

# LYMPHOCYTES TRANSFORMED BY EPSTEIN-BARR VIRUS

## Induction of Nuclear Antigen

### Reactive with Antibody in Rheumatoid Arthritis\*

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Rheumatoid arthritis (RA)<sup>1</sup> is a disease in which immune mechanisms have been considered to play a significant if not the major role in pathogenesis (1-3). There have been many studies which support this hypothesis. The synovial tissue of the peripheral joints is the most important site of tissue injury, and the inflammatory cell infiltrate is composed mostly of lymphocytes and plasma cells with a striking absence of polymorphonuclear leukocytes (4-6). Immunofluorescent studies have shown abundant amounts of immunoglobulin and complement in synovial tissue (7, 8), and synovial fluids contain aggregates of immunoglobulin, rheumatoid factor-gamma globulin complexes, and nuclear antigen-antibody complexes (9). The etiology of RA has been considered by many investigators to be due to the presence of some specific and persistent antigenic stimulation. Much work has been directed towards searching for serum antibodies to bacteria or viruses in the hope that there might be clues pointing towards a specific microbial agent. The results have been conflicting and generally inconclusive (10, 11).

Recently, we demonstrated that the sera of patients with RA contained an antibody which reacted with a cell-associated antigen present in human lymphocyte cell lines. This was demonstrated initially by precipitin lines in immunodiffusion between rheumatoid sera and soluble extracts of tissue culture cells, and the antibody in rheumatoid sera was referred to as RA precipitin (RAP) (12, 13). Subsequently, the antigen was demonstrated by immunofluorescence (14) and it has now been shown to be distributed predominantly in the nucleus of tissue culture B-lymphocyte cell lines. Our hypothesis was that this

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<sup>1</sup> Abbreviations used in this paper: EB, Epstein-Barr; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; FCS, fetal calf serum; HCL, human cell line; RA, rheumatoid arthritis; RANA, RA-associated nuclear antigen; RAP, RA precipitin.

antibody could be directed against a neoantigen in transformed lymphocytes. In this study, we present evidence to show that the nuclear antigen reactive with antibody in the sera of patients with RA could be induced in lymphocytes transformed by Epstein-Barr (EB) virus. The nuclear antigen appears to be different from the Epstein-Barr nuclear antigen (EBNA).

### Materials and Methods

*Transformation of Human Peripheral Blood Lymphocytes with EB Virus.* 200 ml of blood from each of three normal individuals was collected in heparin, and the leukocytes separated by dextran sedimentation. 30 ml of leukocyte-rich plasma was added to 10 ml of leukocyte separation medium (Bionetics Laboratory Products, Kensington, Md.), centrifuged at 2,000 rpm at room temperature for 10 min, and the sedimented leukocytes washed with RPMI 1640 (Associated Biomedic Systems, Inc., Buffalo, N.Y.). After centrifuging at 1,500 rpm for 10 min, the procedure was repeated a second time. The method usually yielded 40 ml containing  $1 \times 10^8$ - $1.5 \times 10^6$  leukocytes/ml.

B-lymphocyte line (B95-8), (supplied by Dr. Berge Hamper, National Cancer Institute) producing Epstein-Barr virus (EBV) was used as the source of virus for transformation. B95-8 cells were cultured in RPMI 1640 with 10% fetal calf serum supplemented with glutamine and nonessential amino acids (Flow Laboratories, Inc., Rockville, Md.) and allowed to reach a peak growth of  $2.5 \times 10^6$  cells/ml. The cells were spun down, resuspended at  $2 \times 10^7$  per ml in RPMI 1640, and sonicated at 4°C. The sonicate was then centrifuged and the supernate filtered through 0.45  $\mu$ m Millipore filter (Millipore Corp., Bedford, Mass.). The virus pool was divided into small portions and stored at  $-70^\circ\text{C}$  to be used as needed.

20 ml of leukocyte suspension containing  $2 \times 10^6$  cells/ml was sedimented by centrifugation and resuspended in 1 ml of RPMI 1640 containing fetal calf serum (FCS). The cells were added to 1 ml of undiluted virus from B95-8 cells, and the suspension left at  $37^\circ\text{C}$  for 30 min to allow adsorption of EBV. Infected cells were pipetted into two plastic 250-ml tissue culture flasks (Lux Scientific Corp., Newbury Park, Calif.), and 15 ml of fresh media containing FCS and supplements were added. Control cells which were not exposed to virus were handled separately and cultured at the same concentrations. Cultures were incubated as stationary suspension cultures at  $37^\circ\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . The cell cultures were frequently examined for cell morphology and given fresh media every 7 days. All three preparations of cells infected with EB virus were noted to undergo changes in cell culture characteristic of virus-induced transformation. By day 7, some large lymphocytes were forming clusters on the bottom of the flask. By day 14, these clusters of lymphocytes were greatly enlarged, and in addition, clumps of cells were present in suspension in the culture medium. From day 20 to 23, the number of large lymphocytes in suspension had increased significantly and the cells could be transferred for subculture on a rotary shaker. In contrast, two uninfected control cell lines showed decreasing numbers of cells on days 20-23 and in subsequent weeks, could not be sustained in culture. Cell cultures were harvested at various times and prepared for immunofluorescent studies by cytocentrifugation on to glass slides.

*Extraction of Soluble Antigen from Cells.* Suspensions of cells (between 500 and 1,000 ml) from each cell line at  $1 \times 10^6$  per ml were centrifuged and extracted by the same method described previously for WiL<sub>2</sub> cells (15). Briefly, this consisted of recovering cells by low speed centrifugation and sonication of isolated cells in a solution of 0.25 M sucrose and 0.04 M calcium chloride buffered at pH 6.2 with 0.01 M phosphate. After centrifugation at 105,000 g for 30 min, the supernate of sonicated cells was used as the cell extract for studies in immunodiffusion in agarose against RA sera.

*Immunological Studies.* Two immunological assay systems were used for detection of antigen which reacted with serum antibody in RA sera (14). Three prototype sera from patients with RA had been previously characterized and were used throughout these studies. These sera reacted with soluble antigens in extracts of WiL<sub>2</sub> lymphocytes by immunodiffusion to give precipitin lines which were immunologically different from precipitin lines caused by antibodies to nuclear antigens such as DNA, nucleoprotein, Sm, and ribonucleoprotein previously described (16). It was also previously demonstrated that the precipitin reaction was not caused by rheumatoid factor (14). In immunodiffusion studies reported here, extracts from EBV-transformed cell lines were

placed in wells adjacent to wells containing WiL<sub>2</sub> extracts, and tested against prototype RA sera. Precipitin lines with extracts of transformed cells showing complete fusion with WiL<sub>2</sub> lines were taken to demonstrate immunological identity of antigens in the transformed cell line extracts.

Immunofluorescence was the second assay system used. The three prototype RA sera were used as the intermediate reagents in the indirect immunofluorescent test with fluorescein-conjugated anti-human IgG as the fluorescent marker. These three RA sera were negative for nuclear staining when tissue sections from organs (mouse or human kidney and liver) were used as substrates but reacted with WiL<sub>2</sub> cells to give nuclear staining of discrete finely speckled character. The specificity of the reaction had been previously demonstrated (14). It had also been shown previously that nuclear staining was most clearly demonstrated when cells were fixed with dry heat at 37°C for 30 min. Other fixatives such as acetone and alcohol resulted in loss of reactivity of this nuclear antigen. The different cell lines used in this study were prepared for immunofluorescence by washing the harvested cells in RPMI 1640 medium. Flat preparations of cells were made by cytocentrifugation on to glass slides and heated at 37°C for 30 min. They were reacted with RA sera for 30 min at room temperature, washed, and reacted with fluorescent anti-human IgG. RA sera were usually used at 1:4 dilution. Several sera from patients with Burkitt's lymphoma were made available for these studies through the courtesy of Dr. Guy de Thè, World Health Organization, Lyon, France.

*Differentiation from EBNA.* Reedman and Klein (15) have described an EBNA in EBV-infected cells, and it was important to determine whether the nuclear antigen reacting with RA sera was similar or different. For determination of EBNA, cell lines were fixed in chilled acetone as described (15). Reference sera containing antibodies to EBNA and sera negative for EBNA antibodies (the latter also used as a source of complement) were supplied by Dr. B. Hampar. Nuclear staining characteristic of EBNA was compared to that given by RA sera.

*Other Human and Simian Cell Lines Infected with EBV and Related Herpes Virus.* Several cell lines of human and simian origin (cotton-topped marmoset, orangutan, baboon, and owl monkey) were obtained from the Frederick Cancer Research Center, Frederick, Md. (17). The human cell lines were umbilical cord lymphocytes, transformed with EBV (HCL-3/B95-8) and herpes virus papio (HCL-3/KMPG-1). Two simian cell lines CP-81 (18) and 594-S (19) were lymphocyte lines established from animals who developed spontaneous lymphoid disease. Three other simian lymphocyte cell lines were from animals who developed lymphoid disease after in vivo inoculation with herpes virus (531-H, 1605-S, and MLC-1 (20)) and another line (B95-8) was derived from in vitro transformation of isolated lymphocytes. With the exception of WiL<sub>2</sub>, all the cell lines were under study in the laboratory of one of the authors (H. Rabin) and were being characterized with respect to the transforming activity and antigenicity of the herpes viruses present in simian cells (17). The cells were prepared for immunofluorescent studies as described above and reacted with RA and serum with EBNA antibody to determine the presence or absence of RA-associated nuclear antigen (RANA) and EBNA, respectively. Immunodiffusion studies were not performed with these cell lines.

## Results

*Immunofluorescent Studies on Cultured Human Lymphocyte Cell Lines.* The indirect immunofluorescent test was used to detect the presence of antigen reactive with antibodies in RA sera. The characteristic pattern of nuclear staining seen in WiL<sub>2</sub> cells is illustrated in Fig. 1 a. In this cell line, which is known to contain EB viral genome, the pattern of nuclear staining by using RA sera and fluorescein-conjugated anti-IgG reagent is demonstrated as distinct fine speckled nuclear staining. This pattern of nuclear staining is unique and can be readily distinguished from nuclear staining produced by antibodies to DNA, histones, or nonhistone proteins such as Sm antigen and ribonucleoprotein. In the human lymphocytes infected with EBV, no staining could be detected in cells taken at day 1 and day 14 of culture. By day 20, nuclear staining began to appear in the EBV-infected cells, showing up as a few distinct fine speckles of nuclear staining, as illustrated in Fig. 1 b. This was present in

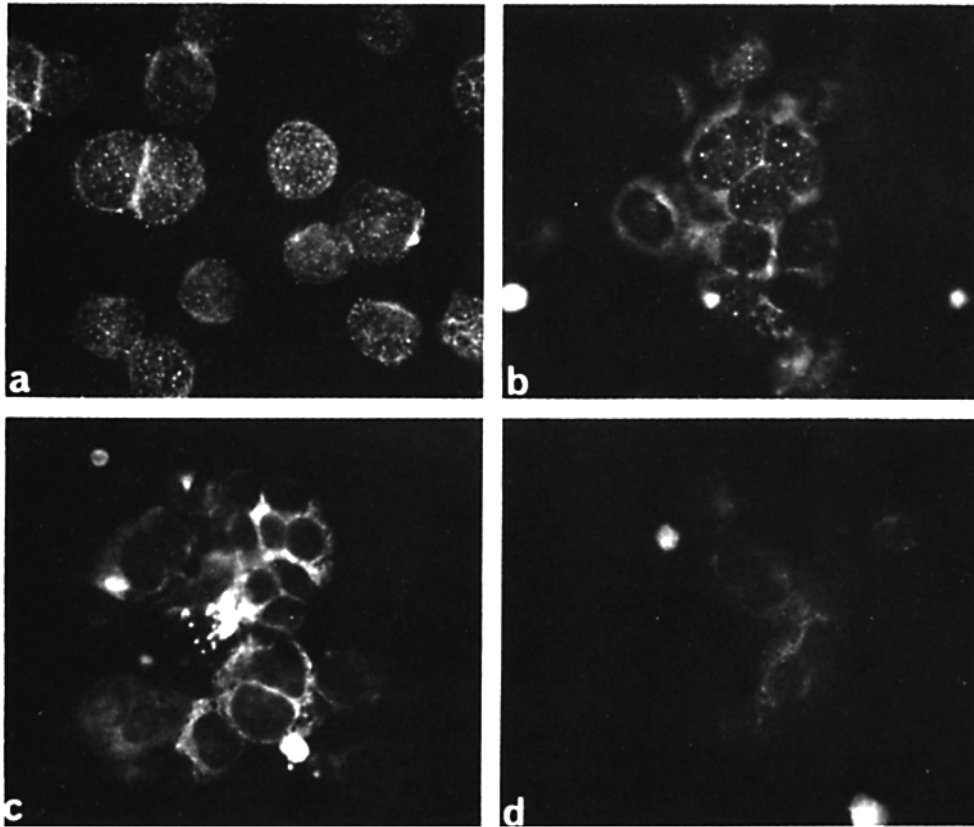


FIG. 1. Indirect immunofluorescence to demonstrate the presence of nuclear antigen reactive with antibody in RA sera. Tissue culture cells were cytocentrifuged on to glass slides, reacted with RA serum containing antibody, and stained with fluorescein-conjugated anti-human IgG. (a) demonstrates the discretely distributed finely speckled nuclear staining observed on WiL<sub>2</sub> cells; (b) was EBV-infected peripheral blood leukocytes at day 20 of culture. A few discretely distributed fine nuclear speckles were seen at this time and became more numerous after the cells became more transformed into continuous lines; (c) were the same EBV-infected cells (day 20) reacted with normal human serum; and (d) was a control noninfected cell culture at day 20, reacted with the same RA serum as in (b), showing absence of nuclear staining.

approximately 10–20% of the infected cells. By day 49, at least 70% of the cells were staining in a pattern similar to the staining seen in WiL<sub>2</sub> cells shown in Fig. 1 a. Fig. 1 c demonstrates that the same EBV-infected cells (at day 20) did not react with normal human serum, and Fig. 1 d shows that the control uninfected cell culture did not show nuclear staining with RA sera. Two other infected cell lines showed similar induction of this nuclear antigen and the corresponding control, uninfected cell cultures were completely negative.

EBV-infected cells at day 49 were extracted as described, and the soluble extract studied in immunodiffusion tests as illustrated in Fig. 2. A RA serum (RAP) containing precipitating antibody to WiL<sub>2</sub> extract also showed precipitin lines with D.J. and K.H. extracts (two EBV-infected cell preparations), and these lines fused in complete immunological identity with the WiL<sub>2</sub> precipitin line. It

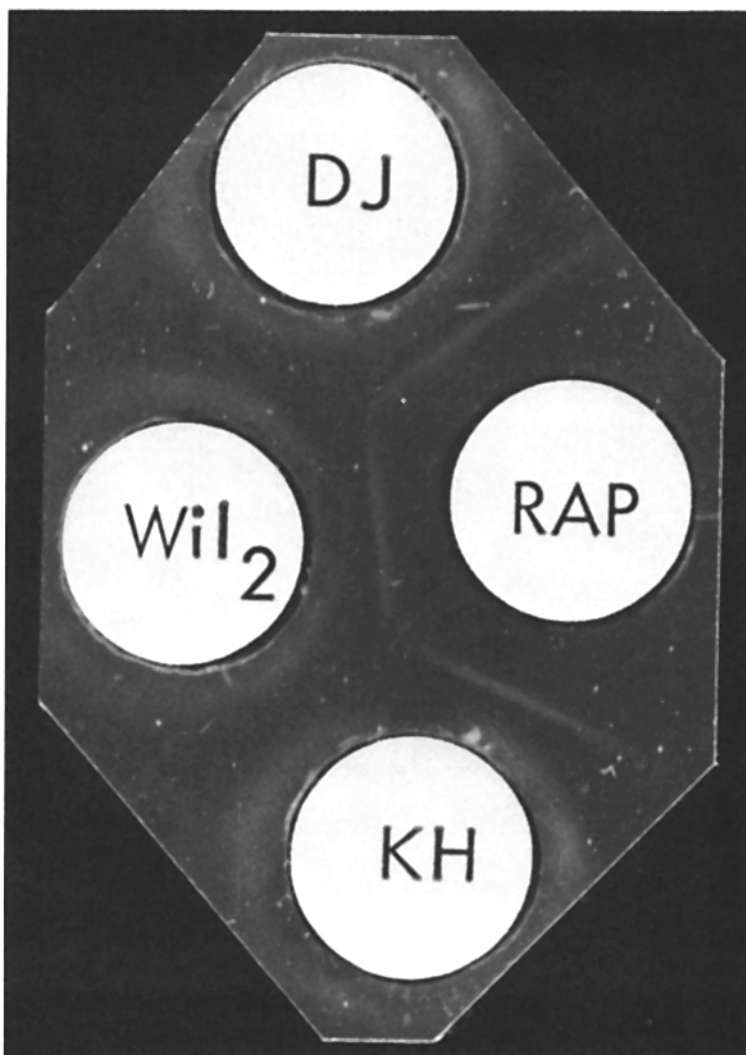


FIG. 2. Immunodiffusion study between RA serum containing precipitating antibody (RAP) and cell culture extracts. D.J. and K.H. were human peripheral blood leukocytes infected with EB virus and cell cultures harvested at day 49. Soluble antigens were extracted in the same manner as that used for the lymphocyte line WiL<sub>2</sub>. Precipitin lines developed between RAP, and the three cell culture extracts and the precipitin lines fused completely with one another to show immunological identity.

was further shown that when RAP serum was absorbed with WiL<sub>2</sub> extract to remove precipitating antibody, the absorbed serum was no longer reactive in immunodiffusion with D.J. and K.H. extracts.

*Studies on Human and Simian Lines Containing EBV and Related Herpes Viruses.* The above studies suggested that the nuclear antigen reactive with serum antibody in RA sera could be induced in human lymphocytes by EBV. The availability of a number of well-characterized human and simian lymphocyte cell lines provided the opportunity to determine whether the nuclear

TABLE I  
EBNA and RANA in Human and Simian Cell Lines

Cell line	Animal	Transforming virus	Lymphocyte cell type	Immunofluorescent staining for	
				EBNA	RANA
WiL <sub>2</sub>	Human spleen	EBV	B	+++	+++
HCL-3/B95-8	Human umbilicus	EBV	B	+++	++
HCL-3/KMPG-1	Human umbilicus	<i>Herpes (H) papio</i>	B	-	-
531-H	Owl monkey-in vitro EBV-induced lymphoreticular hyperplasia	EBV	B	+++	+
B95-8	Cotton-topped marmoset-in vitro EBV-transformed lymphocytes	EBV	B	+++	+
1605-S	Cotton-topped marmoset-in vivo EBV-induced lymphoma	EBV	B	++	+
CP-81	Orangutan with monomyelogenous leukemia	<i>Herpes pongo</i>	Undifferentiated	+	-
594-S	Baboon with lymphoreticular hyperplasia	<i>Herpes papio</i>	B	-	-
MLC-1	Cotton-topped marmoset- <i>H. saimiri</i> induced lymphoma	<i>Herpes saimiri</i>	T	-	-

antigen was induced only by EBV or was also inducible by related herpes viruses of simian origin. It can be noted (Table I) that the human cord lymphocyte line infected with EBV (HCL-3/B95-8) was positive for EBNA and for the nuclear antigen reactive with RA sera, but cord cells infected with herpes virus papio were negative for both antigens. We observed that simian cell lines which had been transformed in vitro with EBV (B95-8) or obtained from animals inoculated in vivo with EBV (531-H and 1605-S) also contained the nuclear antigen reactive with RA sera. On the other hand, simian cell lines carrying herpes-like viruses such as herpesvirus pongo (CP-81, of orangutan origin), herpesvirus papio (594-S, of baboon origin), and herpesvirus saimiri (MLC-1, of squirrel monkey origin) were negative for the nuclear antigen reactive with RA sera. It was noted that the WiL<sub>2</sub> cell line was stronger in immunofluorescence for the RA-associated nuclear antigen than other cell lines positive for this antigen. The cell line MLC-1, which was from a marmoset which developed lymphoma after inoculation with herpesvirus saimiri had been characterized as a T lymphocyte and was negative for either EBNA-like or the RA-associated nuclear antigen. These studies showed a close relationship between RA-associated nuclear antigen and EBV-infected cells, but lack of a relationship with cells infected with herpes-related viruses of simian origin. Although most cells containing RA-associated nuclear antigen also contained EBNA, there was lack of complete concordance, as shown by presence of EBNA-like antigen in cell line CP-81 but absence of RA-associated nuclear antigen.

*Differentiation from EBNA.* A serum containing antibody to EBNA (from Dr. B. Hampar) and negative for precipitating antibody to WiL<sub>2</sub> extract was compared with an RA serum negative for antibody to EBNA but positive for precipitating antibody to WiL<sub>2</sub>. WiL<sub>2</sub> cells which have both EBNA and the RA-associated nuclear antigen were used as substrate in indirect immunofluorescence. Staining for RA nuclear antigen by RA serum was best demonstrated with anti-IgG reagent and was not amplified with anti-C3. In contrast, staining for EBNA by anti-EBNA serum was not demonstrable with anti-IgG reagent but was strongly positive with anti-C3, as described by Reedman and Klein

TABLE II  
*Lack of Relationship between Antibodies to RANA and EBNA*

Sera	Immunofluorescent staining for		Precipitating antibody to RANA
	RANA	EBNA	
RA			
VM	+ (32)	+ (256)	+ (16)
VI	+ (16)	-	+ (4)
TF	+ (16)	+ (512)	+ (4)
Burkitt lymphoma			
1440	-	+ (5,120)*	-
1020	-	+ (2,560)	-
1023	-	+ (80)	-
1053	-	+ (1,280)	-
1003	-	-	-
Normal sera			
CP	-	+ (128)	-
MC	-	+ (256)	-
BB	-	-	-
JV	-	+ (256)	-

\* Antibody to EBNA in Burkitt lymphoma sera were performed through the courtesy of Dr. G. de Thè. EBNA antibody studies for the other sera were performed on WiL<sub>2</sub> cells as were all studies for antibody to RANA. Numbers in parenthesis represent the reciprocal of the highest dilution of sera positive for staining or precipitation.

(15). In addition, the patterns of nuclear staining were completely different in that EBNA staining was dense and clumpy involving the entire nucleus, whereas, RA nuclear antigen staining consisted of discrete, individual speckles as illustrated in Fig. 1.

Further studies which differentiated the RANA from EBNA are presented in Table II. The three prototype RA sera used in these studies were all positive for antibody to RA nuclear antigen by immunofluorescence and immunodiffusion but only two of the three had antibody to EBNA. The lack of relationship between the two nuclear antigens was also demonstrated by sera of patients with Burkitt's lymphoma and certain normal sera. In many instances, high titers of antibodies to EBNA were not associated with any detectable antibodies to RANA, either by immunofluorescence or immunodiffusion.

### Discussion

In an earlier study (14), it was demonstrated that the soluble nuclear antigen reactive with serum antibody in RA sera was present in human lymphocyte cell lines WiL<sub>2</sub> and Raji but not present in a variety of normal human and animal tissues. It was postulated that the nuclear antigen might belong to a class of nuclear "derepressor" proteins present in rapidly proliferating cells. Another possibility was that the nuclear antigen might be a viral-induced protein since WiL<sub>2</sub> and Raji cell lines are B lymphocytes known to carry EBV. In other studies not reported here, we have determined that RA-reactive nuclear antigen is not present in other rapidly proliferating tissue culture cell lines, including

monkey kidney cells (CV-1), human fibroblast cells (WI38), and human T lymphocytes (1301 and Molt4). The present studies suggest that it might be an EBV-associated antigen. This is supported by the demonstration that the nuclear antigen began to appear in human lymphocytes in the early stages of transformation induced by EBV and increased in intensity and in percentage of positive cells as transformation progressed. The nuclear antigen was present in human and simian lymphocytes transformed with EBV, but not in lymphocytes transformed with herpesviruses of simian origin. Human lymphocytes were not strictly necessary for the expression of this nuclear antigen, since it could be detected in simian lymphocytes transformed with EBV.

Our studies suggest that the RANA is different from EBNA. The antigens showed different patterns of nuclear staining and different reactivity to anti-complement and anti-IgG fluorescein reagents. Heat fixation was the best method of preserving the RA-associated antigen for its demonstration by immunofluorescence, a situation different from EBNA which is best demonstrated by fixation in cold acetone. Furthermore, certain sera contained antibodies to EBNA, but no antibodies to RA-associated antigen. RA antibodies are also precipitating antibodies whereas antibody to EBNA has not been demonstrated by precipitation reactions. Once induced, the RANA can be demonstrated to be present at all times in EBV-transformed cells and is therefore different from the EBV-induced transient nuclear antigen recently described (21).

These studies do not tell us if there is an etiological relationship between EBV infection and RA. If a relationship is present, specific host and other factors must play important roles since EBV has been implicated in such diverse diseases as Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis. However, if a persistent infectious agent should be one of the factors causing RA, the role of EBV might merit further study.

### Summary

Sera from approximately two-thirds of patients with rheumatoid arthritis contain an antibody which is reactive with a nuclear antigen present in human B-lymphocyte tissue culture cells. The immunological reaction can be demonstrated by precipitation and immunofluorescence. Evidence is presented that the reactive nuclear antigen is associated with Epstein-Barr (EB) virus-transformed lymphocytes. Normal human peripheral blood lymphocytes did not contain the nuclear antigen reactive with rheumatoid arthritis sera, but after infection with EB virus, they showed increasing amounts of reactive nuclear antigen as the cells were transformed into continuous lines. Several established human and simian lymphocyte cell lines known to carry EB viral genomes were shown to contain rheumatoid arthritis-associated nuclear antigen. Evidence is presented which suggests that the rheumatoid arthritis-associated nuclear antigen is different from the previously described EB nuclear antigen.

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