Demonstration of impaired T cell regulation of Epstein-Barr virus stimulated B lymphocytes in rheumatoid arthritis with HLA identical, disease discordant sibling pairs

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SUMMARY Lymphocytes from patients with rheumatoid arthritis (RA) show an abnormal response after stimulation with Epstein-Barr virus (EBV), a potent B cell mitogen. In vitro IgM production from EBV stimulated lymphocytes was measured over a 21 day period. In keeping with previous studies, RA lymphocytes showed increasing IgM production between 14 and 21 days, whereas IgM production decreased during this period in normal lymphocytes ($p < 0.001$). Experiments on 12 HLA identical, RA discordant sibling pairs were also undertaken. B enriched and T enriched lymphocyte populations were obtained and recombined in both an autologous and homologous manner. The abnormality in IgM production in patients with RA was shown to reside in the RA T cell population ($p < 0.005$), and RA B cells combined with normal T cells behaved similarly to autologous cultures of normal B and T cells. The study shows that impaired immunoregulation of EBV stimulated B cells in RA is secondary to a functional defect in RA T cells, but no difference in the concentration of T suppressor/cytotoxic cells could be found between the disease discordant siblings. The abnormality in immunoregulation appears to be secondary to RA, rather than a product of genes encoded within the major histocompatibility complex (MHC) region, as defined by HLA-DR, A, B, and C typing.

Key words: in vitro IgM production, class I MHC restriction, T helper and T suppressor/cytotoxic lymphocytes.

Rheumatoid arthritis (RA) is a common disabling disorder of unknown aetiology. Cumulative evidence indicates that an altered state of immunity has a central role in the pathogenesis. The polyclonal increase in immunoglobulins and the presence of a variety of circulating autoantibodies suggest that increased B lymphocyte activity is a common feature of the disease. The mechanism whereby increased B cell activity occurs is not known. The polyclonal B cell activity may be secondary to impaired T cell immunoregulation, for which convincing evidence already exists in systemic lupus erythematosus.^{1,2} Conversely, the finding that circulating B lymphocytes in RA show an increased expression of the

CD5 (T_H) phenotypic marker, usually associated with B cell chronic lymphatic leukaemia, suggests that an autonomous expansion of the B cell population may be occurring.³

Epstein-Barr virus (EBV), a ubiquitous DNA herpesvirus, is a potent B cell mitogen.⁴ Several studies have shown that the in vitro response of EBV infected B cells is abnormal in RA.⁵⁻¹⁰ The present study confirms this finding and further analyses the abnormality, employing T and B lymphocyte admixing experiments in histocompatible, but RA disease discordant, siblings. HLA histocompatibility between T and B cells in such experiments is necessary because the cytotoxic response to EBV infected B cells is restricted by class I MHC antigens¹¹ and compatibility at the class II locus avoids the induction of an allogeneic mixed lymphocyte reaction.

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Patients and methods

PATIENTS AND CONTROLS

Three groups were studied. All subjects had evidence of previous EBV infection, as judged by the presence of IgG antibodies to EBV viral capsid antigen.¹² The first group comprised 18 normal controls, the second contained 16 patients with classical, seropositive RA who were not receiving steroids, cytotoxic agents, or disease modifying drugs (i.e., gold, D-penicillamine, etc), and the third group contained 12 pairs of histocompatible, but RA discordant, siblings who had been identified during a family study of multicase RA.¹³ Table 1 shows the details of this last group.

ISOLATION OF MONONUCLEAR CELLS

Heparinised venous blood (40 ml) was diluted with Earl's balanced salt solution and separated on Lymphoprep (Flow Laboratories).¹⁴ Cells were harvested from the interphase, washed, and resuspended in RPMI supplemented with 10% heat inactivated fetal calf serum, 1% glutamine, and 1% antibiotic (RF10), before counting. The cells were used either unseparated or after separation into 'T' and 'B' enriched fractions.

Table 1 Rheumatoid factor (RAHA) status and HLA haplotypes in 12 pairs of histocompatible, RA disease discordant siblings

Siblings	Rheumatoid factor	HLA haplotype			
		A	B	C	DR
1 A	>1/160	31/11	7/35	7/4	4/1
B	Neg				
2 A	1/80	31/2	7/63	-/3	4/4
B	Neg				
3 A	1/320	31/11	7/35	-/4	4/1
B	Neg				
4 A	1/80	1/24	8/39	-/-	4/7
B	Neg				
5 A	1/80	31/2	7/44	-/-	4/2
B	Neg				
6 A	1/40	26/28	40/44		7/-
B	Neg				
7 A	1/80	2/29	40/44	3/-	4/-
B	Neg				
8 A	>1/640	31/2	7/49	-/-	4/4
B	Neg				
9 A	1/640	2/2	8/44	-/5	3/6
B	Neg				
10 A	1/40	2/28	8/44		3/4
B	Neg				
11 A	1/640	2/2	40/44	1/5	1/4
B	Neg				
12 A	1/40	3/3	35/50		1/4
B	Neg				

SEPARATION OF B AND T LYMPHOCYTES

Mononuclear cells (MNC) were incubated in RPMI containing neuraminidase treated sheep red blood cells and fetal calf serum (absorbed against sheep red blood cells) and centrifuged. The B enriched interphase was washed and resuspended in RF10. The T pellet was lysed with ammonium chloride and resuspended in RF10. Both populations were analysed on a fluorescence activated cell sorter. The cell populations were identified with monoclonal antibodies (Becton Dickinson). Table 2 shows the results. No attempt was made to remove adherent cells of the macrophage lineage.

LYMPHOCYTE CULTURES

In the experiments with unseparated peripheral blood MNC the cells were cultured at a concentration of 1×10^6 /ml. In the experiments on histocompatible siblings the B and T cells were recombined both in an autologous and homologous manner at a ratio of 0.25×10^6 /ml B cells and 0.75×10^6 /ml T cells. Cultures of B cells alone (0.25×10^6 /ml) were also established. The cells were incubated for 60 minutes in EBV supernatant derived from a Marmoset B95-8 cell line, spun, and resuspended in RF10 culture medium. Cultures were maintained in triplicate in Falcon tubes at 37°C in a humid atmosphere containing 5% CO₂ in air.

HARVESTING AND ASSAY OF SUPERNATANTS

The culture supernatants were harvested at 7, 14, and 21 days. The supernatants were assayed for IgM production in an enzyme linked immunosorbent assay (ELISA) system. The results are expressed as µg of IgM secreted per 10×10^6 cells.

STATISTICAL METHODS

Non-parametric tests were used. The Mann-Whitney U test was used for unpaired data. The Wilcoxon paired sign rank test was used for paired data obtained from siblings.

Table 2 Lymphocyte subsets expressed as percentage (SD) of total cell population in RA and non-RA siblings.

Cell population		Lymphocyte subsets (%)			
		T (Leu 4)	B (Leu 12)	Th (Leu 13)	Ts (Leu 2)
Unseparated lymphocyte population	Normal sib	75 (11)	10 (3)	53 (8)	30 (13)
	RA sib	78 (11)	12 (8)	53 (9)	32 (9)
T enriched	Normal sib	89 (6)	6 (2)	55 (6)	34 (8)
	RA sib	90 (6)	4 (1)	55 (19)	32 (8)
B enriched	Normal sib	11 (2)	50 (18)		
	RA sib	8 (3)	53 (18)		

Results

In the experiments using unseparated peripheral blood MNC IgM concentration in the supernatants increased during the first 14 days culture in both normal controls and the random RA subjects. IgM concentrations then fell between 14 and 21 days in 15 out of the 18 normal subjects. In contrast, IgM concentrations between 14 and 21 days did not fall in any of the 16 random RA subjects. Table 3 shows the ratios of the concentration of IgM at 21 and 14 days. The difference between the RA group (mean 2.99) and normals (mean 0.75) was significant ($p<0.001$, Mann-Whitney U test).

In the sibling pairs the IgM production from unseparated lymphocyte cultures followed similar patterns to those seen in the normal and random RA groups (Table 3). The mean 21:14 day ratio for the normal siblings was 0.78 ($p=NS$ when compared

with controls) and 1.73 in the RA siblings ($p=NS$ when compared with the random RA group). The difference between 21:14 day ratios in the sibling pairs remained significantly different ($p<0.005$).

Fig. 1 shows the results of a typical autologous

Table 3 Ratio of IgM concentration in supernatants at 21 and 14 days culture in (a) a random RA group, (b) controls, (c) RA siblings, and (d) non-RA siblings

Group	Ratio of IgM concentration at 21:14 days
(a) Random RA	2.99 (0.88)*
(b) Controls	0.75 (0.11)
(c) RA siblings	1.73 (0.80)
(d) Non RA siblings	0.78 (0.74)

(a) ν (b) $p<0.001$; (c) ν (d) $p<0.005$; (a) ν (c) $p=NS$; (b) ν (d) $p=NS$ (Mann-Whitney U test).
*Values are mean (SD).

Fig. 1 Effect of normal and RA T cells on IgM production from EBV stimulated B cells at 14 and 21 days' culture.

Fig. 2 IgM concentration at 21 days in autologous and homologous cultures from HLA identical, disease discordant sibling pairs. B normal + T normal ν B normal + T RA $p<0.005$; B RA + T normal ν B RA + T RA $p=0.01$ (Wilcoxon sign rank test).

and homologous recombination experiment in a sibling pair. Suppression of IgM production occurred with both RA and non-RA T cells, but the effect of RA T cells was considerably less at 21 days.

Fig. 2 shows the results of autologous and homologous recombinations of B and T cells on IgM concentration at 21 days in the 12 sibling pairs. T cells from the normal sibling suppressed IgM production to a greater extent than RA T cells in the presence of both normal B cells ($p < 0.005$, Wilcoxon sign rank test) and RA B cells ($p = 0.01$).

In cultures of B cells alone the IgM concentration from both the RA and the non-RA sibling was not significantly different at 21 days. Both the RA and non-RA B cells produced similar concentrations of IgM at 21 days in the presence of RA T cells. In the presence of non-RA T cells the IgM concentration was again similar for RA and non-RA B cells.

These findings suggest that the major factor responsible for the immunoregulatory abnormality in IgM production from EBV stimulated B cells in RA is the T cell population. Phenotypic characterisation of T cell subsets into T helper and T suppressor/cytotoxic groups, however, failed to show a numerical difference between the RA and non-RA siblings (Table 2).

Discussion

The recognition that sera from patients with RA contained a circulating antibody that reacted with a nuclear antigen (RANA) present only in EBV infected cell lines drew attention to EBV as a potentially relevant agent in RA.¹⁵ Subsequent studies have shown that RANA is related immunologically to Epstein-Barr virus nuclear antigen (EBNA).^{16 17} and monoclonal antibodies against EBNA-1 react with synovial lining cells from patients with RA.¹⁸

Other studies on EBV in RA have concentrated on its role as a B cell mitogen. Stierle *et al* showed that RA B cells infected in vitro with EBV were more likely to form permanent B cell lines than normal B cells.⁸ Tosato and colleagues were unable to confirm this observation, but found that the number of circulating spontaneously transforming B cells were significantly greater in patients with RA than in controls.¹⁹ Various groups have shown that the ability of patients with RA to regulate in vitro EBV infected B lymphocytes is impaired, in terms of immunoglobulin production, proliferation, and cell outgrowth.⁵⁻¹⁰ It seemed probable that this abnormality was due to defective suppressor/cytotoxic regulation.⁶ To analyse the defect further the present study describes the findings of T and B

lymphocyte admixing experiments in RA discordant but HLA histocompatible siblings. Because T cell cytotoxicity to EBV infected B cells is restricted by class I MHC antigens it was necessary to use histocompatible pairings.¹¹

Several findings have emerged from the present study. The EBV stimulated lymphocytes from the normal sibling behaved similarly to those from normal controls, and lymphocytes from the RA sibling behaved similarly to those from the random RA group. This suggests that the impaired immunoregulation in RA is secondary to the disease rather than a product of genes encoded within the MHC, as defined by HLA-DR and class I antigen typing. This is in keeping with the observation of Tosato *et al*, who were unable to show that HLA-DR4 positive normal individuals had an impaired regulatory response.⁵ It remains possible that MHC expression on the cell surface, in terms of density, may vary between the RA and non-RA sibling.

Secondly, the present study showed a significant impairment of the ability of RA T cells to control IgM production from both RA and non-RA histocompatible B cells at 21 days' culture when compared with normal T cells. IgM production from RA and non-RA B cells alone was similar at 21 days. Moreover, IgM production was influenced in the admixing experiments only by alterations of the T cell population and not by the B cell population. We feel that this provides convincing evidence for an acquired T cell immunoregulatory defect in RA.

Thirdly, the functional defect in RA T cell behaviour could not be accounted for by a deficiency in T suppressor/cytotoxic cell numbers. Recent studies have shown a decrease in circulating T suppressor/inducer (CD4 2H4) cells in RA,^{19a} and it is possible that the abnormality in the control of EBV infected B cells in RA is secondary to a deficiency in this cell population. Several other factors may also be operating. Hasler *et al* showed that regulation of EBV induced B cell proliferation was dependent upon interferon gamma, and reduced interferon gamma production occurred in RA T cells in autologous mixed lymphocyte reactions.⁷ Evidence is also accumulating that induction of suppressor cell function is dependent upon other cytokines, in particular interleukin 2 and T suppressor growth factor, acting synergistically.²⁰

The present study does not provide any evidence that EBV has a causal role in the pathogenesis of RA and, if anything, would weigh against a causal effect as disease discordance was observed in histocompatible siblings, all of whom had evidence of prior EBV infection. Moreover, the defect in immunoregulation of EBV induced B cells is not confined to RA, and similar abnormalities have

been detected in multiple sclerosis,²¹ systemic sclerosis,²² and ankylosing spondylitis.²³ The T cell dependent impairment of immunoregulation of B cells in RA could, however, explain the humoral abnormalities observed in this condition. Whether the immunoregulatory defect is in any way specific to RA or varies qualitatively between the differing disorders remains to be established.

Whatever mechanism or mechanisms are operating in the causation of impaired T cell immunoregulation in RA, the use of histocompatible but disease discordant siblings provides a useful laboratory experimental model to dissect these disease related abnormalities further.

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