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# Disturbance of the Epstein–Barr virus-host balance in rheumatoid arthritis patients: a quantitative study

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## SUMMARY

Rheumatoid arthritis (RA), seronegative spondyloarthropathy (SA) and osteoarthritis (OA) patients receiving no steroid or disease-modifying therapy have been monitored, along with healthy controls, for their prevailing level of Epstein–Barr virus (EBV) infection using four independent indices of the EBV-host balance, (i) levels of virus shedding in throat washings as measured by a cord-blood transformation assay of improved sensitivity, (ii) frequency of virus-infected B cells in the circulating blood as measured by the rate of 'spontaneous' transformation in limiting dilution cultures, (iii) antibody titres to viral antigens, and (iv) virus-specific cytotoxic T cell responsiveness as measured in the *in vitro* regression assay. All four parameters indicated significant disturbance of the virus-host balance accompanying RA, the range of values exhibited by RA patients as a group in each case extending beyond the normal control range in the direction of more active infection. However, observations with SA and OA patients suggested that such a disturbance may not be RA-specific.

**Keywords** Epstein–Barr virus rheumatoid arthritis virus-host balance

## INTRODUCTION

The discovery that patients with rheumatoid arthritis (RA) frequently possess serum antibodies to a nuclear antigen, RANA, found exclusively in Epstein–Barr virus (EBV)-transformed B cell lines (Alspaugh & Tan, 1976; Alspaugh *et al.*, 1978) first focused attention upon a possible role for the virus in the pathogenesis of RA (Depper & Zvaifler, 1981). The molecular nature of RANA is still not fully resolved butrecent evidence (Billings *et al.*, 1983; Sculley *et al.*, 1984; T. B. Sculley, personal communication) would suggest indentity with one of the EBV-coded nuclear antigens EBNA 1 or EBNA 2 (Hennessy & Kieff, 1983). Viewed in this light, the greater frequency with which anti-RANA antibodies can be detected in the sera of RA patients as opposed to healthy controls (Catalano *et al.*, 1979, 1980; Venables *et al.*, 1981) appears to be another manifestation of the generally elevated titres of anti-EBV antibodies which are seen in association with RA (Catalano *et al.*, 1979; Alspaugh *et al.*, 1981; Ferrel *et al.*, 1981). This exaggerated serological response is similar, at least in some respects, to that seen in renal allograft recipients (Henle & Henle, 1981) where disturbance of the virus–host balance is an inevitable accompaniment of the impairment of cell-mediated immune response (Crawford *et al.*, 1981; Gaston, Rickinson & Epstein, 1982a).

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Accordingly, there has been considerable interest in monitoring RA patients in terms of those cellular immune functions which are thought to be responsible for controlling persistent EBV infection *in vivo* and which are recognized through their ability to alter the course of virus-induced B cell transformation *in vitro*. At least two forms of control appear to be affected in RA patients. (i) In the early phase of the experimental infection, T cells from healthy adult donors (whether EBV-immune or non-immune) can delay the onset of virus-induced B cell proliferation through secretion of $\gamma$-interferon into the culture medium (Thorley-Lawson, 1981; Andersson *et al.*, 1983); such a delay is not seen in corresponding cultures set up from RA patients (Bardwick *et al.*, 1980; Depper, Bluestein & Zvaifler, 1981) where $\gamma$-interferon production by T cells itself appears to be unusually sensitive to inhibition by prostaglandins secreted by coresident monocytes (Hasler *et al.*, 1983a, b). (ii) At a later phase of the experimental infection, following the initiation of B cell growth, continued proliferation to virus-transformed B cell lines is prevented in cultures from healthy virus-immune donors by a second quite separate and immunologically specific mechanism, namely through the influence of virus-specific cytotoxic T cells reactivated in the culture (Moss, Rickinson & Pope, 1978); in at least some RA patients, this T cell-mediated regression of outgrowth, which has also been described as 'late suppression' (Tosato, Steinberg & Blaese, 1981), is significantly impaired (Gaston, Rickinson & Epstein, 1982b; Moss *et al.*, 1983).

These various immunological parameters only provide *indirect* indices of the EBV-host balance as it exists in RA patients. Not until recently have attempts been made to quantitate the level of EBV infection *directly*, either by measuring virus shedding in throat washings (Aitcheson *et al.*, 1983) or by examining the frequency of virus-infected B cells in peripheral blood (Tosato *et al.*, 1984). The present study represents a much more concerted attempt to monitor both direct and indirect indices of the EBV-host balance in the same individuals using assays of improved sensitivity. Such assays have recently been developed in this laboratory and were first used in prospective studies involving healthy virus carriers and immunosuppressed renal allograft recipients (Yao, Rickinson & Epstein, 1985a; Yao *et al.*, 1985b). Such studies provide the necessary background against which the present results from RA, seronegative spondyloarthropathy (SA) and osteoarthritis (OA) patients can be judged.

## MATERIALS AND METHODS

*Patients and controls.* The following groups of individuals were studied, (a) 33 patients with classic or definite RA (Ropes, Bennett & Cobb, 1958) of whom 26 were positive for rheumatoid factor, age range of patients 36–74 years, (b) 15 patients with SA (11 with ankylosing spondylitis, two with Reiter's syndrome, two with psoriatic arthropathy), age range 35–64 years, (c) 15 patients with OA, age range 28–76 years, (d) 26 healthy adult controls, age range 22–64 years. Patients in groups (a), (b) and (c) were receiving only non-steroidal anti-inflammatory drugs; no patient had received steroids or disease-modifying agents such as gold or D-penicillamine for at least 6 weeks prior to this study.

*Indices of the EBV–host balance.* Full details of the methods employed have been published elsewhere (Yao *et al.*, 1985a,b) and a brief account only is given here.

(i) *EBV shedding.* Throat washings were collected and placed on ice before clearing by centrifugation at 2000 ***g*** for 10 min. The supernatant was then filtered through a 0·45 $\mu$ Millipore membrane and the filtrate ultracentrifuged at 100,000 ***g*** for 2 h at 4°C. The pellet was resuspended to 1/20 original volume in RPMI 1640 supplemented with 2 mM glutamine, 100 iu/ml penicillin, 100 $\mu$g/ml streptomycin and 10% foetal calf serum (culture medium). Half of the volume was stored at −70°C whilst the other half was immediately assayed for its ability to transform cord-blood lymphocytes into permanent lymphoblastoid cell lines. The indicator cells used in these assays were cryopreserved lymphocytes from particular cord-blood samples already found to possess optimal sensitivity to EBV-induced transformation *in vitro*.

(ii) *Spontaneous transformation' assay of circulating virus-infected B cells.* Blood samples of 40–50 ml were taken into heparinized (10 u heparin/ml) syringes and the blood mononuclear (UM) cells were separated by isopycnic centrifugation on Ficoll-Hypaque. The UM cells were seeded into

0·2 ml flat-bottomed microtest plate wells at $2 \times 10^6$, $5 \times 10^5$, $2{\cdot}5 \times 10^5$ cells/well, usually into six replicate wells per cell concentration; in these particular experiments the cell number in the less densely seeded cultures was not augmented with cord-blood UM cells, as had been the case in other studies involving this assay (Yao *et al.*, 1985a, b). All such cultures were maintained in medium containing the immunosuppressive drug cyclosporin A (CSA) at 0·1 μg/ml, a concentration which blocked the *in vitro* activation EBV-specific cytotoxic T cells and allowed the true frequency of 'spontaneous transformation' to be displayed (Rickinson *et al.*, 1984).

(iii) *Anti-EBV antibody titres.* All donors used in these experiments were assayed for serum antibodies to EBV capsid antigen (VCA) and the diffuse component of the EBV early antigen (EA), using standard techniques (Yao *et al.*, 1985).

(iv) *EBV-specific cytotoxic T cell memory.* All donors were also tested for the level of virus-specific T cell immunity using the standard *in vitro* regression assay (Moss *et al.*, 1978), in which donor UM cells are seeded over a range of doubling dilutions in microtest plate wells immediately following their exposure to a potent EBV preparation. T cell-mediated regression occurs preferentially in the more densely seeded cultures and thus the magnitude of the cytotoxic T cell response can be expressed in terms of the minimum number of UM cells required per well for a 50% incidence of the effect. The higher the number of UM cells required, the weaker the cytotoxic T cell response.

*Experimental procedure.* Samples were taken at nine outpatient clinics in all. On each occasion, the group of individuals monitored included some RA, some SA and some OA patients as well as several healthy seropositive donors and at least one healthy seronegative donor. Samples taken on the same day were all processed at the same time and assayed in parallel. Standard cryopreserved samples from particular healthy seropositive donors were included in each batch of assays as internal controls of assay sensitivity.

## RESULTS

### *EBV antibody titres*

Serum antibody testing identified 2/33 RA, 2/15 SA and 2/15 OA patients with no serological evidence of prior EBV infection; 2/26 healthy adult controls were also EBV antibody-negative. These individuals all gave negative results in each of the other assays of the EBV carrier state and were therefore excluded from the following analysis.

As shown in Fig. 1, of the EBV antibody-positive individuals within each group, RA patients showed a slightly higher range of anti-VCA titres (Geometric-mean 1 : 1498, $P < 0{\cdot}02$ by Student's *t*-

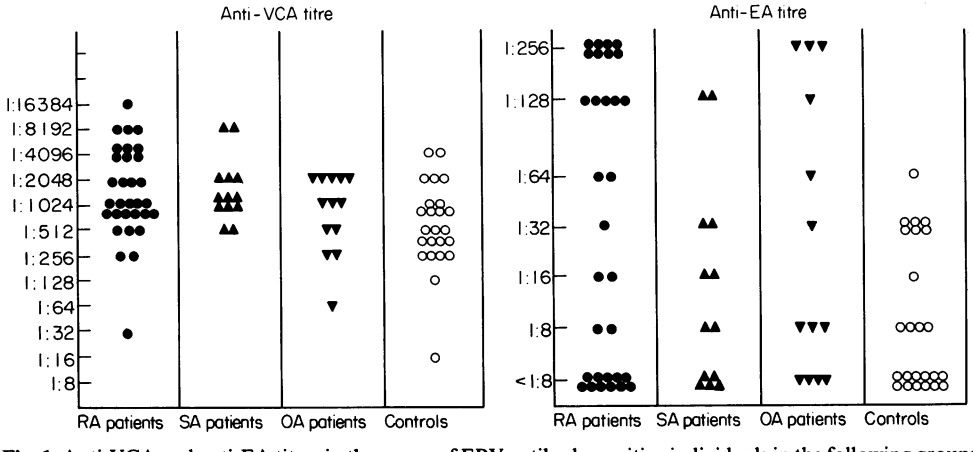


**Fig. 1.** Anti-VCA and anti-EA titres in the serum of EBV antibody-positive individuals in the following groups: (i) RA patients (●), (ii) SA patients (▲), (iii) OA patients (▼), (iv) healthy controls (○).

test) and a markedly higher range of anti-EA titres (mean 1:30, $P<0.01$) when compared with healthy adult controls (mean anti-VCA 1:627, mean anti-EA 1:9). Although the SA and OA patient groups were smaller, some elevation of anti-VCA titres for SA patients (mean 1:1487, $P<0.05$) and of anti-EA titres for OA patients (mean 1:23, $P<0.1$) was apparent here also. It should perhaps be noted that in many previous studies the presence of anti-EA antibodies in serum has been taken to indicate reactivation of a persistent EBV infection; in fact, it is clear from this and from other work (Gaston *et al.*, 1982a, b) that a sufficiently sensitive immunofluorescence technique will allow anti-EA antibodies to be detected at low titres in a proportion of healthy control donors.

*EBV-specific T cell immunity*

Since T cell-mediated regression of B cell outgrowth is preferentially observed in cultures of EBV-infected UM cells seeded at high initial concentrations, the concentration at which the phenomenon titrates out can be taken as a measure of prevailing virus-specific T cell immunity (see Materials and Methods). The results of regression assays for each of the patient groups and for control donors are summarised in Fig. 2. The majority of individuals in each patient group gave values within the control donor range ($4\times10^4$–$6\times10^5$ UM cells required per culture well for a 50% incidence of regression) but 6/31 RA patients, and 3/13 SA patients were clearly significantly different from normal controls (RA versus control, $P<0.01$; SA versus control $P<0.05$) in giving no evidence of regression even at the highest input cell number (i.e., $>6\times10^5$ UM cells required per culture well).

*EBV shedding*

Table 1 presents the results obtained when patients and control donors were tested for the presence of transforming virus in throat washings. The detection of active virus shedding in 58% seropositive control donors, on the basis of a single throat washing sample per donor, is entirely in accord with results recently obtained in a much more extensive analysis of healthy virus carriers (Yao *et al.*, 1985a). In this earlier study many seropositive control donors were found to shed low levels of virus in throat washings, so that successive samples taken from such donors typically revealed a negative test on one occasion of testing followed by a positive test with transformation of only a very small proportion of indicator wells on another. The incidence of detectable virus shedding amonst RA patients in the present work was slightly higher (74%) than amongst healthy controls and the total number of positive test wells observed in the RA patient assays was also correspondingly increased.

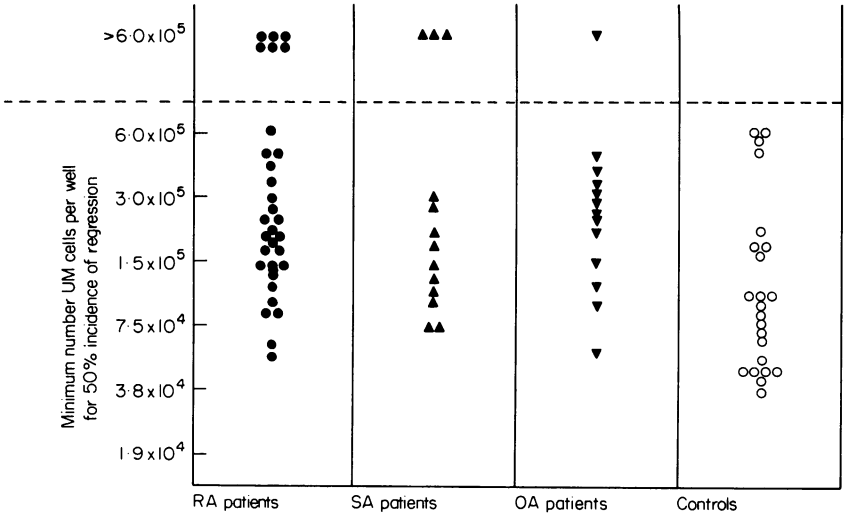


**Fig. 2.** Regression assay results obtained from EBV antibody-positive individuals in each of the groups already outlined in Fig. 1. Results are expressed as the minimum of UM cells per well required for a 50% incidence of regression, so that high numbers reflect weak T cell responsiveness.

**Table 1.** Detection of EBV in the throat washings* from RA, SA and OA patients with serological evidence of prior EBV infection

| | Frequency of detectable shedding | | Frequency of positive indicator cultures‡ | |
|---|---|---|---|---|
| Patient group | Incidence | Percentage | Incidence | Percentage |
| RA patients | 23/31 | 74 | 156/372 | 42 |
| SA patients | 9/13 | 69 | 51/156 | 33 |
| OA patients | 6/13 | 46 | 35/156 | 22 |
| Healthy controls† | 14/24 | 58 | 94/288 | 33 |

* Results obtained with concentrated throat washings using an 8-week cord-blood lymphocyte transformation assay of optimal sensitivity.

† Healthy EBV-antibody positive donors served as controls; like the patients, each control was sampled and tested on a single occasion only.

‡ Significant differences are as follows: RA versus controls, $P<0{\cdot}05$; RA versus OA, $P<0{\cdot}001$.

Although the transformation efficiency of throat washings was not tested by serial dilution, it was interesting to note that 6/31 RA patients gave evidence of throat washing-induced cord blood cell transforming in every indicator well, foci of growth appearing within the first half of the 8-week assay period (data not shown). Such a very high level of virus shedding was only matched by 1/24 control donors (Yao *et al.*, 1985a). The smaller groups of SA and OA patients tended towards the control-group values of virus shedding, with only one very-high-level shedder in each group.

*'Spontaneous transformation' of cultured UM cells*

Cultured UM cells, seeded over a range of initial concentrations in CSA-containing medium, were monitored for spontaneous outgrowth of EBV-carrying B cell lines, the results being shown in Table 2. The majority of donors in all groups gave evidence of spontaneous outgrowth and the overall incidence of positive test wells was not markedly different between RA, SA and OA patients and healthy controls, although comparison of results from RA and from SA patients with those from OA patients did achieve statistical significance. In particular, there were two RA patients and one SA patient whose individual rates of spontaneous outgrowth exceeded those seen with any of the control donors in this study (data not shown) or in earlier work (Yao *et al.*, 1985a).

## DISCUSSION

Prospective studies with healthy virus carriers (Yao *et al.*, 1985a) have recently emphasized several important features of the virus carrier state. (i) In any one individual, each parameter of the virus-host balance is remarkably stable, (ii) the level of EBV persistence *in vivo*, measured either by virus replication in the throat or by virus-infected B cell numbers in the blood, differs markedly and consistently between individual asymptomatic carriers, and (iii) those differences in levels of infection which exist between asymptomatic carriers are not necessarily apparent from a comparison of their anti-viral antibody titres or levels of virus-specific T cell immunity.

Analysis of the persistent EBV infection in renal allograft recipients (Yao *et al.*, 1985b) furthermore showed just how sensitive the virus–host balance is to immunosuppressive drugs. For this reason, all the patients monitored in the present work were not receiving steroids, nor indeed any disease-modifying agents such as gold or penicillamine which might also affect the position of the virus–host balance quite apart from any effect of the disease state *per se*. It must be remembered that many of the previous reports cited in Introduction did not put such a limitation on the selection of patients and the results obtained may well be influenced by that fact.

**Table 2.** Spontaneous transformation of cultured lymphocytes* from RA patients, SA and OA patients with serological evidence of prior EBV virus infection

| Patient group | Frequency of detectable specific transformation | | Frequency of positive indicator cultures§ | |
|---|---|---|---|---|
| | Incidence | Percentage | Incidence | Percentage |
| RA patients† | 22/29 | 76 | 144/870 | 17 |
| Sero-negative arthropathy patients | 12/13 | 93 | 61/390 | 16 |
| OA patients | 7/13 | 54 | 38/390 | 10 |
| Healthy controls‡ | 15/24 | 63 | 102/720 | 14 |

* All cultures were set up and maintained for 8 weeks in medium containing CSA at 0·1 μg/ml in order to reveal the true incidence of spontaneous outgrowth of EBV-transformed B cell lines (Rickinson *et al.*, 1984).

† Results for 29/31 of the EBV antibody-positive RA patients only. In the two other cases there was no spontaneous transformation but these patients' B cells also proved unusually refractory to experimentally induced transformation even when exposed to a potent EBV preparation *in vitro*; in such circumstances, negative spontaneous transformation results cannot be interpreted as indicating low levels of EBV-infected B cells *in vivo* (Rickinson *et al.*, 1984).

‡ Healthy EBV antibody-positive donors served as controls; like the patients, each was sampled and tested on a single occasion only.

§ Significant differences are as follows: RA versus OA, $P < 0{\cdot}001$; SA versus OA, $P < 0{\cdot}05$.

Whatever parameter of the virus carrier state was chosen for analysis, the majority of RA patients fell within the rather broad range which healthy seropositive donors as a whole occupy. In each case, however, a minority of the patients with RA lay outside this range in the direction of increased virus infection and relaxed host control. This could not be attributed to the slightly increased age range of RA patients compared with controls since the RA patients also differed from the more closely age-matched OA group. In accordance with previous reports (Moss *et al.*, 1978; Gaston *et al.*, 1982b) therefore, virus-specific T cell surveillance was not detectable in certain patients. Moreover 6/31 RA patients shed unusually high amounts of the virus in the throat and, of these, two also had unusually elevated numbers of virus-infected B cells in the blood. It would seem that the virus-host balance is indeed altered slightly in RA patients as a group. Viewed in the context of earlier work (Yao *et al.*, 1985b), however, the extent of the change is certainly less than that noted for instance in renal allograft recipients on chronic baseline immunosuppressive therapy; almost half such immunosuppressed patients were found to be high virus shedders and, of these, many had unusually high numbers of circulating virus-infected B cells.

It seems most likely that all individuals with active RA undergo some disturbance of the virus-host balance. In a non-prospective study of this kind, however, disturbance is only obvious for those patients where one or more parameters of the virus-host balance move outside the rather broad range defined by the control donor group. Thus, if one can extrapolate from the observations on renal allograft recipients (Yao *et al.*, 1985b), RA patients whose natural (pre-disease) virus-host balance happened to lie towards one extreme of the normal range will be those for whom the development of the disease is associated with a movement outside of that range. The actual *magnitude* of the change may well be influenced by several factors, of which disease activity is perhaps the most important. Certainly, in a related study in this laboratory (Gaston *et al.*, 1985), patients whose RA was sufficiently severe to necessitate hospitalisation showed a higher incidence of impaired regression (60%) when compared to the patients described on the present report; moreover, a group of patients whose disease activity declined during treatment showed a concomitant increase in their strength of regression. Differences in the criteria used to select patients might therefore very well explain why studies in some other centres (Tosato *et al.*, 1981, 1984) have reported more dramatic changes in the EBV–host balance in association with RA.

Finally, the present report indicates that disturbance of the EBV–host balance is not unique to patients with RA. Although the other patient groups were relatively small, some SA patients in particular were sustaining unusually high levels of persistent EBV infection, very possibly as a direct consequence of their disease. Larger groups of SA and OA patients should now be analysed in order to examine the point more carefully. It is noteworthy, however, that abnormalities of the immune response to EBV have also been reported in multiple sclerosis; such patients show both higher titres of antibodies to EBV capsid antigen (Sumaya, Myers & Ellison, 1980) and an impaired EBV-specific cytotoxic T cell response (Craig *et al.*, 1983).

The possible relationship between EBV and RA has aroused much interest, and certainly the ability of the virus to establish a persistent infection in which B cells are polyclonally activated makes this agent an attractive candidate in the aetiology and pathogenesis of the disease. The present study indicates, however, (i) that the alteration in the virus–host balance in RA patients is relatively mild (perhaps becoming marked in association with particularly active phases of disease) and (ii) that such alterations are not confined to RA but may also be noted in patients with other arthropathies. Considering the results as a whole, it is arguable that quantitative changes in the EBV carrier state of RA patients occur as a consequence rather than as a cause of the persistent inflammatory response which characterizes arthritis.

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