Elevated Levels of Antibodies to Epstein-Barr Virus Antigens in Sera and Synovial Fluids of Patients with Rheumatoid Arthritis

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ABSTRACT The frequencies and levels of antibodies to Epstein-Barr virus (EBV)-specific antigens were determined in paired sera and synovial fluids from patients with rheumatoid arthritis (RA) and in sera from patients with other connective tissue diseases; i.e., systemic lupus erythematosus, progressive systemic sclerosis, and osteoarthritis (OA). The specimens were also tested for the presence of antibodies to RA-associated nuclear antigen. Compared to healthy controls, the patients' sera showed increased frequencies of elevated antibody titers (≥320) to Epstein-Barr viral capsid antigen, a correspondingly enhanced (twofold to threefold) geometric mean titer, and an increased frequency of antibodies at elevated titers (≥10), usually to the restricted component and rarely the diffuse component of the early antigen complex. Levels of antibody to the EBV-associated nuclear antigen were within the normal range. Enhancement of antibody titers was more pronounced in seropositive RA patients (i.e., positive for rheumatoid factor) than in those who were not. Enhancement was also found in systemic lupus erythematosus and progressive systemic sclerosis. Antibody to RA-associated nuclear antigen was detected at an increased frequency only in the group of seropositive RA patients (90%), as compared to 8–15% in the other connective tissue diseases and 6–8% in healthy controls. The antibody titers in the synovial fluids equaled or were at most twofold higher or lower than those in the sera. In addition, levels of EBV-specific antibodies were studied serially over a period of 6–10 mo in patients with RA and OA. Parameters of disease activity were determined and compared to antibody levels. EBV-specific antibodies in sera of OA patients remained constant and within normal limits throughout the study. Although EBV-specific antibodies were often elevated in RA patients, they also remained constant, with the exception of three patients, who showed gradual increases in one of the four antibodies, which did not correlate with disease activity.

INTRODUCTION

Previous studies have demonstrated that sera from patients with seropositive rheumatoid arthritis (RA);[1] i.e., patients positive for rheumatoid factor (RF), frequently contain antibodies to an antigen called RA-associated nuclear antigen (RANA) (1, 2). Later studies demonstrated that RANA was associated with Epstein-Barr virus (EBV) infection (3), suggesting a possible role of the virus in the pathogenesis of RA.

Several reports have noted that the levels of antibodies to EBV-specific antigens were not elevated in sera (4–6), or synovial fluids (7) from RA patients.

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[1] *Abbreviations used in this paper*: ANA, antinuclear antibodies; BL, Burkitt's lymphoma; CMV, cytomegalo virus; D, diffuse component; EA, early antigen; EBNA, EBV-associated nuclear antigen; EBV, Epstein-Barr virus; GMT, geometric mean titer: HSV, herpes simplex virus; MV, measles virus; NPC, nasopharyngeal carcinoma; NSAID, nonsteroidal anti-inflammatory drugs; OA, osteoarthritis; PSS, progressive systemic sclerosis; R, restricted components; RA, rheumatoid arthritis; RANA, RA-associated nuclear antigen; RF, rheumatoid factor; SLE, systemic lupus erythematosus; VCA, viral capsid antigen; VZV, varicella-zoster virus.

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However, in our initial study (8), we demonstrated elevated titers (≥320) of antibodies of Epstein-Barr viral capsid antigen (VCA) in 64% of 28 seropositive RA patients. Elevated anti-VCA titers have been found also in systemic lupus erythematosus (SLE) (9) and in a variety of immunosuppressive conditions (10).

The present study was undertaken to determine the relevance of antibodies directed against VCA (11), the diffuse component (D) and restricted components (R) of the EBV-induced early antigen (EA) complex (12), and the EBV-associated nuclear antigen (EBNA) (13) in RA.

METHODS

Patients. Sera from 48 patients with seropositive RA and 13 patients with seronegative RA were studied. All patients fulfilled the American Rheumatism Association criteria for classical RA (14). Matching synovial fluids were tested from 10 seropositive and 9 seronegative RA patients. For comparison, sera were studied from 12 patients with osteoarthritis (OA) with matching synovial fluids from 3; 29 patients who met the criteria of SLE (15); 10 patients with a diagnosis of progressive systemic sclerosis (PSS) based on typical skin changes and the absence of polymyositis and signs of SLE; three patients with other arthritides (crystal-induced arthritis; Beçhet's disease; juvenile RA-Sjögren's syndrome) with matching synovial fluids; 24 patients with Burkitt's lymphoma (BL) and 16 patients with nasopharyngeal carcinoma (NPC) (kindly donated to M. A. Alspaugh by Dr. G. de-The, who also supplied the EBV-specific serological data); and sera from three groups of 16, 71, and 80 apparently healthy individuals, collected at the Louisiana State University, the Scripps Clinic and Research Foundation, and The Children's Hospital of Philadelphia.

28 patients with RA and 12 with OA were studied serially over a period of 6–10 mo. Their ages ranged from 18–87 yr. There were 5 males and 23 females. 21 patients were Black and 7 were Caucasians. The duration of disease was 6 mo to 25 yr. Disease activity was determined monthly by Dr. David Rosenstock at the Louisiana State University Medial Center clinics as previously described (16), with the exception that the number of aspirin tablets (acetylsalicylic acid) per day and fatigue after x hours were omitted. 14 patients were on nonsteroidal antiinflammatory drugs (NSAID): Naproxen, hydroxychloroquine, Tolmetin, and two other NSAID, (Caprofen and Proquazone, currently under investigation (Hoffmann-La Roche, Inc., Nutley, N. J.) Four were on gold (sodium aurothiomalate or sodium aurothioglucose) and NSAID, one was prednisone and a NSAID, and nine were on combined steroids, gold, and NSAID. Patients were on maintenance regimens throughout the study.

Serological test procedures. IgG-coated latex particles (Rheumaton, Wampole Laboratories, Cranbury, N. J.) were used for the detection of RF. Studies demonstrating the lack of association between anti-RANA and RF (1, 17) and EBV antibodies and RF (8) have been reported previously. Samples of sera from patients with seropositive RA were kindly tested for the presence of IgG-RF by Dr. Dennis Carson before and after removal of RF by affinity chromatography (17). Levels of C_3, IgG, IgA, and IgM in patients' sera were determined at least once in all sera and sequentially in 19 patients over a 6–10-mo period by the radial immunodiffusion method (Meloy Laboratories, Inc., Springfield, Va.). The age, sex, and race of each patient was considered. Antibody to RANA was measured by double immunodiffusion as previously described (1), using WI-L2 extract at a concentration of 6 mg/ml. Sera were tested undiluted for screening studies. The indirect immunofluorescence techniques for the titration of VCA-specific IgG antibodies or of antibodies to the D and R of the EA complex have been described in detail (11, 12). The EB_3 cell line was used for the anti-VCA assay. A virus preparation from the P_3H_3 cell line was used for the superinfection of Raji cells for the induction of D and R antigens. An EBV-negative cell line (Bjab) was used for control cells to determine the presence of antinuclear antibodies (ANA). Sera from individuals who had not been infected with EBV were used as negative control sera. Antibody to EBNA was measured by the anticomplement immunofluorescence method according to Reedman and Klein (13). The immune-adherence hemagglutination assay (18) was used to titrate antibodies to varicella-zoster virus (VZV), herpes simplex virus (HSV), cytomegalo virus (CMV), and measles virus (MV).

RESULTS

Table I demonstrates the incidence of elevated titers of antibodies to EBV-specific antigens and their geometric means, as well as the incidence of antibodies to RANA in patients with connective tissue diseases (seropositive and seronegative RA, OA, SLE, and PSS) and, for comparison, patients with EBV-associated diseases (BL and NPC) and apparently healthy controls. On the basis of earlier data, the following titers were considered "elevated": anti-VCA and anti-EBNA, ≤320, and anti-D or anti-R, ≥ 10 (12, 19).

It is seen from the table that, compared to healthy controls, RA patients show an increased frequency of elevated anti-VCA titers, a twofold increased GMT, an increased incidence of detectable antibodies to EA, mostly anti-R, but no apparent increase in the anti-EBNA levels. The EBV-specific serologic activity of RA patients is far less increased than that seen in the EBV-associated diseases. The increase in EBV-specific antibody activities is more pronounced in seropositive than in seronegative RA patients, who differ also in the frequency of detectable antibodies to RANA, i.e., 90 and 8%, respectively. Anti-RANA was present in 19 and 33% of sera from the BL and NPC patients tested, but in no more than 8% of the control sera.

Affinity chromatography was used to remove IgG and IgM-RF from three RA sera. The IgG-VCA antibody was reduced in one serum but was unaffected in the two others. Anti-RANA activity was retained in all three.

As for the other connective tissue diseases, sera from patients with SLE and PSS showed a higher incidence of elevated anti-VCA and, in the case of PSS, also anti-EBNA titers and, correspondingly, somewhat higher GMT of these antibodies than the sera from the seropositive RA patients. Some SLE and PSS sera did have ANA, which were noted on Bjab control cells. This generally did not affect the VCA, D and R staining, which showed through the ANA background. Anti-

TABLE I

Frequency and Levels of Antibody Titers to EBV-specific Antigens and RANA in Sera from Patients with Connective Tissue Diseases and Controls

Disease	Number of patients	Antibodies to RANA ≥neat	VCA ≥320	VCA GMT	D ≥10	R ≥10	EBNA ≥320	EBNA GMT
		%	%		%	%	%	%
RA								
Sero +	48	90	56	226	4	38	0	32
Sero −	13	8	36	168	0	21	0	25
OA	12	8	33	187	0	17	8	52
Other	3	0	0	80	0	0	0	20
SLE	29	15	76	271	3	41	nt	nt
PSS	10	10	80	392	0	70	40	65
BL	24	19	92	1,278	0	79	33	168
NPC	16	33	100	1,075	83	nd	42	72
Normal individuals								
LSU	16	6	25	94	0	25	6	33
SCRF	71	8	nd	nd	nd	nd	nd	nd
CHP	80	nd	7	104	1.2	9.8	5	39

GMT, geometric mean titer.
nd, not done.
nt, not testable.
LSU, Louisiana State University.
SCRF, Scripps Clinic & Research Foundation.
CHP, Children's Hospital, Philadelphia.

EBNA titers could not be assessed in most SLE sera because they had high titers of ANA. The sera from the OA patients gave results comparable to those obtained with sera from seronegative RA patients. Antibody to RANA was found at low frequency in sera of other connective tissue diseases, i.e., 8% in OA, 15% in SLE, and 10% in PSS.

Table II compares the serologic data obtained with

TABLE II

Frequency of Antibodies to EBV-specific Antigens and RANA in Paired Sera and Synovial Fluids

Disease	Specimen	Antibodies to RANA ≥Neat	VCA ≥320	VCA GMT	D ≥10	R ≥10	EBNA ≥320
		No. positive/No. tested					
RA							
Sero +	Serum	8/10	5/10	226	0/10	4/10	0/10
	Synovial fluid	9/10	3/10	160	0/10	3/10	0/10
Sero −	Serum	0/9	4/9	160	0/9	2/9	0/9
	Synovial fluid	0/9	3/9	117	0/9	2/9	0/9
OA	Serum	1/3	2/3		0/3	2/3	0/3
	Synovial fluid	1/3	2/3		0/3	2/3	0/3
Other	Serum	0/3	0/3		0/3	0/3	0/3
	Synovial fluid	0/3	1/3		0/3	0/3	0/3

GMT, geometric mean titer.

TABLE III

Comparison of Antibody Levels to Various Viruses in Paired Sera and Synovial Fluids from Patients with Rheumatoid Arthritis

Antibody to				
EBV (VCA-IgG)	VZV	HSV	CMV	MV
serum/synovial fluid				
1,280/640	10/10	40/40	160/80	40/40
640/320	80/40	40/40	20/20	80/40
640/320	40/20	80/40	40/40	10/10
160/160	<10/<10	<10/<10	<10/<10	<10/<10
160/160	<10/<10	80/40	<10/<10	<10/<10
320/160	40/20	<10/<10	<10/<10	<10/<10
80/40	20/10	80/40	<10/<10	<10/<10
40/40	40/20	160/80	<10/<10	160/80
40/40	40/20	160/160	<10/<10	40/40

paired sera and synovial fluids from 10 seropositive and 9 seronegative RA patients as well as 6 patients with other arthritides. The antibody titers to EBV-specific antigens detected in the synovial fluids were either identical to those obtained from the matched serum, or at most twofold lower, accounting for the minor differences in the GMT recorded. Furthermore, as shown in Table III, if the patients had detectable serum antibodies to VZV, HSV, CMV, or MV, the synovial fluids also contained equal, or at most twofold lower, titers. The pairs of sera and synovial fluids in Table III were selected to represent high, medium, and low anti-VCA titers.

Table IV shows the results obtained with a partially different set of paired sera and synovial fluids in tests for RF and antibodies to VCA and RANA. Again, the synovial fluid titers generally matched those seen in the sera or were at most twofold higher in some, or lower in other specimens.

28 patients with RA and 12 patients with OA were studied serially over a period of 6–10 mo. EBV antibodies remained within the normal ranges in OA patients and never showed significant titer changes (greater than or equal to fourfold). The RA patients fell into three groups with respect to EBV serology: (*a*) high constant anti-VCA, ≥320; (*b*) low constant anti-VCA, ≤160; and (*c*) change from low to high anti-VCA. The division of patients into these three groups could not be attributed to the patients' drug regimens or the duration of disease. Of the 16 patients in group 1, 7 patients had low levels of anti-R, 3 had low levels of anti-D, and 6 had no anti-EA (<10). None of the patients' elevated anti-D or anti-R antibodies (>10) showed changes greater than twofold. However, anti-D rose in one patient slowly stepwise from a titer of 20 to 80 (Fig. 1), but this did not correlate with disease activity. Anti-EBNA levels remained rather constant and within the normal range in 12 patients, but there were 4 patients who had very low levels. These lower levels did not correspond with a longer duration of disease. Of the 11 patients in group 2, 3 had elevated anti-R, which did not change significantly, and 8 had no anti-EA (<10). Anti-EBNA remained within the normal range and did not fluctuate more than twofold throughout the study. Fig. 2 shows a representative case from group 2. There was only one patient in group 3. Anti-VCA gradually rose in this patient in a stepwise fashion from a titer of 80 to 640 over a period of 10 mo, but this did not correlate with disease activity (Fig. 3).

56% of these patients had at least one elevated immunoglobin (predominantly IgG) within serial sets. Immunoglobulin levels often changed (42%) between normal and elevated levels from one month to the next, but did not correlate with viral antibodies, which were constant.

TABLE IV

Antibody Titers in Paired Sera and Synovial Fluids from Patients with Seropositive Rheumatoid Arthritis

Rheumatoid factor	Anti-RANA	Anti-VCA (IgG)
	serum/synovial fluid	
320/640	64/64	160/160
320/640	32/32	1,280/640
160/160	32/32	160/80
80/160	8/8	160/160
40/40	16/16	160/80
160/80	4/8	80/40
40/320	32/16	80/160
80/40	neg/4	320/160
80/160	neg/neg	320/160
160/160	16/16	640/640
Average ratio		
0.9	1.0	1.6

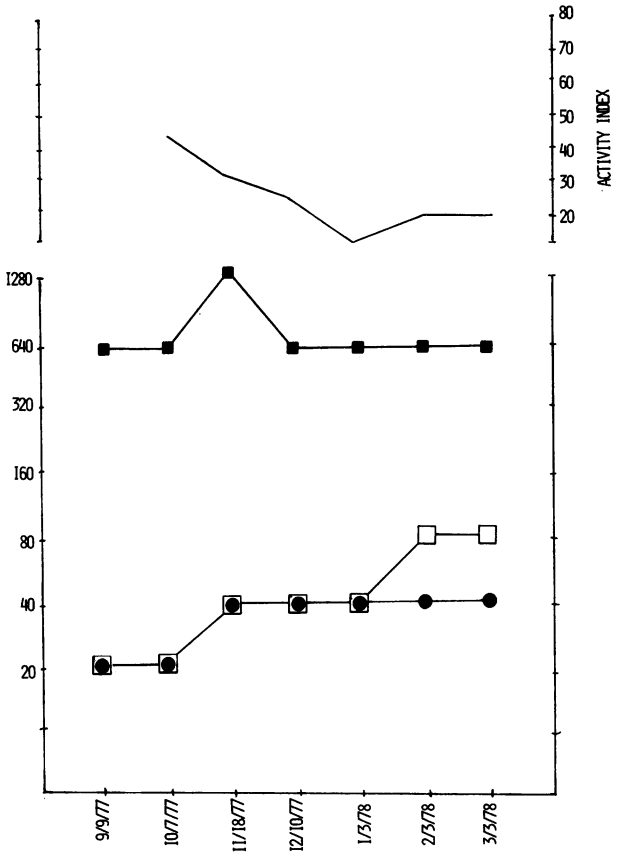

FIGURE 1 Monthly EBV-specific antibody levels in a 66-yr-old male with seropositive RA, who was maintained on a combined drug regimen of gold, prednisone, and Naproxen throughout the study. Anti-VCA was constantly elevated throughout the study. Anti-R was consistently not detectable (<10). Anti-D rose stepwise from a titer of 20 to 80. Anti-EBNA remained nearly constant and within the normal range. The changes in EBV-specific antibodies did not appear to correlate with the activity index. ●, Anti-EBNA; □, anti-D; ■, anti-VCA.

DISCUSSION

The data presented confirm and extend our previous observation (8) that, contrary to earlier reports (4–6), RA patients, especially when seropositive, show an increased incidence of elevated titers of antibodies to VCA as well as an increased incidence of antibodies to the R of the EA complex. The anti-EBNA titers of the RA patients, however, fell, contrary to another report (19), within the range observed in the controls. Failure to differentiate between seropositive and seronegative RA could explain the discrepant results of the earlier reports on antibodies to VCA and R; and, drug therapy, disease activity, or race (75% of these patients were Black) may explain the differing results for anti-EBNA. The results obtained here should not be construed to mean that EBV-specific antibodies are involved in the pathogenesis of RA because (*a*) the GMT of anti-VCA in the seropositive RA patients was only twice as high as that of healthy controls and not nearly as high as those observed in the EBV-associated diseases, i.e., BL and NPC (12); (*b*) similar or even slightly more pronounced enhancement of anti-VCA titers and of the incidence of antibodies to the R are observed in other connective tissue diseases, that is in SLE, as reported earlier by Rothfield et al. (9), and in PSS; (*c*) an increased frequency of elevated anti-VCA titers and of antibodies to the EA complex as compared to controls is also observed in a variety of other nonmalignant or malignant illnesses, all having immunosuppressive effects or requiring immunosuppressive therapy and thus thought to activate the latent persistent viral carrier state that regularly ensues after primary EBV

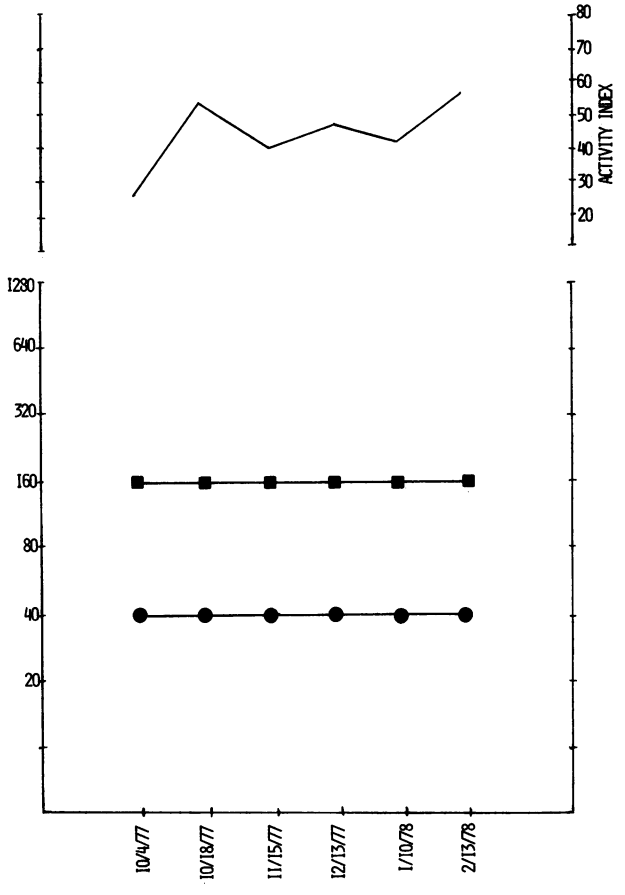

FIGURE 2 Monthly EBV-specific antibody levels in a 62-yr-old female with seropositive RA, who was maintained on Carprofen. Anti-VCA remained constant and within the normal range throughout the study. Anti-D and anti-R titers remained <10. Anti-EBNA remained constant and within the normal range. Changes in the activity index were not accompanied by changes in EBV-specific antibody titers. ●, Anti-EBNA; ■, anti-VCA.

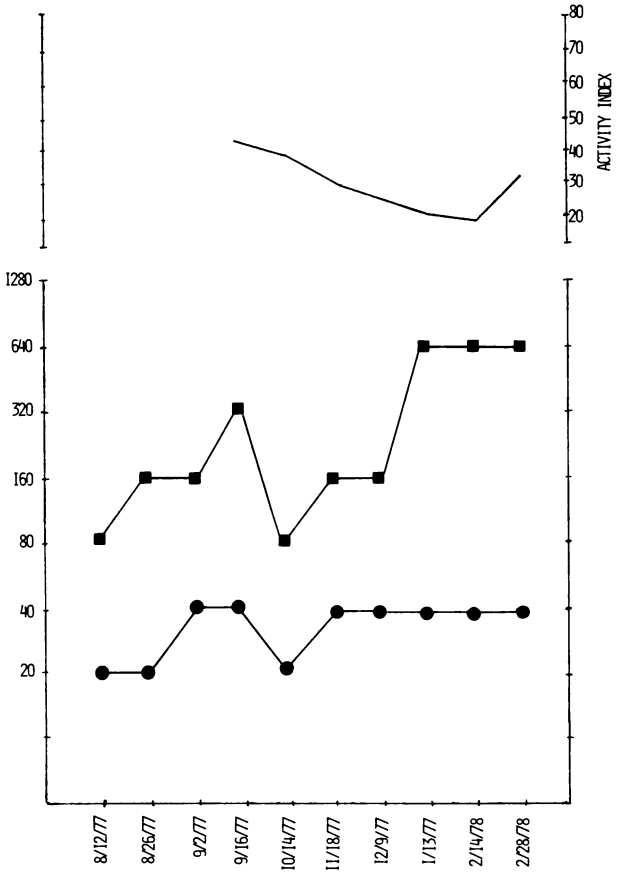

FIGURE 3 Monthly EBV-specific antibody levels in a 51-yr-old male patient with seropositive RA, who was maintained on a combined drug regimen of gold, prednisone, and Naproxen throughout the study. Anti-VCA rose from a titer of 80 to 640 over a period of 10 mo. Anti-D and anti-R titers were consistently <10. Anti-EBNA fluctuated very little and remained within the normal range. The EBV-specific antibodies did not appear to correlate with the activity index. ●, Anti-EBNA; ■, anti-VCA.

infections (10); and (*d*) EBV-specific antibodies do not change with disease activity.

Comparative titrations of paired sera and synovial fluids from seropositive or seronegative RA patients yielded essentially similar levels of EBV-specific antibodies in both specimens, with at most a twofold reduction in titer in some of the synovial fluids. These results are in keeping with previous studies by Cramer et al. (7). Similar results were also obtained in the present study in tests for antibodies to VZV, HSV, CMV, and MV. The synovial fluid titers seem to reflect merely the serum antibody levels and not local antibody production, and the slight decreases in synovial fluid titers seen in some cases are most likely due to technical variations and possibly nonspecific factors such as the viscosity of the specimens rather than antigen-antibody complex formation within the joints. Also, the RF and anti-RANA titers of paired sera and synovial fluids were of similar orders, but the latter tended to be slightly higher. This minor difference between the anti-viral and RA-associated antibodies might be attributable to the different techniques used for their assays.

In this study, antibodies to RANA were detected in the majority (90%) of seropositive RA patients but in 8–15% of other arthritides and 6–8% of healthy controls. This frequency in seropositive RA is higher than in previous reports (67%) and may be attributed to the population of patients or their drug regimens. Anti-RANA was found at intermediary frequencies in BL (19%) and NPC (33%).

The data presented suggest that EBV-specific antibodies may not play a major role in RA. This does not, however, preclude the possibility that persistent EBV infection may be important in the disease, since high levels of anti-RANA are found in ~90% of patients with seropositive RA, and RANA is associated with a non-productive EBV infection.

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