel disease ($p = 0.003$), 29 patients with osteoarthritis ($p = 0.001$), and 43 healthy individuals ($p = 0.002$). Reactivity was not observed to a control peptide possessing a nonhomologous amino acid sequence. On evaluating clinical details with serological activity, no correlation with disease duration ($p = 0.128$), sex ($p = 0.768$), or rheumatoid factor status ($p = 0.576$) was found.

Conclusion. A significantly elevated IgG antibody response to an HERV-K10 Gag matrix peptide was observed in patients with RA, suggesting that the exposure of HERV-K10 may cause a secondary, antigenic driven immune response in RA. (First Release Sept 15 2014; J Rheumatol 2014;41:1952–60; doi:10.3899/jrheum.130502)

Key Indexing Terms:
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Rheumatoid arthritis (RA) is an autoimmune disease involving chronic inflammation of the synovial membrane culminating in the pathological destruction of joint cartilage and bone[1]. This can be mediated through autoantibodies targeting host proteins such as IgGFc, collagen type II, and citrullinated proteins that also provide useful clinical markers of disease[2,3]. The etiology of RA remains unknown, although a genetic predisposition, together with other triggers including viral and bacterial agents, are considered to be contributory factors. Patients infected with the exogenous human immunodeficiency virus can develop clinical manifestations of RA[4], as can animals infected with Maedi-visna virus and caprine arthritis encephalitis virus[5].

More recently in humans, endogenous retroviruses have been implicated in the etiology and pathology of RA[6,7]. Human endogenous retroviruses (HERV) constitute 8% of the human genome and are records of ancestral infectious agents which have become integrated into our DNA and then passed through successive generations in a Mendelian manner[8]. The genome of many HERV families has become defective over time through genetic mutations, although HERV-K10, a member of the HERV-K (HML-2) family, has

retained intact open reading frames within *gag*, *pol*, and *env* genes. As a consequence, HERV-K10 is capable of producing viral products[9] and particles in RA synovial tissue[10,11]. Overall, a number of serological and molecular investigations have considered HERV-K10 as a possible trigger of RA or a means of augmenting disease pathogenesis[12,13].

One study demonstrated a significant antibody response (principally IgM) to the Gag region of HERV-K10 in patients with RA as compared to controls[14]. The Gag region itself incorporates both matrix and capsid proteins, which assemble to form viral particles[9,15]. Importantly, the matrix protein lies directly beneath a lipid envelope, which on disruption facilitates its exposure to both adaptive and innate immune systems. Consequently, a segment of the Gag matrix may provide a reservoir of antigenic material that could evoke and drive a secondary (IgG) immune response to this endogenous virus.

We have previously described the use of bioinformatic algorithms[16] to predict antigenic peptides of proteins and viruses that can be tested in ELISA[17]. In our study, we have determined a highly antigenic peptide on the matrix protein of HERV-K10 and assessed the mean IgG antibody response to this peptide in patients with RA, inflammatory bowel disease (IBD), osteoarthritis (OA), and healthy individuals. Further, we assessed antibody levels to HERV-K10 with age, disease duration, and sex.

MATERIALS AND METHODS

Patients. Patients with RA were consecutively recruited from local outpatient rheumatology clinics (Heartlands Hospital, Birmingham, and New Cross Hospital, United Kingdom). Blood was taken from 30 patients with RA of an average age of 63 years (age range of 40–89 yrs) and disease duration equating to 75 months. All patients with RA satisfied the American College of Rheumatology criteria for diagnosis of rheumatoid arthritis and had clinically active disease as defined by serological and clinical variables. Rheumatoid factor (RF) status was also determined (Bio-Rad). Blood samples were also taken from 43 healthy donors (N) with an average age of 49 years (range from 22–80 yrs). An IBD group (23 patients) that did not have inflammatory arthritis was used as a control disease of unknown etiology: average age 51 years (range from 23–78 yrs). Samples were also collected from 29 patients with OA with clinically active disease: average age 67 years (range from 29–92 yrs). All samples were collected with full ethical approval and patients' consent.

Development of HERV-K10 Gag matrix peptide. Bioinformatic analysis was performed on HERV-K10 using established algorithms[16]. In brief, the sequence of HERV-K10 (accession no. M14123) was extracted from the National Center for Biotechnology Information/Genbank online database (www.ncbi.nlm.nih) and *in silico* analysis performed using online software programs (www.expasy.ch/cgi-bin/protscale.pl). Algorithms included hydrophilicity, residue polarity, solvent accessibility, and flexibility index. Based on these 4 variables, a highly antigenic region within the Gag matrix was identified and a 15-amino-acid peptide (RIGKELKQAGRKGNI; denoted by our group as MAG1: Matrix Gag peptide) was generated. N-terminal biotinylated and nonbiotinylated peptide (95% purity) was developed by Severn Biotech Ltd.

ELISA system for anti-IgG antibodies to MAG1. Biotinylated peptide MAG1 [400 ng/well in 50 μl phosphate buffered saline (PBS), PBS 0.15 M, pH 7.2] was coated onto 96-well plastic Reacti-Bind NeutrAvidin Coated ELISA Plates (Thermo Scientific Pierce) preblocked with bovine serum albumin (BSA). Serum samples were tested at 1/200, 50 μl/well, in diluent: 0.1% BSA, 0.05% Tween, 20 in PBS, and incubated at 37°C for 1 h. Plates were washed × 3 using an automatic plate washer (Wellwash 4 MK2, Thermo Labsystems) with wash buffer [PBS/Tween 20, 0.1% (detergent: polyoxyethylene 20 sorbit-monolaurate, BDH, 663684B)]. An affinity-purified $F(ab)_2$ horseradish peroxidase antihuman IgG conjugate (STAR97P, Serotec) was used (50 μl/well in diluent) to detect peptide-bound antibody (1 h, 37°C). Following washing (× 3), substrate 50 μl/well (tetramethylbenzidine: 3,-3′, 5,-5′, S T0440, Sigma) was added and plates were incubated for 4 min in the dark at room temperature. The colorimetric reaction was subsequently stopped with 2N hydrochloric acid and the optical density read at 450 nm (Multiscan MS spectrophotometer, Thermo Labsystems). Patients' sera were also assessed using nonbiotinylated MAG1 that required 96-well HBX2 immunol high-binding plates (Dynex Technologies).

Validation of reactivity to MAG1 in RA. A negative control peptide (GKTCPKEIPKGSKNT; defined as CON1[14,18]) that exhibited no amino acid sequence homology to MAG1 was also used in our ELISA system. To indirectly confirm the importance of the matrix protein, a second control peptide (RRSPRGRASHS) was used. This amino acid sequence, denoted as CON2, was derived from a different reading frame to MAG1 and tested on patients with RA and healthy controls. In essence, CON2 represented an alternative antigenic target to the Gag matrix. Preliminary data of general reactivity to CON2 using OA and healthy subjects (data not shown) suggested a probable immune response in all individuals. The immunogenicity of MAG1 was ascertained through testing in ELISA of an IgG-purified terminal bleed derived from a rabbit following the immunization and repetitive boosting[19] of this peptide (Severn Biotech Ltd.). In addition, the reactivity profile of purified IgG from 2 patients with RA was also assessed in this system.

Statistical analysis. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc.) on log-transformed serological data where necessary to provide a normal distribution for variable tests including ANOVA (with Bonferroni and Tukey's multiple comparison test where appropriate, and Student t test). Correlation of patient information was performed using a 2-tailed Pearson's test and assessed by regression analysis. Chi-square was used where appropriate. Statistical significance was accepted if $p < 0.05$.

RESULTS

We hypothesized that an antigenic peptide of HERV-K10 matrix could facilitate a secondary, antigen-driven immune response to this virus. Further, in correlating serological activity with patient details, we hoped to ascertain its value as a clinical marker of disease. Finally, we considered that the IgG antibody response in our control samples to MAG1 may provide an insight as to baseline reactivity of this ubiquitous virus.

IgG class anti-MAG1 antibodies (HERV-K10 Gag matrix) in RA. A total of 30 RA sera and serum samples from individuals with inflammatory (23 IBD) and noninflammatory (29 OA) conditions and 43 healthy controls were tested in ELISA for IgG reactivity to HERV-K10 matrix peptide MAG1. Optical density (OD) values for samples tested in each group are shown in Figure 1a. Multivariate group analysis (ANOVA) identified a significantly higher mean antibody level ($p < 0.05$) in 1 group, but found a markedly skewed distribution of all data (Figure 1b). This anomaly was corrected by log transformation and provided a normal distribution (Figure 1c) to facilitate variable statistical evaluation. Posthoc analysis identified a significant

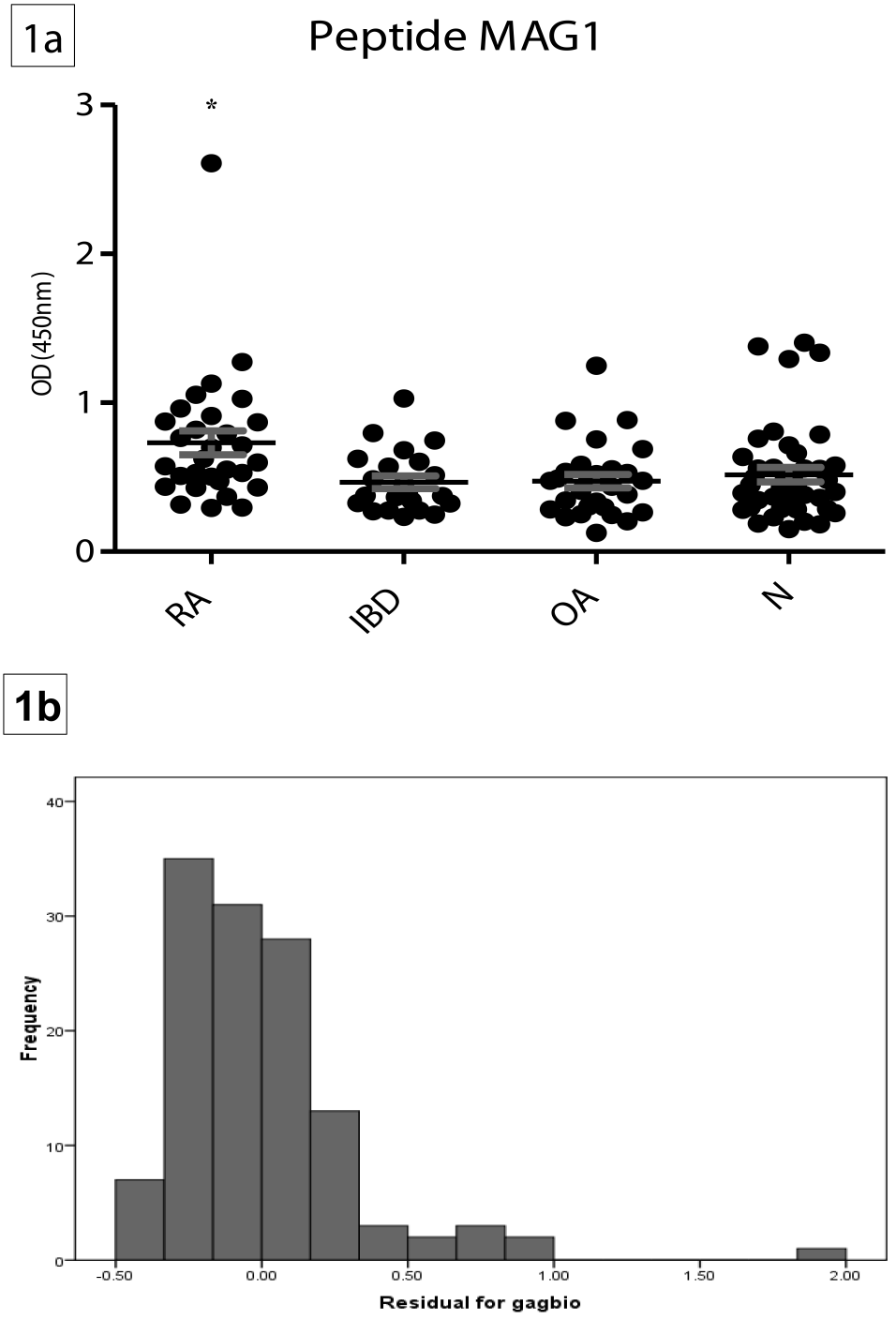
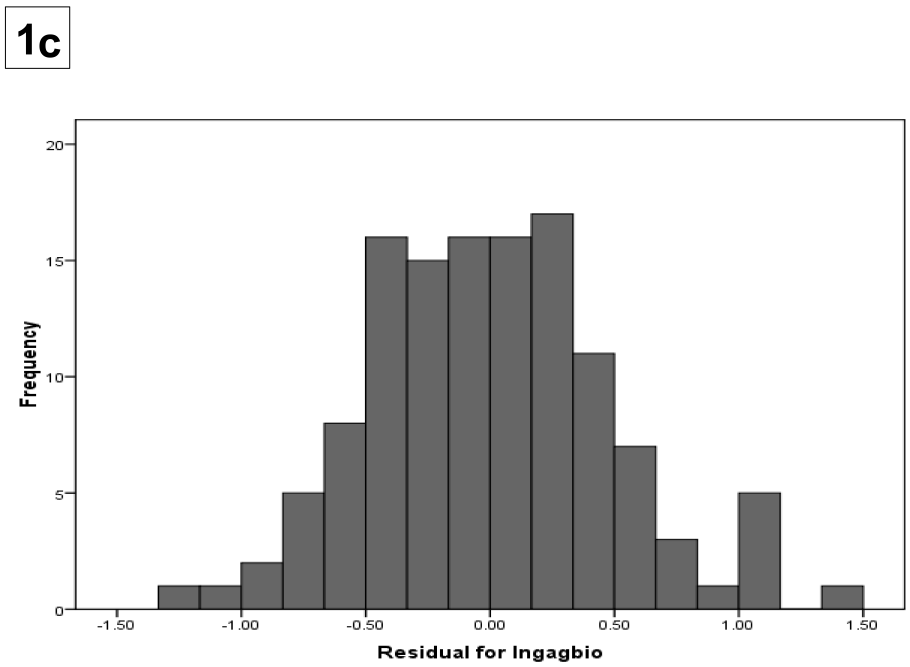


Figure 1. (1a) Serological response to matrix peptide MAG1 in patients with RA, IBD, OA, and healthy controls. Inserts (1b) and (1c) highlight respective sample distributions prior to and after log transformation. Stratification of original OD units (< 0.25, 0.25–0.49, 0.5–0.749, > 0.75) highlighted a significant trend (chi-square = 17.258, p = 0.045) toward the number of samples (RA: 0, 8, 10, 12; IBD: 2, 13, 6, 2; OA: 3, 16, 6, 4; N: 6, 21, 9, 7, respectively) with higher OD value in RA relative to controls. RA: rheumatoid arthritis; IBD: inflammatory bowel disease; OA: osteoarthritis; OD: optical density; N: healthy controls.

difference between the mean IgG antibody response in patients with RA as compared to IBD ($p < 0.003$), OA ($p < 0.001$), and healthy controls ($p < 0.002$) to peptide MAG1 (Table 1). Figure 1a also highlighted variation in optical



density (OD) levels about the mean for all groups. Similarly, a marked overlap in anti-HERV-K10 antibody levels was noted between groups, although stratification of data (Figure 1) revealed a significant trend ($p < 0.045$) toward the number of RA samples with high OD values in comparison to controls. Our data also revealed that the mean OD to MAG1 was significantly dependent for each group ($p < 0.0001$ for RA, IBD, OA, and healthy controls) when using biotinylated as compared to nonbiotinylated peptide.

Validation of reactivity to MAG1 in RA. In patients with RA, a serological response was observed to the HERV-K10 peptide sequence MAG1, but not to the nonhomologous control sequence peptide CON1 (the mean OD equating to background values: 0.01–0.05 OD units; Table 1). An additional control peptide, CON2, was used, which derived its amino acid sequence from a different reading frame to peptide MAG1. Here, our data showed no significant difference ($p = 0.005$) between the mean antibody reactivity of 17 patients with RA and an equivalent number of controls (Table 2). This result contrasted with the enhanced reactivity observed in patients with RA over healthy controls to peptide MAG1. A polyclonal antibody (PoMAG1) raised in a rabbit to the Gag matrix peptide exhibited positive OD values (i.e., > 0.05 OD units) over a wide range of concentrations (Figure 2) and demonstrated a similar titration profile to IgG purified antibodies derived from 2 patients with RA.

Clinical associations of patients with RA with IgG reactivity. The IgG antibody response to matrix peptide MAG1 was assessed against clinical data obtained from patients with RA. Our data showed very weak positive correlation with age ($R^2 = 0.018$) within the RA cohort ($p = 0.647$; Figure 3a) and when evaluated ($R^2 = 0.040$) against age-matched and sex-matched OA controls ($p = 0.40$; Figure 3b). Similarly,

Table 1. Comparison of antibody reactivity to peptides in patients and controls. Statistical analysis based on logarithmically transformed OD values. Antibody reactivity in all groups to CON1 (nonhomologous peptide: GKTCPKEIPKGSKNT) was negligible, and reactivity to nonbiotinylated MAG1 was just above background (0.05 OD units). Overall, there was no significant difference between groups. Multivariate analysis identified a significant difference between biotinylated and nonbiotinylated groups ($p = 0.001$).

Group	n	Peptide–MAG1, Mean OD (SEM)	OD Range	p	Peptide–CON1, Mean OD (SEM)	Nonbio–MAG1, Mean OD (SEM)
RA	30	0.732 (0.058)	2.610–0.293		0.041 (0.003)	0.119 (0.006)
IBD	23	0.465 (0.067)	1.029–0.233	0.003*	0.028 (0.004)	0.064 (0.013)
OA	29	0.473 (0.059)	1.249–0.126	0.001*	0.098 (0.004)	0.135 (0.009)
N	43	0.517 (0.049)	1.405–0.151	0.002*	0.073 (0.007)	0.157 (0.027)

*Statistical difference determined through posthoc analysis of patients with RA as compared to IBD, OA, and N to MAG1. OD: optical density; RA: rheumatoid arthritis; IBD: inflammatory bowel disease; OA: osteoarthritis; N: healthy controls.

Table 2. Antibody reactivity to MAG1 in RA and clinical variables. Control peptide CON2 (RRSPRGRASHS) related to a Gag non-open reading segment of endogenous virus HERV-K10 present in the human population[15]: 27 patients with RA and 27 healthy controls were tested using an alkaline phosphatase conjugate/substrate system[23]. Mean OD values are given with standard errors in parentheses. No significant difference was found between male and female patients within RF-positive or -negative groups (p = 0.128).

Variable	n	OD (SEM)	Analysis	p
Control peptide CON2	27	RA: 1.163 (0.081)	t test	0.804
	27	N: 1.130 (0.104)		
RF	16	RF+: 0.762 (0.127)	ANOVA	0.576
	10	RF–: 0.643 (0.076)		
Sex	13	Male: 0.645 (0.078)	ANOVA	0.768
	17	Female: 0.818 (0.127)		
RF and bone radiograph positive	18/23 (78%)		chi-square	0.001*

*Significant (p < 0.05). RA: rheumatoid arthritis; OD: optical density; RF: rheumatoid factor.

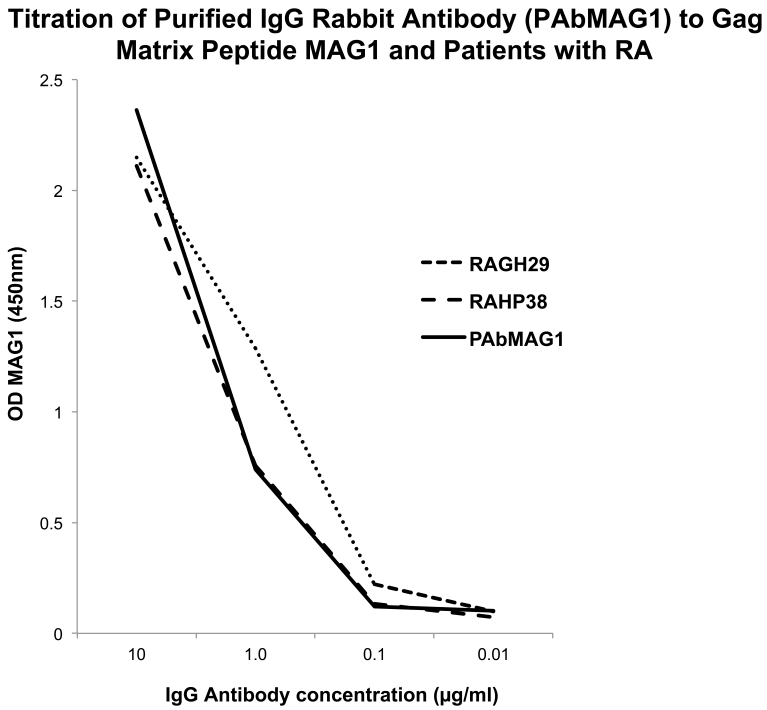
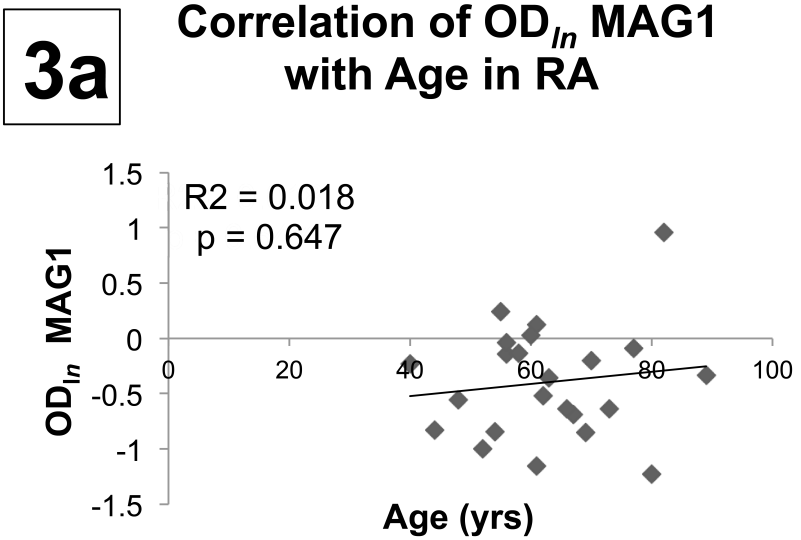
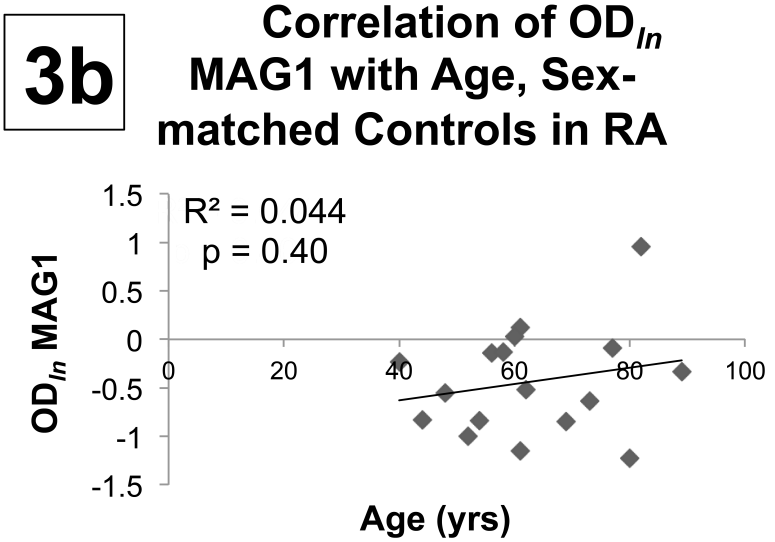


Figure 2. Titration of a rabbit polyclonal IgG purified antibody (PAbMAG1) to the HERV-K10 matrix peptide, and the titration profile of IgG from 2 patients with RA GH29 and HP38. RA: rheumatoid arthritis.

there was weak correlation with disease duration (R^2 = 0.139, p = 0.128) for the RA samples tested (Figure 3c). Patients who were RF-positive also exhibited no significant difference (p = 0.576) in terms of mean reactivity to HERV-K10 MAG1 (OD 0.762 SEM ± 0.127) in comparison to patients who were RF-negative (OD 0.643 SEM ± 0.076; Table 2). Further, analysis of RF-positive individuals with regard to sex revealed a mean OD that was weakly raised in females but did not reach statistical significance (p = 0.768). We also observed a significant association (p = 0.001) for patients with RA who were RF-positive and possessed bone lesions following bone radiograph analysis (Table 2).

IgG class anti-MAG1 antibodies in disease controls and healthy subjects. The antibody response in sera from inflammatory and noninflammatory diseases, plus healthy controls, provided a baseline level of reactivity to MAG1





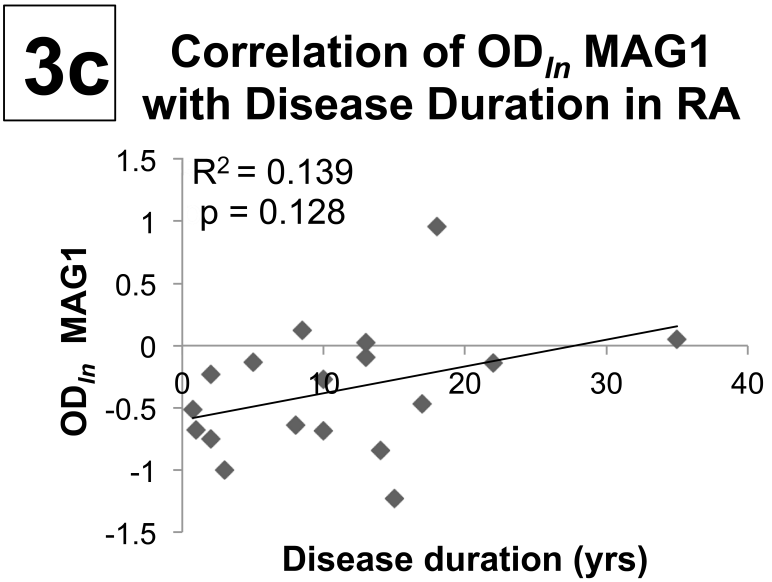


Figure 3. Correlation of RA serological activity (log transformed OD values) to (3a) Gag matrix peptide MAG with age, (3b) age-matched and sex-matched controls, and (3c) disease duration. RA: rheumatoid arthritis; OD: optical density.

(Table 1). Differences between mean serum OD levels (IBD 0.465 SEM ± 0.067, OA 0.473 SEM ± 0.059, and healthy controls 0.517 SEM ± 0.049) were not significantly different (overall, $p > 0.05$; Table 3). Further, there was no correlation for each group between serological activity to peptide MAG1 and age ($p \geq 0.05$).

DISCUSSION

The etiology of RA remains unknown, but may involve a viral agent in the context of a predisposing genetic background[20]. The detection of human endogenous retroviruses in RA has gained interest following a number of molecular and serological investigations of clinically active patients[21,22,23]. To this end, HERV-K10, a member of the HLM-2 Class II endogenous retroviral family, has been the most closely associated HERV with RA. A previous study[14] assessed the serological response (chiefly IgM class antibodies) to a HERV-K Gag product in patients with RA and other inflammatory diseases and controls. The authors reported a significant antibody response in patients with RA that was substantiated by quantitative reverse transcriptase PCR. The latter showed higher levels of HERV-K10 messenger RNA in RA samples with basal level in controls that were consistent with the ubiquitous presence of the virus in the human population[8]. However, it remains to be determined whether the IgG antibody response to HERV-K10 Gag matrix protein (essential for viral particle formation) is specifically elevated in patients with RA in comparison to other inflammatory and noninflammatory diseases and healthy controls. These data may provide evidence of a secondary immune response to a HERV viral component that facilitates an antigen-driven immune response *in vivo*[24].

In using our ELISA system, we found a significantly higher mean IgG antibody response to HERV-K10 MAG1 in patients with RA as compared to patients with IBD, OA, and healthy individuals. Variation in OD values within the RA group was evident, but consistent with serological studies investigating endogenous retroviruses in other diseases[6,21]. This may reflect individual immune responses (as noted for all groups to peptide CON1) and/or polymorphisms within HERV-K10[25]. To our knowledge, no definitive or substantial population studies of endogenous viral antibodies in healthy subjects and various diseases have been published for direct comparison with our particular study. Moreover, the overlap noted in antibody levels to HERV-K10 in RA and control groups may well reflect the ubiquitous expression of this virus in the general population, with its presence as a multicopy virus (30–50 copies per haploid genome) and contributing factors as yet unknown. In taking the IBD mean OD as an arbitrary cutoff, 70% of patients with RA proved positive in this assay system. Overall, the immunoreactivity to MAG1 was specific because all serum samples exhibited little to no antibody reactivity against the control peptide CON1, which exhibited no amino acid sequence homology to MAG1[14]. A polyclonal antibody, PoMAG1, was also successfully raised in a rabbit that had been immunized with the Gag matrix peptide. Indeed, the high titer (indicative of a large amount of antibody produced against this peptide) for the IgG fraction was similarly shown by 2 IgG preparations from 2 RA samples. Thus, it may be concluded that the HERV-K10 viral matrix region containing the sequence MAG1 was sufficiently immunogenic and antigenic to induce and perpetuate an immune response in patients.

The enhanced IgG antibody response in our patients with RA suggested a secondary immune response that was presumably antigen-driven *in vivo* by a continuous supply of viral matrix protein. This class of antibody is associated with affinity maturation (of antigen-binding sites) and may explain the tenacity of antibody binding within our ELISA system. However, it was noted that nonbiotinylated MAG1 compromised the reactivity of serum samples. This was rectified by using a biotinylated peptide that enhanced antibody accessibility to its epitope[26]. The validity of the viral matrix protein was also demonstrated by using a peptide sequence (CON2) that was derived from an alternative reading frame to MAG1. In theory, peptide CON2 provided an alternative antigenic target that would not be differentiated by RA and control sera, and would indirectly support the antigenic role of MAG1 in patients with RA. This notion was substantiated by our data, which showed an equivalent mean serological response to CON2 in both groups ($p > 0.05$).

Within the RA group, we evaluated serological data against patient details at the time of sample collection. Overall, no correlation was found with age, disease duration, sex, or RF status (Table 2). Further, there was no difference in the mean IgG antibody levels to MAG1 between men and women with RA. This result was surprising because women are at least 3 times more likely to develop RA than men[1], and in cell model systems, HERV-K10 mRNA and protein expression has been enhanced through hormonal stimulation[27]. A correlation of patients with seropositive RA with initial bone radiograph screening, however, was consistent with clinical observa-

Table 3. Anti-HERV-K10 reactivity with age in disease and healthy controls. Statistical analysis based on logarithmically transformed OD values.

Patient Group/ Control	Correlation R^2	Regression Coefficient	p*
IBD	0.087	–0.007	0.171
OA	0.11	–0.011	0.079
N	0.08	0.002	0.773

*Statistical different ($p < 0.05$). OD: optical density; IBD: inflammatory bowel disease; OA: osteoarthritis; N: healthy controls.

tions[28,29]. While the Disease Activity Score (DAS) was not recorded at the time of sample collection, it was interesting to note that Reynier, *et al*[30] observed a significant association between DAS and HERV-K10 viral load in their cohort of patients.

Data from IBD, OA, and healthy individuals provided a level of baseline antibody reactivity to HERV-K10. This finding was consistent with other studies[14,21] highlighting the ubiquitous presence of this virus in the general population. Moreover, we noticed little variation in antibody levels to HERV-K10 with age in our control samples, a finding that may provide a useful index of nominal antibody reactivity to this virus in future autoimmunity studies.

The majority of the patients with RA studied was receiving disease-modifying drugs, including immunosuppressive agents. Given that antibodies to HERV peptides were elevated in these patients with RA, it is unlikely that disease-modifying drugs suppress the immune reaction to HERV, which suggests that antibody reactivity does not reflect a true biomarker of disease[31]. A study with a larger number of patients would be required to determine whether therapeutic options for RA have differential effects on antibody response. It is plausible that a reduction in immune surveillance could influence antibody levels in RA, but in our study, patients with IBD were also receiving immunosuppressive agents. Hence, it would seem reasonable to assume that any impairment in immune surveillance (and thus enhanced antibody levels to HERV-K10) would equally apply to IBD and RA groups.

In our study, antibody levels to HERV-K10 were generally lower in disease controls and healthy subjects despite some overlap with RA. It would be interesting to assess in a further study of preclinical or early RA subjects whether antibodies to MAG1 became elevated before diagnosis, akin to the detection of antibodies to citrullinated proteins[32], which provide an early indication of RA in comparison to RF. If proven, the IgG antibody response to Gag matrix peptide MAG1 could then provide an associate marker of disease that may have a clinical application in the early diagnosis of RA. One mechanism for the role of HERV-K10 in RA is molecular mimicry[33]. This proposes an unwanted immune reaction to host protein(s) while mounting an immune response to virus. Interestingly, a preliminary alignment search of HERV-K10 matrix peptide MAG1 highlights an amino acid sequence (GKELK) similar to IgG1Fc (GKEYK), a key target for RF[16,34]. The finding of a unique epitope shared by virus and host protein could provide a novel molecular mimic that has the potential to trigger and/or augment an immune response in RA[35,36]. A Basic Local Alignment Search Tool search of our 15-amino-acid peptide (RIGKELKQAGRKGNI) also revealed some similarity to a sequence found in *Leptospira interrogans* (RISKELREAGRTMAP), associated with Weil disease. However, none of our patients with RA presented or were diagnosed with this condition. Evidently, the pathogenicity of antibodies to our peptide sequence remains to be determined in a suitable animal model. Undoubtedly, the precise role of HERV-K10 in RA is likely to be complex, involving a breakdown of immune tolerance[37], additional B and T cell epitopes, Toll-like receptors of the innate immune system mediated by nucleic acids[38,39], and envelope glycoproteins[40].

We consider that additional multicenter studies reflecting larger population sizes of early and established RA are necessary to validate our serological findings of antibodies to HERV-K10 in RA. Moreover, inflammatory arthritis conditions including psoriatic arthritis[41] and gout would also be required to ascertain whether the elevated antibody levels to HERV-K10 Gag matrix were specific to RA. Longitudinal studies of individuals prior to and after receiving therapy who are RF- positive or negative will also help establish the rise and stability of antibody levels to HERV-K10. Moreover, its relevance to other immunological variables (e.g., IgG, DAS, and its diagnostic/prognostic value against anticyclic citrullinated peptide antibodies in individuals prone to RA) should be investigated. Additional controls may also prove useful (e.g., patients with systemic lupus erythematosus)[42]. Other antigenic peptides adjacent to MAG1 within the Gag matrix would also help consolidate our observations. Our work may contribute to our understanding of HERV-K10 as a relevant marker of this disease (as opposed to a general inflammatory marker) and facilitate research on endogenous retroviruses in other autoimmune conditions.

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