Leading article: Epstein-Barr virus infection and autoimmunity in rheumatoid arthritis

The Epstein-Barr virus (EBV) is an attractive candidate pathogen for rheumatoid arthritis (RA) because it is persistent, ubiquitous, and has profound effects on the immune system (for review, see Tosato and Blaese). Its persistence is due to its ability to remain latent in two cell types—the B lymphocyte and the salivary gland epithelium—both of which could be envisaged as possible sources for some of the immunological abnormalities in RA. In this review I will examine the link between EBV and RA and attempt to establish whether the link is (a) aetiological, (b) represents an abnormal response to the virus, or (c) whether the serological response to one EBV encoded antigen, which has been termed rheumatoid arthritis nuclear antigen (RANA), gives a clue to the pattern of autoimmunity in RA.

The link between EBV and RA

The link between the virus and the disease may be found in striking parallels between EBV infection and immunopathological events which occur in rheumatoid arthritis.

(a) The virus is a polyclonal activator of B cells, which induces autoantibody and rheumatoid factor production both in vitro and in vivo.

(b) Lymphocytes from patients with RA show evidence of impaired regulation of EBV infection in vitro.

(c) Antibodies to EBV determined antigens are raised in sera from patients with RA.

Epstein-Barr virus as a polyclonal activator

The Epstein-Barr virus has long been known for its ability to transform B cells into permanent cell lines. Its transforming ability is now thought to be related to DNA rearrangements involving the myc oncogene, which in turn accounts for its association with Burkitt's lymphoma and nasopharyngeal carcinoma. EBV is also a polyclonal activator, which stimulates B cells to produce immunoglobulins including rheumatoid factors. Infectious mononucleosis is caused by acute EBV infection, and the serological findings can be regarded as an in vivo model of the response to the virus. Infectious mononucleosis is associated with rheumatoid factors and other autoantibodies which are strikingly similar to those found in RA. These include antinuclear antibodies and precipitating antibodies to cellular antigens of the type seen in rheumatoid vasculitis. Sera from patients with RA and infectious mononucleosis also contain antibodies to cytoskeletal antigens, which react with vimentin and cytokeratin.

Impaired regulation of EBV infection

Slaughter et al reported that lymphocytes from patients with RA cultured in vitro were more likely to transform spontaneously (without adding exogenous EBV) and grow out into permanent cell lines. This has been confirmed by a number of investigators and has been ascribed to defective T cell control of EBV infected B cells. The suggestion that such defective control also operates in vivo was supported by the finding that RA B cells contain an increased number of copies of the EBV genome, detected by limiting dilution analysis.

The serological response to EBV determined antigens in RA

In naturally occurring EBV infection antibodies to three major classes of antigen are seen—the viral capsid antigens (VCAs), the early antigens (EAs), and Epstein-Barr nuclear antigens (EBNAs). All three antigens are encoded by EBV DNA but only VCA is present on the virus itself. The function of the EAs and the EBNAs is unknown, though it is thought that they are involved in cell transformation and replication of the virus. In infectious mononucleosis antibodies to all three antigens are seen in the acute and convalescent phases, and after recovery IgG antibodies to EBVCA persist for life and are frequently used in epidemiological surveys of previous infection. These surveys have shown that seroconversion for EBV is often asymptomatic and, in London, the prevalence of IgG anti-EBVCA reaches 50% by the age of 12 and 92% in adults aged 25 or over.

Some studies have reported approximately two-fold raised titres of anti-EBVCA both in adults with RA and in children with juvenile chronic arthritis, though others have found anti-EBVCA titres in RA were the same as those in controls. One study which compared anti-EBVCA antibody titres...
in early RA found them to be normal at presentation,\textsuperscript{21} though they tended to become raised later in the disease, suggesting that the slight difference in anti-EBV titre was acquired either as a result of the disease or its treatment, rather than being due to active EBV infection at the onset of the disease. Two studies found a higher prevalence and titre of antibodies to early antigen,\textsuperscript{16,17} and one found significantly increased titres of antibodies to EBNA.\textsuperscript{18}

The strongest evidence of a link between EBV and RA was suggested by the description of a fourth, and apparently distinct, antigen, RANA, which was encoded by EBV and which appeared to be a specific target for antibodies in RA. RANA was first described as the SS-C antigen, which, like SS-A and SS-B, was present in the B lymphoblastoid cell line WI-L2\textsuperscript{22} and detected by immunodiffusion. Anti-SS-C was thought to help in the definition of subsets of Sjögren’s syndrome, anti-SS-A and SS-B being associated with primary disease and anti-SS-C with Sjögren’s disease with RA. When it appeared that anti-SS-C was equally common in RA without Sjögren’s syndrome its name was changed to rheumatoid arthritis precipitin (RAP).\textsuperscript{23} Sera containing RAP also reacted by immunofluorescence with WI-L2 cells giving fine discrete speckles in both nucleus and cytoplasm in cells fixed by air drying. The antigen identified by this technique was called rheumatoid arthritis nuclear antigen.\textsuperscript{24} In more recent studies both RAP, antibodies detected by immunodiffusion, and anti-RANA detected by immunofluorescence have acquired the unifying title of anti-RANA. This assumed, without good evidence, that the two methods were detecting the same antibody-antigen system.

**EBV in the aetiology of RA**

The first studies showed some diagnostic specificity of anti-RANA for RA. When originally described, anti-RANA (as RAP) was detected in 67% of patients and 8% of controls\textsuperscript{23} (Table). Catalano et al using a more sensitive microimmunodiffusion technique found a higher prevalence in RA sera (94%) but a corresponding increase in normal sera (25%).\textsuperscript{19} By immunofluorescence on Raji cells Ng et al found anti-RANA in 94% of patients with RA and in 16% of healthy controls and claimed that the antibody was a marker for seropositive and seronegative disease.\textsuperscript{25} With the published data available at that time, such a claim would seem to have some justification, and the suggestion by Vaughan that EBV was a ‘major candidate’ in the aetiology of RA may have reflected the thoughts of a number of investigators.\textsuperscript{26} Subsequent studies, however, found an increasing prevalence of anti-RANA in sera from healthy controls (Table). In our studies we found anti-RANA by immunodiffusion in 54% of healthy adults,\textsuperscript{27} and in a later study of juvenile chronic arthritis, in 50% of healthy EBVCA positive children.\textsuperscript{28} Slovin et al found such a high prevalence in normal sera (85%) that they suggested that this term rheumatoid arthritis precipitin was a ‘misnomer’.\textsuperscript{29} It is difficult to reconcile these discordant results, though Alsophaugh et al suggested that a high protein concentration of the cell extract in the immunodiffusion system used in some studies may account for anti-RANA precipitins in control sera.\textsuperscript{30} Subsequent studies, which have tended to use immunofluorescence, still find a relatively high prevalence of anti-RANA in sera from controls.\textsuperscript{31–33} One study, which prospectively examined various serological parameters in an early arthritis clinic, found that anti-RANA, far from being a disease marker, showed only marginal predictive value for the diagnosis of RA.\textsuperscript{34}

Further evidence against an aetiological role was suggested by prevalence studies of EBV infection, defined by the presence of antibodies to EBVCA, which showed no difference between RA and the normal population.\textsuperscript{27,35} Moreover, these studies found that the patients who were negative for antibodies to EBVCA did not differ clinically from those who were positive for the virus. Studies in children, in whom the expected prevalence of EBV infection is lower, have found a corresponding decrease in the prevalence of anti-VCA in those

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<th>Method\textsuperscript{a}</th>
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\textsuperscript{a}Method for detecting anti-RANA antibodies: ID=immuno-diffusion. IF=immunofluorescence.

\textsuperscript{b}Approximate ratio of titres of anti-RANA antibodies in RA sera to those in normal sera was calculated from the median titre of each group, assuming that sera negative for anti-RANA antibodies were positive at one dilution below the screening dilution. In all studies differences in anti-RANA prevalence or titre between RA and normal sera were statistically significant. NR=not reported.
with juvenile chronic arthritis, including the subgroup with classical, seropositive, erosive disease.28

Defective control of EBV infection in RA

The gradual rise in the reported prevalence of anti-RANA antibodies in healthy sera and the negative results from seroepidemiological studies of anti-EBVCA antibodies have led to a corresponding fall in interest in the possibility that EBV could be of aetiological importance in RA. Defective T cell control of EBV infected B cells is easily explained by the generalised T cell defect and therefore not specific for the virus. Similarly, rheumatoid factor secretion by stimulated B cells can also be obtained with polyclonal activators such as pokeweed mitogen. Many recent studies now assume that the relatively high prevalence and titre of anti-RANA merely reflect an increased response to EBV due to the defective T cell control of B cells in the disease. Hyper-responsiveness to all EBV antigens is not a general finding, however. Antibody titres to EBVCA or to EBNA are either normal or only marginally raised, and one study which examined anti-VCA titres in disease controls also found similarly raised anti-EBVCA titres in patients with scleroderma.16 By contrast, all studies which have examined titres of antibodies to RANA have found them to be significantly raised above normal. This suggests that of the phenomena linking EBV to RA, raised anti-RANA titres is the only one which remains unexplained.

Characterisation of RANA and autoantibodies in RA

The high titres of anti-RANA antibodies in RA imply a selective response to only one EBV antigen, which in turn emphasises the importance of characterising RANA further. In their early studies Alspaugh et al went to great lengths to distinguish between RANA and EBNA.24 Apart from the differences in the fixation requirements, staining patterns, and cellular distribution of the two antigens there were also differences between their antibodies. Anti-RANA was not found in sera from five patients with Burkitt’s lymphoma, of whom three had very high titres of anti-EBNA antibodies, and several groups have reported anti-EBNA negative RA sera containing anti-RANA. Similarly, Catalano et al identified several sera containing anti-EBNA antibodies which were anti-RANA negative and vice versa (in one serum) in a longitudinal study of patients with infectious mononucleosis.26 They interpreted these findings as indicating that anti-RANA antibodies were dependent on in vivo EBV infection but separate from anti-EBNA.

The distinction between RANA and EBNA was later complicated by the description of a least two antigens, EBNA-1 and EBNA-2, which were recognised by anti-EBNA positive sera.37 EBNA-1 was a highly polymorphic protein whose molecular weight varied in different cell lines from approximately 68 to 90 kilodaltons, the variation being due to a sequence of approximately 20 000 daltons molecular weight termed the internal repeat three (IR3), which consisted entirely of alternating sequences of the amino acids glycine and alanine. In a study by Billings et al,38 confirmed by our laboratory,39 it was found that anti-RANA antibodies reacted with the EBNA-1 polypeptide by immunoblotting. An explanation for differences between RANA and EBNA was that their respective antibodies reacted with different epitopes on the same polypeptide. A further report described synthetic peptides corresponding to sequences in EBNA-1 in enzyme linked immunosorbent assays (ELISA) for measuring specific antibodies.40 Several peptides reacted with RA sera, but the highest levels of binding and the greatest difference between RA and controls were with a peptide, P62, containing glycine and alanine with the sequence GAGGGAGGAGAGGGAGGA, corresponding to part of the IR3 region on EBNA-1. This suggested that P62 contained the epitope binding to anti-RANA antibodies. Sculley et al confirmed that RA sera reacted with EBNA-1 but confused the picture by suggesting that EBNA-2 was the principal target for anti-RANA antibodies, based on correlations with antibodies to RANA by immunofluorescence.41 42

We have shown43 that EBNA-2 plays no part in the anti-RANA reaction by demonstrating RANA in a cell line, P3HR-1, in which the EBNA-2 gene has been deleted.44 Furthermore, antibodies purified by affinity chromatography with the peptide P62 reacted with RANA by immunofluorescence. Our study also suggested that although P62 may play a part in the RANA precipitin reaction, other epitopes, possibly even on host proteins, are necessary to produce a precipitin. In our study, like that of Rhodes et al,45 we found higher titres of antibodies to P62 in RA sera than in controls. The same population of RA sera reported previously contained almost identical levels of antibodies to EBVCA. This suggested that, like anti-RANA, the sequence containing glycine and alanine represented a selective target for the antibody response in RA.

In our previous study we found that titres of anti-RANA antibodies fell if RA sera were absorbed with cell extracts which did not contain the EBV genome.27 We suggested two possible explanations for this phenomenon, which could also account for the raised anti-RANA antibodies in RA. Firstly,
that RANA is a host protein induced by EBV, or secondly, that the titre of the RANA precipitin is increased by cross reactive autoantibodies. The first explanation is clearly incorrect as it is now known that the gene for EBNA-1 (and hence RANA) is encoded by EBV. On the other hand, there is considerable evidence giving indirect support for the second mechanism. In a study of autoimmunity in infectious mononucleosis Rhodes et al found that Ig antibodies reacting with the EBNA-1 polypeptide by immunoblotting also reacted with several other polypeptides in cell extracts not containing EBV: in other words autoantibodies. Furthermore, they showed that these reactions could be inhibited by P62, suggesting that the epitopes present on this short sequence were shared by both EBNA-1 and the autoantigens. The similarity between the autoantibody response in infectious mononucleosis and RA (cytokeratins and vimentin) raises the possibility that the autoantibodies in RA are apparently enhancing the anti-P62, and hence anti-RANA titres, by similar cross reactions. This hypothesis is also supported by amino acid sequence homology between EBNA-1 and structural proteins, a particularly striking example being cytokteratin, which contains several glycine triplets alternating with serine. Another possibility is suggested by a recent study that demonstrated a host protein in the cytoplasm of cells in the lining layer of rheumatoid synovial membranes, which reacted with a monoclonal antibody to EBNA-1, suggesting a cross reaction related to an antigen within the synovial membrane.

It is also possible that anti-RANA antibodies and autoantibodies in RA are due to polyclonal activation. Activated B cells secrete rheumatoid factors and antibodies which react with repeat sequences. In this case the glycine alanine copolymer of EBNA-1 would simply represent one of many targets for the immune response in RA. This would explain the selectivity of anti-EBV antibodies for RANA. It also accounts for the similarity with the autoimmune profile of infectious mononucleosis, in which B cells are activated by EBV, though there is little evidence that EBV is the activator in RA. Such a hypothesis should prompt a search for an agent in RA with similar properties to those of the EBV, or for disease mechanisms, not necessarily due to a virus, which could result in such polyclonal activation.

The story of RANA and its antibodies represents a remarkable development from a controversial claim of a serological reaction specific for RA with what appeared to be a unique EBV determined antigen, into the findings of an exaggerated serological response to one epitope on EBNA-1, which can now be defined by a synthetic peptide. During the course of the evolution of these findings the virus has lost credibility as an aetiological agent, and even the suggestion that in vivo control of EBV infection in RA is defective has little evidence to support it. These studies suggest that EB virus, although not necessarily of aetiological importance, may be an important probe for the analysis of the finer specificities of autoimmunity in RA. With further work on the epitopes recognised by the antibodies, the elusive nature of autoimmunity in RA may be clarified and possibly simplified, and this may in turn lead to a clearer understanding of the aetiopathogenic mechanisms in the disease.

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