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DETECTION OF CYTOMEGALOVIRUS, EPSTEIN–BARR VIRUS AND HERPES VIRUS-6 IN PATIENTS WITH RHEUMATOID ARTHRITIS WITH OR WITHOUT SJÖGREN'S SYNDROME

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SUMMARY

The frequency of latent viral infection by cytomegalovirus (CMV), Epstein–Barr virus (EBV) and herpes virus-6 (HHV-6) was investigated in patients with RA with or without Sjögren's syndrome (SS) and in normal controls. Virus presence was determined by polymerase chain amplification of DNA isolated from peripheral blood mononuclear or polymorphonuclear cells and/or saliva-derived mononuclear/epithelial cells. Anti-viral antibodies and autoantibodies were also assayed. Patients with RA both with and without SS were found to have a significantly increased frequency of latent viral infection (two-fold higher, $P = 0.035$ for EBV and seven-fold higher, $P = 0.018$ for HHV-6) compared to normal controls but only in cells isolated from saliva. The increased frequency of virally infected cells from the saliva of patients with RA, regardless of the SS status, when compared to normal controls may reflect the ongoing inflammatory process, the impact of therapy and/or a less effective local immune responsiveness.

KEY WORDS: Rheumatoid arthritis, Sjögren's syndrome, Cytomegalovirus, Epstein–Barr virus, Herpes virus-6.

IT is well recognized that RA is often accompanied by secondary Sjögren's syndrome (sSS), only distinguishable from the primary disease which lacks characteristic manifestations of other rheumatic diseases [1]. The prevalence of sSS in RA has been reported to be up to 31% [2]. SS is a chronic, slowly progressive disease of unknown etiology. Since SS is characterized by a lymphocytic invasion of the exocrine glands, viruses that are lymphotrophic in nature and that colonize these glands have been of prime interest as possible etiological agents or modifiers of the pathogenesis of SS.

Of particular interest are the viruses of the herpes family namely Epstein–Barr virus (EBV), cytomegalovirus (CMV) and recently, herpes virus-6 (HHV-6). These viruses are ubiquitous in all populations, and can reside in their infected host cells, usually lymphocytes, in a latent state for many years, (possibly even the lifetime of the individual). Periodically the viruses are reactivated [3]. Since these viruses are known to colonize salivary glands and the upper respiratory tract, a number of researchers have studied the association of these viruses with primary SS [4–21]. The presence of viral antigens and/or genome in tissue and circulating antibodies specific for the virus have been investigated. Although the interpretation of the findings has been controversial, it appears that these viruses are frequently present in patients with primary and possibly secondary SS and can be detected specifically in the pathological salivary tissue. Our objective was to determine the presence of the three herpes family viruses in peripheral blood and in the cells isolated from whole saliva of patients with RA both with and without sSS in order to determine the frequency of infection and possible association with sSS status. We also characterized the presence of autoantibodies associated with SS and RA in the two groups of patients. Our findings indicate that cells isolated from whole saliva from patients with RA, regardless of their sSS status, are more frequently latently infected by one or more of the herpes family of viruses, than are cells isolated from the saliva of normal individuals.

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SUBJECTS AND METHODS

Twenty randomly chosen patients with RA, fulfilling the ACR criteria [22] and 16 normal individuals were included in the study. Normal controls were nurses and laboratory staff (Table I). SS status was determined using both a questionnaire for symptoms of dry eyes and dry mouth [23] as well as functional and histopathological measurements (Schirmer's test, lip biopsy) [24, 25]. At the time of study the sera were tested for presence of ANA and antibodies directed against Ro/SS-A, La/SS-B, CMV and HHV-6. The anti-CMV and HHV-6 antibodies were detected by ELISA or IFA using Vironostika and HHV-6 kits (Organon Teknika, Scarborough, Ont.; Stellar BioSystems Inc., Columbia, MD) respectively. Antibodies to Ro/SS-A and La/SS-B were detected by ELISA (Therestest, Chicago, IL). No serology for EBV was done since the IFA methods available are less quantitative than ELISA.

Whole stimulated saliva was collected into tubes containing 10 ml of phosphate buffered saline (PBS),

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TABLE I
Detection of viral genome in peripheral blood mononuclear cells polymorphonuclear cells and/or salivary mononuclear and epithelial cells isolated from patients with RA, with and without SS and in normal controls

Patient/group	CMV*	EBV*	HHV-6*
(A) RA with SS			
MV	–	–	+
AK	–	+++	–
IC	–	–	+
SS	+	++	+
JST	–	+	++
JSE	+	+	++
NB	+	+++	++
SL	+	–	++
AJ	–	+	–
% positive	44	67	78
(B) RA no SS			
JK	+	+++	–
MM	++	+	+
CG	+	++	–
JH	+	+++	+
NN	+	+	+
MF	+	+	++
CM	–	++	++
CD	+	–	+
FG	–	–	–
LG	+	+	+
% positive	73	73	64
(C) Normal			
1	–	+	–
2	+++	+	+
3	+	+	+
4	+	–	–
5	+	++	–
6	+	++	++
7	–	–	–
8	–	+	±
9	–	+	+
10	–	++	–
11	+++	++	–
12			–
13			–
14			–
15			–
16			–
% positive	55	82	31

*By PCR amplification and hybridization—not detected at any site, detected in: + one site; ++ two sites, +++ three sites.

for a 10-min period. After reduction of the mucopolysaccharides by 100 µl additions of 0.01M β-mercaptoethanol until the mucus dissolved, the salivary mononuclear and epithelial (S-ME) cells were centrifuged and washed three times in PBS. To the saliva supernatant, an equal volume of 20% polyethylene glycol 3350 (PEG) (Sigma Chemicals) was added and the mixture was incubated at 4°C for 16 h. The precipitate obtained after centrifugation at 12 000 rpm for 15 min was redissolved in 1 ml PBS and extensively dialysed in PBS.

Heparinized peripheral blood was separated by Ficoll density gradient centrifugation into mononuclear cells (PBMCs) and polymorphonuclear cells (PMNCs). After density gradient separation, the Ficoll was removed and the red blood cells in the pellet containing the PMNCs, were lysed by the addition of 45 ml of lysis buffer (155 mM NH_4Cl, 10 mM NH_4HCO_3, 0.1 mM EDTA, pH 7.4). After incubation for 15 min at 4°C, the leukocytes were centrifuged and then washed twice in HBSS.

All of the viruses studied were cultured in established cell lines. These include B95–8 for EBV, MRC-5 (infected with the Davis strain of CMV) for CMV and MOLT-4 (infected with HHV-6) for HHV-6. For negative controls for the viral genome studies, the RAMOS cell lines was used for EBV, an uninfected MRC-5 line for CMV and either B95–8 or MOLT-4 (uninfected with HHV-6) were used for HHV-6. All cell lines and viral stocks originated from American Type Culture Collection (Rockville, MD).

Large molecular weight DNA was isolated from the cell pellets of the clinical specimens and positive and control cell lines using standard phenol/chloroform extractions as previously described [26]. Aliquots of 1 µg of the samples were used for the viral genome amplifications. Primers and probes for the *Bam* W region of EBV, the major immediate early gene of CMV and positions 17627–17429 of HHV-6 (Sheldon Biotechnology, Montreal PQ) were used for the amplification and detection of the specific virus products as previously described [26–29]. For EBV the primers [27] were: 5' ccagaggtaagtggactt; 3' gaccggtgccttcttagg; and probe aatttttctgctaagcccaacactc. For CMV the primers [28] were: 5' ggcgcctttaatatgatgggaggatgtttgcag; 3' ggcaagttctgcagtgccgccatggcctga, and probe gagcctttcgaggagatgaagtgtattggg. For HHV-6 the primers [29] were: 5' ctcgagtatgccgagacccctaatc; 3' aagcttgcacaatgccaaaaaacag and the probe aactgtctgactggcaaaaacttttt. Rigorous controls were included to minimize false positives and negatives. All amplifications were done on three separate occasions and the products were analysed for each amplification in duplicate. All of the amplified DNA products for each tissue and virus were spotted onto the same sheet of nitrocellulose, to enable comparative analysis between the normal controls and the two groups of RA patients. After hybridization with the ^{32}P-labelled viral specific probe [26], the blots were exposed to X-ray film (Fig. 1). The density of the dots on the film were quantitated by laser densitometry (Syscan-5000, United States Biochemical Corp, Cleveland, OH). After subtraction of the background (area of film not directly exposed to blot) the values were recorded. Amplifications, where the negative control and the no-template control were consistently negative were used in the study. Dots from the same amplification of the positive control for each virus studied were used to standardize the blots from experiment to experiment. Only values above the negative cut off that were consistently positive for the replicates were considered positive. Since the conditions used in the amplifications lead only to semi-quantitative results, the data were analysed as positive or negative for the presence of the virus genome. Each site (PBMCs, PMNCs or S-ME) and virus was examined separately. Since the amount of viral DNA to host cell DNA present can be variable, the same amount of input DNA from all sources was used in the amplifications. Data are

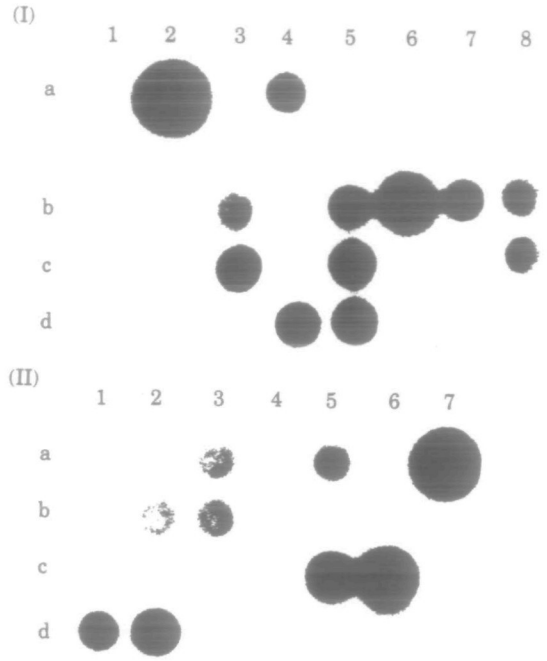

FIG. 1.—Detection of the amplified Bam W region of EBV by dot blot analysis in the saliva (I) and peripheral blood mononuclear cells (PBMCs) (II) of normal controls and patients with RA. I. (Saliva) Dot a-2 is the positive control (P3HR-2), a-4 is the negative control (RAMOS). Dots in row b are from normal controls and rows c and d from RA patients. Only dots b-6, c-3, c-5, d-4, d-5 were consistently positive. II. (PBMCs) Dot a-7 is the positive control (P3HR-2), a-5 is the negative control (RAMOS), remaining dots in row a and in b and c from patients with RA, row d from normal controls. Only dots c-5, c-6 and d-2 were consistently positive. All amplifications were as specified in Methods and were from 1 μg DNA except the positive control (0.1 μg).

expressed as the number of sites in an individual where viral DNA was detected.

The presence of IgM or IgA RFs were determined in serum (diluted 1:100 and 1:200) or the PEG precipitate of the saliva (diluted to OD280 0.200 and 0.100). By diluting the PEG precipitate to a fixed protein concentration, a relative comparison of detection of RF to total protein, given the variability in the production of the saliva (especially important for those with sSS) was possible. IgM RFs were detected by ELISA as previously described [30]. For the detection of the IgA RF, two ELISA systems were used. In brief, purified Fc fragments (5 μg/ml) from a IgG1 myeloma protein were used to coat 96-well plates. After a 16 h incubation at 4°C, unbound Fc was washed off the plates with PBS-0.05% Tween 20. Aliquots of 100 μl of the sera or PEG precipitates, diluted as above, were added and the plates were incubated for 4 h at room temperature (RT). The plates were washed with PBS-Tween and, either 100 μl of biotinylated $F(ab')^2$ fragments of goat anti-alpha chain specific antibodies (Tago, Markham, Ontario) or, HRP-conjugated anti-human alpha (Southern Biotechnology Assoc. Inc., Birmingham AL) was added and incubated for 2 h at RT. The plates were washed three times with PBS-Tween. For the biotinylated antibodies, HRP-conjugated avidin D (1/5000, Vector, Dimension, Mississauga, Ontario) was added and after incubation for 30 min at RT, the plates were washed three times. For both systems, the substrate, *o*-phenylene diamine was added and when sufficient colour developed (~30 min), the reaction was stopped by addition of 4 M H_2SO_4. The plates were read at OD_{492} by an ELISA plate reader (Fisher Chemical, Montreal, Quebec).

The amount of total Ig in the PEG precipitates was quantitated by ELISA. In brief, 96-well plates were coated with $F(ab')_2$ fragments of rabbit anti-total human Ig (Cappel, Organon Teknika) for 16 h at 4°C. After washing three times in PBS–0.05% Tween 20, 100 μl aliquots of the PEG precipitate, neat or diluted 1/10 were added and incubated for 2 h at 37°C. Bound Ig was detected, after washing in PBS-Tween, by incubation for 2 h at 37°C with HRP-conjugated goat anti-total Ig (Cappel). After an additional three washes, substrate was added and the plates were processed as above. Comparison to a standard total Ig curve, included on each plate, enabled quantitation.

Statistical comparisons were made using Instat (GraphPad, San Diego, CA) software programs. Values are expressed as mean ± S.E.M.

RESULTS

The demographic details of the patients with RA revealed that the RA patients with sSS had a significant impairment in tear flow (Schirmer's test 9.12 ± 1.02 mm) compared to RA patients without SS (33.38 ± 9.25 mm) ($P<0.0001$). The patients with sSS were slightly older, with a mean age of 67.9 ± 2.7 yr, compared to 63.3 ± 4.3 yr for those without sSS and had disease of twofold longer duration (18 ± 4.3 yr) than the patients without sSS (9.8 ± 1.8 yr). Although not all of the RA patients lacking symptoms of sSS (questionnaire, Schirmer's test normal) had lip biopsies, where it was done, only normal tissue with no obvious lymphocytic infiltrate was present. Six of nine RA patients with sSS and four of 10 without sSS were taking prednisone with the mean dose 6.25 mg and 3.87 (with or without sSS, respectively). ANA (five of nine with sSS, two of 10 without sSS) and La/SS-B, (one of nine with sSS, five of 10 without sSS) were found in both patient populations, however, anti-Ro/SS-A antibodies were restricted to the patients with sSS (2 of 9).

Analyses of RFs present in the sera indicated that 50–67% of patients had IgM and IgA RFs irregardless of sSS states. Additionally, 14 to 30% of the saliva samples were found to have IgA and/or IgM RFs, whereas no normal control tested had detectable RFs of either isotype (sera or saliva). There was a significant association (odds ratio 17.5) between the presence of RFs of the IgM and IgA isotypes (Fisher's Exact test, $P = 0.009$). Both IgA RF assays (standard and avidin/biotin ELISA) gave comparable results (data not shown).

The genome(s) of at least one of the three viruses studied was detected in almost every individual (Table I, Fig. 1). In fact, only two individuals, RA patient FG

and normal control 7 did not have detectable viral genomes of any of the three viruses in any compartment tested. EBV genome was the most frequently detected of the viruses, in both of the patient populations and in the normal individuals. The EBV genome appeared to be most disseminated in an individual as it was detected in all three of the sites tested twofold more frequently, compared to the other viruses. Only for HHV-6 was there a twofold increased frequency of detection in the RA patients compared to the normal controls. There was no apparent association between either the age of the patient, or disease duration and the number of different viral genomes present in the two patient populations.

Since one of the tissues that is pathologically affected in SS is the salivary glands, it was of interest to determine the number and type of viral genomes present at that site. As can be seen from Table II, all three viruses were detected at this site, predominantly in the RA patients with SS. Only the frequency of EBV detection in the S-ME cells was the same as in peripheral blood for both groups of patients. In contrast, in the normal individuals, EBV detection was much lower in S-ME cells than in peripheral blood. Both CMV and HHV-6 were less frequently detected in S-ME cells than in peripheral blood in RA patients as well as in normal controls. Although there was no difference in the frequency of EBV genome detection in the cells isolated from the saliva in the two groups of RA patients, both CMV and HHV-6 were detected at a higher frequency in those with SS. Some individuals in all groups did not have detectable viral DNA in the cells isolated from saliva. No virus genome was detected in two of the RA patients with SS, in two of the RA patients without SS, and in seven of the normal controls. When the RA patients were grouped together, there was a significant increased frequency in detection of EBV and HHV-6 in the patients, compared to the normal controls.

Anti-CMV antibodies were detected in seven of nine RA patients with SS and five of nine without SS and 13 of 17 normal individuals. Although there was a rough association of virus genome presence and positive serology for antibodies to the virus in question (60% concordance for CMV, 70% for HHV-6), both instances where positive detection of the viral genome in the absence of anti-viral antibodies and vice versa were identified (data not shown). This was seen for both CMV and HHV-6. There was no significant difference in the titre of anti-CMV antibodies between the two groups of RA patients (RA + SS, OD 0.94 ± 0.08; RA, no SS, 0.76 ± 0.10; cut off for positive value 0.23 OD). The frequency of anti-CMV antibodies in RA patients was similar to the control population. Because the control population was tested at a different time (twofold higher cut off value for positivity) no direct comparisons of titre differences are possible (titre 1.56 ± 0.08 OD; cut off for positive 0.404 OD). No saliva sample was found to have detectable IgG or IgM anti-CMV antibodies when the cut off for serum was used to assign positivity, even in PEG precipitates that were not diluted. When the mean OD plus 2 S.D. for anti-CMV antibodies in saliva of normal controls was used as a cut off, only one patient with RA without SS (LG) had detectable anti-CMV antibodies (IgG/IgM) in the saliva (data not shown). This individual did not have detectable CMV genome in the S-ME cells.

TABLE II
Detection of viral genomes in salivary mononuclear and epithelial cells isolated from patients with RA and from normal controls

		No. of patients with virus genome detected					
Patient/group	(No.)	CMV	(%)	EBV	(%)	HHV-6	(%)
RA with SS	(9)	1	(11)	6	(67)	5	(56)
		N.S.*		N.S.*		N.S.*	
RA no SS	(10)	0	(0)	7	(70)	3	(30)
RA, all	(19)	1	(5)	13	(68)	8	(42)
		N.S.*		$P = 0.035$		$P = 0.018$	
Control	(11)	0	(0)	3	(27)	1†	(6)

*Not significant, Fisher's exact test. †Sixteen tested.

DISCUSSION

RA and SS are complex diseases, with both genetics and environmental factors implicated in the pathogenic processes. These diseases have a spectrum of symptoms, variable time of onset and rates of disease progression. It appears from our study, where the patients were randomly chosen, that sSS appears to affect older individuals with longer standing RA disease. This is in contrast to what has previously been published [2] where there was no appreciable difference in age or disease duration. The two groups of patients were comparable, however, in terms of frequency of RFs of both the IgA and IgM isotypes (50–60%) as well as anti-viral antibodies in serum. Previous studies had shown the presence of IgA RFs in primary SS with frequencies of 21–60% in serum and saliva depending on the study [31–33]. However, no comparisons were made with patients with rheumatic disease. In our study, the frequency of RFs was fourfold lower in saliva than in serum, even with the PEG enrichment. There was no appreciable difference in the RF frequency in the two groups of RA patients.

Investigation of the autoantibodies typical of primary SS, revealed that those with sSS, did have a higher frequency of ANA and in particular anti-Ro/SS-A antibodies than those with RA without SS, but was roughly equivalent to that of primary SS as was previously reported [2]. Surprisingly anti-La/SS-B antibodies were more frequent in the RA population without sSS, but this could be due to the small sample size.

Viral etiology and primary SS has been debated widely with in some cases, conflicting results. In our study of sSS in the setting of RA, we determined the presence of viral genome detection using the very sensitive technique of PCR, in several cellular compartments (peripheral blood and the cells from saliva) as well as the presence of anti-viral antibodies in patients with and without SS and in normal controls. It appears that RA in general, carries a slightly increased overall risk for HHV-6 viral latent infection ($P = 0.08$) but not

EBV or CMV. From other studies HHV-6 is ubiquitous with up to 97% of populations infected as monitored by antibody [14, 34]. Much less, however, is known about HHV-6 latency [35].

If one focuses on the cells isolated from whole saliva, there is, however, an apparent increased risk of colonization and latent infection by all three of the herpes viruses at that site, in patients with RA regardless of their sSS status, when compared to normal controls. The frequency of detection of EBV genome by PCR in the saliva was found to be slightly lower than reported by Mariette *et al.* [21] for patients with primary SS (86 *vs* 68% for the RA patients in our study), however, the frequency in the saliva of the normal controls was the same (*29 vs* 27% in our study). Little information has been reported for virus detection by PCR in RA, except in a study by Saito *et al.* [17] where they found that there was a much lower frequency in RA (9%) compared to primary SS (96%). In their study no clinical information was provided about the patients with RA. Their analysis was calculated as the number of genome copies in 100 μl of saliva. It is possible that the different methods of collecting saliva and our standardization at the level of DNA may account for the differences. Detection of EBV genome by the slightly less sensitive method of *in situ* hybridization in salivary gland biopsy tissue of patients with RA and sSS revealed significantly higher frequencies of EBV genome in the patients compared to normal controls (27% *vs* 0) [20].

The herpes family viruses studied in the current report are lymphotrophic with the HHV-6 and CMV infecting T cells, [36–38] where B cells are the primary lymphoid host for EBV [39] although importantly, EBV has been reported to infect the salivary epithelial cells as these cells also express CD21 the receptor for EBV [40]. CMV as well as being lymphotrophic lytically infects cells of the fibroblast lineage. Detection of the viral genomes in the PMNCs is interpreted as due to the scavenger activity of these cells. Interestingly, Saltzman and co-workers detected high levels of CMV DNA in granulocytes in patients with AIDS [41]. CMV has been recently detected in the synovial membrane in 11 of 83 patients with RA [42].

It is likely that there can be several explanations for the increased frequency of detection of these viruses in the cells isolated from saliva of patients with RA. Firstly, due to the ongoing inflammatory process, the lymphocytic infiltration characteristic of SS may carry along with it the small percentage of lymphocytes that are latently infected with the viruses in question. Secondly, there may be an impaired immunity which allows the viruses to colonize at a higher frequency in patients with RA compared with normal individuals. This impairment could be due to the rheumatoid and/or the Sjögren's disease process or due to the use of remittive therapy. With the progression of the disease, it is possible that the local immunity becomes further compromised, which could allow further colonization, due to the inability to produce neutralizing antibodies. Thirdly, the state of activation of the viruses (latent/lytic) in the salivary tissue of patients with RA may vary compared to normal individuals. Further studies are needed to determine the expression of stage specific viral proteins and their correlation with disease.

Our study demonstrates that the local environment may affect in some way, the accumulation of latent viruses, however, the presence of these 'latent' viruses cannot be necessarily correlated with pathology at the site. Further studies are required to determine whether patients with RA with recent onset disease or on NSAIDs alone would have a similarly compromised mucosal environment.

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