

IN VITRO EFFECTS OF EPSTEIN-BARR
VIRUS ON PERIPHERAL
BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH
RHEUMATOID ARTHRITIS AND NORMAL SUBJECTS*

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There are two reasons for studying the possible role of the Epstein-Barr virus (EBV) in relation to rheumatoid arthritis (RA). The first is the recent demonstration by Alspaugh and co-workers that EBV infection of normal peripheral blood lymphocytes induces the development in these cells of a nuclear antigen against which most patients with RA have precipitating antibodies (1). The second reason is that EBV is a naturally occurring, polyclonal activator of antibody production by human B lymphocytes (2, 3).

In the present experiments, we have asked to what extent RA and normal subjects have B lymphocytes which can be induced by EBV to secrete anti-IgG antibodies. At the same time, we have determined the degree to which lymphocytes from RA patients spontaneously transform in the absence of EBV infection. EBV nucleic acid can be detected in most permanent lines of human B cells (4). In most cases, therefore, EBV plays an essential role in the in vitro transformation of human B cells to permanent lines. If RA lymphocytes undergo morphologic transformation and become permanent lines more readily than do normal lymphocytes, one might wonder whether or not they either contain more EBV genomes per cell than normal lymphocytes, or are abnormally responsive to EBV infection.

Materials and Methods

Source. 50 ml of heparinized blood was obtained from 11 patients with seropositive RA. These patients met the American Rheumatism Association criteria (8) for diagnosis and were all currently under treatment at the Scripps Clinic and Research Foundation. 10 other samples of the same volume were obtained from healthy persons having no prior diagnosis of a rheumatic disorder and using no medications.

Virus. EBV was obtained from the B95-8 marmoset lymphoblastoid line, processed as described by Miller and Lipman and Thorley-Lawson et al., and frozen until use (6, 7).

Cell Separation and Culture Procedure. Mononuclear cells were isolated from 50 ml heparinized blood by Ficoll-Hypaque sedimentation (8) followed by five washes in serum-free medium, and resuspension in RPMI-1640 and 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) at a concentration of 10^7 cells/ml. 1 ml of EBV was used to infect 1×10^7 cells. An equal volume of RPMI-1640 with 10% fetal bovine serum was added to duplicate control cells. Cells

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were gently agitated at 37°C for 1 h and then resuspended to a concentration of 1×10^6 cells/ml. Cells were again agitated gently at 37°C for 20 min, then centrifuged at 500 *g* for 10 min at 37°C. The supernatant fluid was removed to be assayed later as a zero time control for total IgG and IgM anti-IgG production. Control and infected cells were cultured at concentrations of 1×10^6 /ml in duplicate Corning T25 flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) at final vol of 10 ml in RPMI-1640 containing 10% fetal bovine serum. The supernatant fluid was removed from each culture by centrifugation at weekly intervals and frozen at -20°C until subsequently assayed for total IgM and IgM anti-IgG production. Cell pellets were resuspended in an equal volume of fresh medium and replaced in the original flasks.

Transformation Assay. The outgrowth of transformed lymphocytes was assayed visually according to the system of Thorley-Lawson et al. (7). In this system a score of one represents dead cells; two, living but not transformed cells; three, enlarged cells and cells in clumps; and four, proliferating cells overrunning the culture.

Radioimmunoassay. IgM anti-IgG in the culture supernates was analyzed by solid phase radioimmunoassay exactly as previously described, using pooled human IgG (Sigma Chemical Co., St. Louis, Mo.) as antigen (9). The cpm anti-IgG bound were converted to nanograms of IgM anti-IgG per milliliter using a standard curve containing known quantities of a purified monoclonal IgM (k) anti-IgG (IgM-Si) isolated from the serum of a patient with cryoglobulinemia (10).

Total IgM in the culture supernates was also analyzed by solid phase radioimmunoassay, performed exactly the same as the IgM anti-IgG assay except that the tubes were coated with 5 µg/ml of purified unlabeled anti-IgM rather than 60 µg/ml human IgG. Again, counts were transformed to nanograms IgM per milliliter using a standard curve containing the same monoclonal IgM (k) protein as used for the IgM anti-IgG assay.

Depletion Experiments. Human Cohn Fraction II (Sigma Chemical Co.) was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.) according to the manufacturer's directions. To 1 ml of a 50% slurry of IgG-Sepharose was added 2 ml of undiluted supernatant fluid. After shaking overnight at room temperature the IgG-Sepharose was removed by centrifugation and the supernate was assayed for total IgM and IgM anti-IgG.

Results

IgM Anti-IgG Secretion. Fig. 1 shows the amount of IgM anti-IgG as measured by radioimmunoassay released into the supernatant fluids at 6-day intervals by cells from patients with RA and normal controls, either infected with EBV, or uninfected. The points of significance are: (a) not only the lymphocytes of patients with RA, but also those of some normal individuals made anti-IgG when infected with EBV, although the rheumatoid lymphocytes made significantly more than did the normal lymphocytes; (b) the peak of anti-IgG secretion was seen at 18 and 24 days after the initiation of culture in both rheumatoids and normals; (c) while the lymphocytes of normal donors produced little or no anti-IgG in the absence of EBV infection, the lymphocytes from RA patients produced small but significant quantities in an early (6-12 days) peak.

Total IgM Secretion. As shown in Fig. 2, EBV-infected lymphocytes produced more IgM than uninfected cells over the 30-day period of the experiments. Of note, whether infected with EBV or uninfected, both the normal and RA lymphocytes produced similar amounts of IgM with the same time course. Thus, it follows that the lymphocytes from patients with RA made more IgM anti-IgG relative to the amount of IgM being produced in the culture than did cells from normal subjects. As shown in Fig. 3, when measured by radioimmunoassay, $30 \pm 8\%$ of the IgM in the plasmas of the RA patients had anti-IgG activity, and this percentage was generally the same

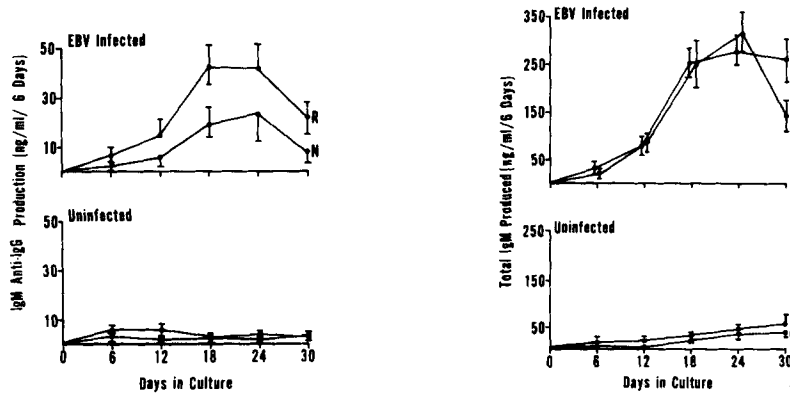


FIG. 1. IgM anti-IgG production by peripheral blood mononuclear cells from patients with arthritis (R) and normal subjects (N). Cells from 10 patients with RA (R, ●) and 9 normal subjects (N, ○), either infected with EBV (upper panel) or uninfected (lower panel) were cultured at a concentration of 10^6 cells/ml in RPMI-1640 medium with 10% fetal bovine serum. At 6-day intervals the cells were centrifuged and the culture media removed for assay of IgM anti-IgG by radioimmunoassay. Each point represents the mean \pm SEM amount of anti-IgG produced per milliliter by the respective patient groups over the previous 6 day interval.

FIG. 2. Total IgM production by peripheral blood mononuclear cells from patients with rheumatoid arthritis (R) and normal subjects (N). This experiment was performed using the same culture fluids as described in Fig. 1, except that total IgM was determined by radioimmunoassay.

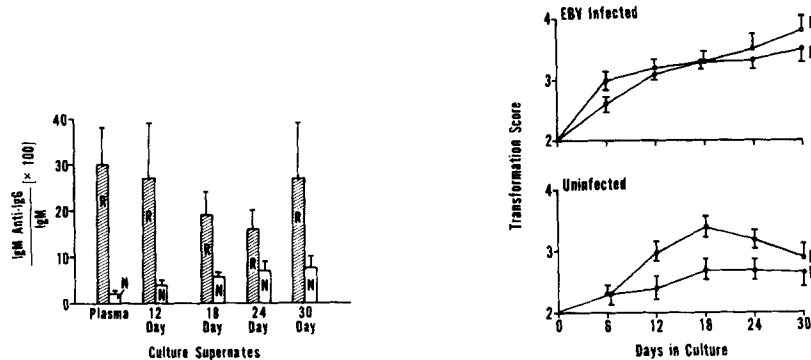


FIG. 3. Percentage of IgM with anti-IgG activity in plasma and in culture supernates from EBV infected mononuclear cells from patients with rheumatoid arthritis (R) and normal subjects (N). This figure was derived using the data from Figs. 1 and 2, and by assaying the mean \pm SEM IgM and IgM anti-IgG in the plasma from the same patients whose cells had been placed in culture.

FIG. 4. Transformation state of rheumatoid and normal peripheral blood mononuclear cells in tissue culture. The transformation state of either EBV infected (upper panel) or uninfected (lower panel) peripheral blood mononuclear cells from patients with rheumatoid arthritis (R, ●) or normal subjects (N, ○) was assayed visually by the method of Thorley-Lawson et al. (7). Grade 2 = living but untransformed cells. Grade 3 = transformed cells in clumps. Grade 4 = dividing cells over-running the culture.

in the various supernates of the cultured cells. On the other hand, $2 \pm 1\%$ of the IgM in the normal plasmas was detected as anti-IgG. This percentage was nearly the same in the supernatants of the 12-day cultures but gradually rose to $7.5 \pm 2\%$ by 30 days.

Specificity of the IgM Anti-IgG. Greater than 90% of the anti-IgG antibody detected in 24-day supernates of EBV-stimulated RA mononuclear cells was removed by absorption with IgG-Sepharose. On the other hand, only 50-70% of the IgM anti-IgG from two randomly selected supernates from EBV-stimulated normal mononuclear

cells harvested at the same time was specifically removed by immunoabsorption, suggesting that this antibody was of lower affinity than the anti-IgG in the RA supernatants. Approximately 1–2% of the IgM in all samples was detected as IgM anti-IgG which could not be absorbed.

Cellular Transformation. Fig. 4 shows the transformation indices for rheumatoid and normal lymphocytes for the first 30 days after initiation of culture. One culture from an RA patient and one from a control died early in the course of the experiment, leaving 9 derived from normals and 10 from RA patients. It is from these that the data in the figure were compiled. In the EBV infected groups, both normal and rheumatoid lymphocytes showed rapid development of transformation with similar time courses. On the other hand, noninfected cell cultures from patients with RA transformed more rapidly, and to a higher degree, than did normal lymphocytes. This was most marked at 12–18 days. The differences between the noninfected rheumatoid and normal cultures at any single time point were not significant. However, when all points from days 18–30 were subjected to variance analysis, a significant difference between the two groups was found ($F = 11.6, P < 0.01$). Furthermore, only 2 of 10 cultures of normal lymphocytes, but 6 of 11 cultures of RA lymphocytes, developed into continuous B lymphoid cell lines. The time interval for the establishment of the permanent lines ranged from 3 to 10 wk.

Discussion

The studies reported herein show that in vitro infection of peripheral blood lymphocytes with EBV can induce the synthesis of IgM anti-IgG antibody, not only in patients with RA but also in normal subjects. The autoantibody production probably results from polyclonal activation of B lymphocytes, as indicated by a concomitant increase in total IgM secretion. Consistent with this interpretation is the apparently lower average affinity of the anti-IgG from the normal cells compared with that from the RA cells. In the normal donors there is no in vivo process operative to promote the proliferation of clones of cells producing higher affinity anti-IgG, as is probably the case in the RA patients. The finding, however, that all normal lymphocytes made some anti-IgG after EBV infection, even though at far lesser quantities than in RA, suggests that B-cell tolerance for IgG does not exist in any normal person.

In patients with RA, the percentage of IgM with anti-IgG activity was high and was similar in the plasma and in the supernates of EBV transformed lymphocyte cultures. Here, the EBV infection led to increased anti-IgG secretion as a result of cell division and a concomitant increase in the absolute number of anti-IgG reactive B cells. Similar results would be expected if other diseases associated with high titers of anti-IgG activity were studied. In normal subjects, on the other hand, the fraction of IgM with anti-IgG activity was low in the plasma and rose in the culture supernates after EBV infection although never approaching the amount seen in RA. Thus, normal individuals probably have fewer B cells in the peripheral blood capable of IgM anti-IgG production than do RA patients. Furthermore, the majority of these cells are not fully expressed in vivo but can respond to a maturation event, or derepression, that takes place in the EBV containing cultures.

Our experiments also show that peripheral blood B lymphocytes from RA patients frequently transform in tissue culture in the absence of superinfection with EBV. This phenomenon may not be specific for RA, but might also occur in other chronic

inflammatory diseases associated with B-cell activation. Our current studies are investigating this possibility. Nevertheless, with the exception of certain neoplastic states, human B cells usually must already contain EBV genetic material if they are to transform and develop into permanent cell lines (4) and this may be of particular interest in RA. The mechanisms controlling the frequency with which transformation occurs are not fully understood. Contributing factors may include the number of EBV genomes per cell, the sensitivity of these genomes to activation, and the presence or absence of virus-specific suppressor cells (7, 11, 12).

One must always be cautious in interpreting the results of *in vitro* experiments, which are necessarily restricted and seldom reflect precise conditions in a whole organism. However, polyclonal B-cell stimulation by clinical EBV infection apparently occurs in infectious mononucleosis, as evidenced by IgM anti-IgG and heterophil antibodies, and elevated total serum IgM concentrations which sometimes persist for up to 2 yr after clinical infection (13). Possible outcomes of clinical infection with EBV that have previously been considered are: recovery with establishment of a latent state, malignant transformation, and immunodeficiency (14). Both genetic and environmental factors apparently influence the ultimate result (15). It does not seem unreasonable to consider autoimmune disease as another theoretically possible result.

Summary

Peripheral blood mononuclear cells from 10 patients with rheumatoid arthritis and 9 control subjects were cultured *in vitro* for 30 days with and without infection by Epstein-Barr virus. All cultures showed polyclonal stimulation of B cells as indicated by rising levels of IgM in the culture supernates, reaching maximal at 18–24 days, and with no quantitative or kinetic difference between the RA and control cells. IgM anti-IgG was also produced in both groups and maximally at 18–24 days, but in greater quantity by the RA lymphocytes. The anti-IgG made by the RA lymphocytes was more easily absorbed by solid phase IgG than was the anti-IgG made by the normal lymphocytes and thus was judged to be of higher affinity. RA lymphocytes uninfected with EBV had higher transformation scores than did the normal controls and developed spontaneously into permanent cell lines in six instances.

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