HYPOTHESIS

HLA-B27 associated spondyloarthropathy, an autoimmune disease based on crossreactivity between bacteria and HLA-B27?

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Abstract

Most autoimmune diseases are associated with certain HLA types. Therefore, spondyloarthropathies (SpA) strongly associated with HLA-B27, are also often classified as autoimmune diseases. This study questions whether SpA indeed fulfils the criteria of an autoimmune disease. The Medline database was searched for all reports between 1966 and April 1998 on the presence of autoimmune reactivity in SpA patients. This search yielded 45 articles on this subject. Only eight articles study T cell reactivity. Twelve reports were found on the assessment of antibodies crossreacting between bacteria and HLA-B27. In the 45 studies demonstrating autoimmune reactions in SpA patients proper controls matched for HLA-B27, sex and age were nearly always lacking. Therefore, it is concluded that the frequency of increased autoreactivity in sera from patients and controls is not significantly different, and that this lack of autoreactivity does not justify classification of SpA as an autoimmune disease. As cross-reactive antibodies against bacteria and HLA-B27 were equally present in sera from patients and controls, the pathogenetic significance of molecular mimicry between various bacteria and HLA-B27 is questionable. Furthermore, the regions of the B27 molecule that are supposed to be crossreactive with bacteria, differ in one or more amino acids among the distinct B27 subtypes. Although these differences strongly influence the binding of antibodies to the B27 molecule, there was no relation between the degree of crossreactivity of certain subtypes and the association of these subtypes with SpA. In conclusion, there is no evident proof that SpA is an autoimmune disease attributable to cross-reactivity between bacteria and HLA-B27. (*Ann Rheum Dis* 1999;58:598–610)

The spondyloarthropathies (SpA) are a group of diseases including ankylosing spondylitis (AS), reactive arthritis (ReA), and Reiter's syndrome (RS).¹ One third of the HLA-B27 posi-

tive AS patients has also acute anterior uveitis (AAU), whereas more than half of the HLA-B27 positive patients with AAU fulfils the criteria for SpA. A remarkable high association between AS, ReA, RS and AAU and the expression of the MHC class-I molecule HLA-B27 is reported.^{2–6}

The assumption is often made that B27 associated diseases are autoimmune diseases (AID).^{7–9} Because a number of bacterial species have been associated with the initiation of ReA, and because a high prevalence of *Klebsiella pneumoniae* and of antibodies to *Klebsiella* in AS patients has been reported, it has been suggested that bacteria play a part in the pathogenesis of B27 associated SpA.^{7–10} The term “molecular mimicry”, introduced in 1964, describes the resemblance of antigenic epitopes of microbial constituents and autologous material.^{11 12} Molecular mimicry between viral proteins and host cell proteins has been suggested to be one of the causes of AID.^{13 14} Therefore, the hypothesis has also been made that molecular mimicry between bacteria and HLA-B27 plays a pathogenetic part in HLA-B27 associated SpA.

Various theories are proposed to explain the association of SpA with B27. Several of them involve some form of molecular mimicry. In these theories B27 functions as a T cell restriction element, as an antigen, or both as an antigen and a T cell restriction element: (1) B27 presents a bacterial derived peptide to B27 restricted CD8 positive CTL crossreacting with self peptides presented by B27.¹⁵ (2) B27 molecules would present a peptide derived from B27 itself with sequence similarity with bacteria.^{16 17} (3) Peptides derived from the B27 molecule would be presented by MHC class II molecules to CD4 positive T cells that previously have been activated by bacterial antigens.^{18 19} (4) Antibodies induced by bacteria may crossreact with B27 molecules, thereby initiating an autoimmune response.²⁰

In several studies data had been obtained that supported the idea that possible crossreactions between bacteria and the B27 molecule may be responsible for the induction of autoimmune reactions directed against the B27 molecule. Using polyclonal anti-sera raised in various animals, many groups studied a

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possible crossreactivity between several bacterial species (*Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Shigella sonnei*, *Yersinia enterocolitica*, *Chlamydia trachomatis*) and HLA-B27.^{21–32} Possibly because of non-specific binding in the serological methods used, such crossreactions were not observed by all research groups.^{33–36} Using more specific monoclonal antibodies reactive with B27 or bacteria, it was demonstrated that crossreactivity between *Yersinia enterocolitica*, *Shigella sonnei*, *Shigella flexneri*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and HLA-B27 may occur. Using polyclonal anti-*Klebsiella* sera some evidence was obtained that suggested the existence of a B27 associated crossreactive epitope that was specifically present on lymphocytes of AS patients, and only on B27 positive lymphocytes of healthy persons after incubation of these lymphocytes with a *Klebsiella* culture-filtrate derived factor.^{26 31 32 37} Later, serological recognition of B27 was shown to be influenced by oxidation of the free sulphhydryl group of the cysteine at position 67 of the B27 molecule, something that can be achieved by interaction of B27 with homocysteine (a product produced by bacteria). Thus, certain factors were shown to be able to modify the serological properties of B27, which in people might lead to recognition of B27 as non-self.^{37–39} All these findings support the hypothesis that B27 associated SpA may be an AID, and that they may be caused by molecular mimicry between B27 and bacterial components.^{35 40–42} It is, however, not at all clear whether the published data validate the classification of SpA as an AID attributable to molecular mimicry between HLA-B27 and bacteria.

The criteria for AID are not clearly defined. This has resulted in a tendency to consider some diseases of unknown origin as AID, even though the role of autoimmune phenomena in such diseases in humans are not clear at all.⁴³ The term “autoimmunity” was first introduced at the beginning of this century by Ehrlich, who formulated the concept of “Horror autotoxicus”.⁴⁴ His concept is often misunderstood as meaning that autoantibodies may not be formed. The true meaning however is that autoantibodies may be formed and can be found, but that in healthy people mechanisms exist that somehow prevent their action and thus inhibit the development of AID.⁴⁴

Sera from normal animals and humans of any age contain a wide variety of autoantibodies, including antibodies reactive with molecules involved in the immune system. Thus, the presence of autoantibodies in people with particular diseases, does not indicate that such antibodies play a part in the pathogenesis of these diseases, or that these diseases can be regarded as AID. The presence of autoantibodies only points to autoimmune reactivity and not necessarily to autoimmune disease. The induction of autoantibodies might be secondary to disease and have been induced for example by tissue damage because of non-autoimmune mechanisms.^{45–50}

Nevertheless, autoantibodies in high titres can be useful diagnostic markers for certain

diseases. Their presence, however, does not prove that autoimmune reactions really are the cause of diseases. Direct evidence as defined by Rose⁵¹ is “the demonstration that a self reactive antibody is the immediate cause of injury or dysfunction”. This has been demonstrated for the autoimmune forms of haemolytic anaemia, thrombocytopenia, leucopenia and for diseases where antibodies to receptors are involved, for example, Graves’ hyperthyroidism and myasthenia gravis. According to Rose,⁵¹ indirect evidence for a pathogenetic role of autoantibodies requires the identification and isolation of the appropriate autoantigen. Subsequent immunisation of suitable animals with this antigen should reproduce the lesions of the disease. According to Rose, most of the so called autoimmune diseases are, however, only defined on the basis of circumstantial evidence, including the presence of other autoimmune disorders in the same person or in the family, associations with particular HLA haplotypes and a markedly skewed T cell receptor V-gene use. Feltkamp,^{43 52} considering that all autoimmune diseases are characterised by autoimmune reactions, favours a more practical definition of an autoimmune disease: “An autoimmune disease is characterised by a significantly increased frequency of autoantibodies in significantly increased titres, compared to healthy local controls”. Because for several human autoimmune diseases the preponderance of women is striking and sex hormones can modify immune responses, and as age and the hygienic quality of life of a population influence the chance for developing various autoimmune diseases, it is important to include local controls that are matched for sex and age in studies assessing AID.^{43 53 54}

Considering the above information the following definition can be used. “Autoimmune diseases are diseases in which immune reactivity directed against autologous antigens is significantly increased when compared to normal individuals, whilst this reactivity is the primary cause of tissue or cell destruction”. Regarding SpA, the aforementioned definitions mean that HLA-B27 associated SpA only can be designated as AID if the presence and nature of autoimmune reactions can be demonstrated. Thus, in SpA patients autoreactivity should be at least more often present in higher levels than in well defined controls, who should have to be matched for sex and for age. Assessment of autoantibodies and autoreactive T cells in cases and controls should have to be performed with the same techniques with the same sensitivity. As the MHC background has been shown to influence the susceptibility to certain autoimmune diseases, and as in B27 transgenic mice antibodies and T cell responses to B27 could not be induced by immunisation with B27 derived peptides compared with non-transgenic mice, controls should also have to be matched for HLA-B27.⁵⁵ If sera or T cells are used to study whether they react both with HLA-B27 and bacteria, additional experiments have to be performed to confirm that crossreactive antibodies or T cells are present in the sera, to exclude the possibility that the crossreactivity was

Table 1 Frequencies of presence of autoreactivity to antigens other than HLA-B27 in SpA patients and controls

Immunoreaction	Reference	Year	Humoral resp. Ig/A/G/M	Cellular assays
Anti-69 kD protein	62	1991	Ig	
Anti-anter.longit.ligament	90	1983		Stim.
Anti-articular cartilage	91	1976	Ig	MI
Anti-C1q	92	1992	G, A	
Anti-cardiolipin	63	1987	G+M	
	64	1989	Ig	
	57	1995	G,M,A >16 units	
Anti-cart.link protein	93	1988	Ig	
Anti-collagen n1;n2;n3; α	94	1980		Stim.
d1	95	1980		Stim.
n1;n2	96	1980	G	
n2	97	1981	G, M	
d2;n2	98	1984	G	
C2 peptides	99	1994		CTL cloning
n2	61	1997	G,A,M	
n	60	1997	A	
Anti-DNA ss; ds	63	1987	G, M	
	69	1992	Ig >1:10	
Anti-HSP60	56	1996	G Mean Abs.	
Anti-intervertebral disc	90	1983		Stim.
Anti-keratin	100	1994	A,G,M Mean Abs	
ANCA	101	1995	G, A	
ANF Liver	65	1967	Ig $\geq$ 1:16	
Liver	66	1967	Ig	
Liver	67	1967	Ig $\geq$ 1:16	
Leucocytes	58	1977	Ig	
Granulocytes; liver	68	1979	Ig 1:40	
Leucocytes	59	1981	Ig 1:20	
	69	1992	Ig >1:40	
Hep-2	101	1995	G	
	102	1993		
APF	103	1995	Ig 1:80 1:100	
	104	1979	Ig 1:80	
	105	1990	Ig 1:80	
Anti-sacroileac joint cart.	90	1983		Stim.
Anti-salivaryduct	106	1967	Ig	
Anti-synovial membrane	91	1976	Ig	MI
Anti-thyroglobulin	107	1979	Ig	
<i>Immunoreaction</i>	<i>Reference</i>	<i>Osteoarthritis</i>	<i>Degenerative joint disease</i>	<i>Gout</i>
Anti-69 kD protein	62			
Anti-anter.longit.ligament	90			
Anti-articular cartilage	91			
Anti-C1q	92			
Anti-cardiolipin	63			
	64			
	57			
Anti-cart.link protein	93	7/29		
Anti-collagen n1;n2;n3; α	94		0;0;0;3/10	0;2;1;3/10
d1	95		3/15	
n1;n2	96			
n2	97			
d2;n2	98			
C2 peptides	99			
n2	61			
n	60			
Anti-DNA ss; ds	63			
	69			
Anti-HSP60	56			
Anti-intervertebral disc	90			
Anti-keratin	100			
ANCA	101			
ANA Liver	65	0/8		
Liver	66	0/15		0/13
Liver	67			
Leucocytes	58			
Granulocytes/liver	68			
Leucocytes	59			
	69			
Hep-2	101			
	102			
APF	103			
	104		2/32	
	105			
Anti-sacroileac joint cart.	90			
Anti-salivaryduct	106	0/43		0/6
Anti-synovial membrane	91			
Anti-thyroglobulin	107			1/2 SF

↑ = Increased mean absorbance in ELISA. Bold type* = significant ($p < 0.05$) if compared with healthy controls. + = Significantly increased frequency of autoimmune reactions among B27 associated SpA compared with controls (healthy or suffering from other rheumatic disorders). A = IgA, Abs = absorbance in ELISA assay, Arthr = arthritis, Cart = cartilage, Deg = degenerative, ds = double stranded, G = IgG, Ig = immunoglobulin, m = mean, M = IgM, MI = migration inhibition, NS = not significant, resp = response, SLE = systemic lupus erythematosus, SF = synovial fluid, ss = single stranded, Stim = lymphocyte stimulation.

attributable to coexistent antibodies and T cells with different specificities for bacteria and B27.

In this study data from literature were collected to answer the question whether SpA

can be designated as an AID, and whether crossreactivity between HLA-B27 and bacteria plays a part in the pathogenesis of B27 associated SpA.

[illegible]

The Medline database was searched with Ovid software for English language literature that appeared between 1966 and April 1998 using

the following subject headings: spondylitis, ankylosing; arthritis, reactive; Reiter's syndrome. This resulted in 5082 references. This search was further limited to those with the

following text words present in the title, abstract, or the subject headings: autoantigen or autoantigens; autoantibody or autoantibodies; autoreactive or autoimmune; serology or culture; antigen or antigens; antibody or antibodies; *Salmonella* or *Yersinia* or *Chlamydia* or *Klebsiella* or *Shigella* or *Campylobacter*; infection or infections; T cells or T lymphocytes; bacteria or bacterial. This resulted in 2516 references. All titles of these 2516 reports were used to exclude those reports that did not cover the subject of our study. In total, 1515 references were not appropriate. Usually, the above searched text words were only mentioned in other contexts. The remaining 1001 references were imported in a database using Reference Manager software. The abstracts of these 1001 articles were read to find those studies in which the presence and nature of autoimmune reactions in B27 associated SpA was studied. Again, the fall out usually consisted of reports in which the above searched text words were only presented in other contexts. Subsequently, reviews and case reports were excluded. Finally, only 45 articles reported on studies that were relevant for the question whether B27 associated SpA are AID, and 12 articles reported on studies that were relevant for the question whether SpA is caused by molecular mimicry between bacteria and B27.

Results

The results are presented in the following three paragraphs: Autoreactivity against antigens other than HLA-B27; Autoreactivity against HLA-B27; Crossreactivity between bacteria and HLA-B27.

AUTOREACTIVITY AGAINST VARIOUS ANTIGENS OTHER THAN HLA-B27

The 32 articles reporting on the measurement of autoimmune responses in B27 associated SpA, encompassed 30 studies on AS, six on RS and four on ReA. Of these 32 reports, 27 reported on autoantibody responses and only six on T cell autoreactivity. The antibodies studied, cover a wide range of different autoantigens including phospholipids, cartilage link protein, collagen like C1q, keratin, thyroglobulin, heat shock protein, perinuclear factor, nuclear antigens and neutrophil cytoplasmic antigens (ANCA).

In six studies, marked with “+” in the column “conclusion” in table 1, the frequency or level of autoimmune reactions were significantly increased among B27 associated SpA patients compared with controls (healthy or suffering from other rheumatic disorders).⁵⁶⁻⁶¹

Immunoreaction against a 36 kDa protein from *Drosophila melanogaster* was found to be increased in B27 positive and negative AS patients when compared with RA and healthy controls who were not matched for HLA or sex.⁶² When antibodies that reacted with the 36 kDa antigen were eluted from the 36 kDa protein after incubation with pooled AS patient sera, it was found that these antibodies reacted with a 69 kDa protein isolated from human lymphocytes or HeLa cells. Also pooled sera

from AS patients reacted with this 69 kDa protein. However, because in these studies pooled sera were used, it is not clear how many individual patients that recognise the 36 kDa *Drosophila* protein also recognise the 69 kDa human protein, and how many patient and control sera react with this 69 kDa protein.

Levels of antibodies to HSP60, present in sera from RA, SLE, Reiter's patients and healthy controls, were reported to be slightly increased in sera from Reiter's patients in one study.⁵⁶ The controls were not matched for HLA, sex or age.

One study reported a higher incidence of IgG anti-cardiolipin in AS patients compared with normal controls matched for age and sex, but not for HLA.⁵⁷ However, in two other studies in which no matches for HLA, sex and age were performed, the frequency of raised levels of anti-cardiolipin antibodies in sera from AS patients was not different compared with rheumatoid arthritis (RA) patients or healthy controls.^{63 64}

In only one of seven studies in which the presence of antibodies against collagen was analysed, AS patients had slightly increased levels of anti-collagen antibodies.^{60 61} Controls were not matched for HLA, sex or age.

In three studies it was reported that no anti-nuclear antibodies (ANA) in AS patients and controls at a dilution of more than 1:16 were found, whereas 15% to 30% of RA patients were positive.⁶⁵⁻⁶⁷ At a dilution of 1:16 approximately 60% of 18 AS patients and 77% of 144 non-rheumatic disease controls were positive for ANAs, while 90% of 184 RA patients were positive. No HLA, sex, or age matches were performed. Vasey and Kinsella,⁵⁸ testing for leucocyte reactive (LR) ANAs by measuring reactivity with granulocyte nuclei, found that 60% of 125 AS and 47% of 124 RA patients was positive. These percentages were significantly higher than for patients with inflammatory disorders (12% of 34 positive) and healthy controls (9% of 122 positive). Among 74 patients with miscellaneous disorders 40% was positive. Although no matches for HLA, sex, or age were used in this study, no correlation was found between the presence of B27 and LR-ANAs. The LR-ANAs in AS patients, although not granulocyte specific, were specific for leucocytes and did not react with other human or non-human substrates. This might explain the discrepancy with the negative results in the previous studies,⁶⁵⁻⁶⁷ in which liver tissue instead of granulocytes was used as substrate. LR-ANA positive sera from 50% of the RA patients and all SLE patients reacted also with adrenal and thyroid tissue as substrates.⁵⁸ Rosenberg *et al*⁶⁸ found that only 6% of 88 AS patients and 13% of 52 psoriatic arthritis (PsoA) patients had ANAs reactive with granulocytes, whereas all 25 psoriasis patients and 91 chest disease controls were negative. Measuring ANAs reactive with liver tissue they found that at serum dilutions of 1:40 17% of 36 AS and 3% of 32 PsoA patients were positive. These frequencies are rather low compared with other reports,⁶⁵⁻⁶⁷ except in the study of Vasey and Kinsella⁵⁸ in which none of

Table 2 Frequencies of the presence of anti-B27 antibodies in sera from SpA patients and controls

B*2705 residues:	Reference	AS patients		RS patients		Healthy controls		Titre	Match
		B27*	B27 ?	B27*	B27 ?	B27*	B27 ?		
69-84	Schwimmbeck <i>et al</i> ^{20 70 71}	7/24*		18/34*		0/22		1:10	B27
69-84	Tsuchiya <i>et al</i> ^{74 75}	14/50	14/60	1/5	1/8	0/11	1/25	1:50	B27
		2/26		0/6	0/8	0/6	0/38	Norway	Sex
								1:50	100% male
								N.Mexico	
68-75	Ewing <i>et al</i> ⁷⁷	↑ Ig* n=50				n=22		1:2000	B27
69-84	de Vries <i>et al</i> ⁷⁸	↑ Ig* n=81 79% male		↑ Ig n=38 84% male		n=32 50% male	n=49 79% male	1:50	Sex 79% male
67-83	Tani <i>et al</i> ⁷²	↑ A*,G*,M* n=52 90% male					n=50 50% male	1:50	—
67-83	Fielder <i>et al</i> ⁷³	↑ A,G*,M n=97					n=25	1:50	—

↑ =Increased mean absorbance in ELISA. Bold type* = significant (p<0.05) if compared with healthy controls. A = IgA, G = IgG, M = IgM, n = number of subjects studied.

the sera from AS patients reacted with liver tissue. Rosenberg *et al*⁶⁸ used fixed leucocytes as substrate, whereas Vasey and Kinsella⁵⁸ used unfixed leucocytes. However, Rosenberg *et al*⁶⁸ using unfixed leucocytes similar to those used by Vasey and Kinsella, were still not able to confirm the findings of Vasey and Kinsella.⁵⁸ In these studies no HLA, sex or age matches had been performed. In a later study, Kinsella *et al* again found a higher incidence of LR-ANA in AS patients than in normal controls (45% of 31 AS patients, 7% of 31 controls at 1:10 dilution, and 23% of 31 AS, 0% of 31 controls at 1:20 dilution).⁵⁹ In this study, patients and controls were matched for sex and age (± 2 years), but HLA matches were not performed. All AS patients were B27 positive and almost all controls were B27 negative, but there was no concordance between LR-ANA and B27 positivity in North American natives. Wong *et al*⁶⁹ testing only three patients with B27 positive AS and their family members for ANAs reactive with Hep-2 cells, found that at a dilution of 1:40, all persons had ANA reactivity within normal range.

AUTOREACTIVITY AGAINST HLA-B27

Schwimmbeck *et al*^{20 70 71} used peptides from residues 69-84 of HLA-B*2705, as substrate in an ELISA system to detect antibodies against B27. Eighteen of 34 (53%) B27 positive RS patients and 7 of 24 (29%) B27 positive AS patients tested at a dilution of 1:10 were positive. None of the 22 B27 positive healthy controls were positive. Thus, patients were shown to have higher titres of antibodies against B27 than controls who were matched for HLA, but not for sex or age (table 2).

Tani *et al*⁷² tested sera from Japanese patients at a dilution of 1:50 for antibodies to the 67-83 B*2705 peptide with an ELISA, and found that active AS patients had significantly higher antibody levels against the B*2705 peptide in all antibody subclasses, and that inactive AS patients had only raised IgG subclass antibody levels compared with healthy normals and RA patients. Antibody levels against a control peptide were not different in the different groups. All AS patients were B27 positive, but the controls were not matched for HLA, sex or age. Of the RA patients 90% were women, while 90%

of the AS patients and 50% of the controls were men. Fielder *et al*,⁷³ using the same method and the same peptides, found that active AS patients had significantly higher IgG subclass antibody levels than RA patients and controls who were also not matched for HLA, sex or age. The levels of IgM and IgA subclasses were similar in patients and controls.

Tsuchiya *et al*^{74 75} used the same technique as Schwimmbeck with the 69-84 peptide from B*2705. Patients and controls from Tromso (Norway) were sex (only men were included) and age matched. The mean age of the patients was 38 years, ranging from 20 to 69, and of the controls 40 years, ranging from 21 to 62. Fourteen of 60 (23%) AS patients of whom 83% were B27 positive, one of eight (13%) RS patients of whom 63% were B27 positive, and one of 25 (4%) normal controls of whom 44% were B27 positive, had antibodies against the B27 peptide 69-84. The difference of the positive ratio between AS and controls was only significant when no HLA matches were performed. However, when the same studies were performed on patients from New Mexico, no significant differences were found between patients and controls who were not matched for age. In a study using the lymphocyte migration inhibition test for patients as well as for controls, no significant differences were found between the migration inhibition (MI) induced by a B27 peptide and the MI induced by a control peptide.^{74 75} When values of MI induced by the B27 peptide were compared with those induced by blank wells containing no peptides at all, it appeared that the proportion of normal controls responding to the B27 peptide was larger than that of patients, although the difference was not statistically significant (p>0.10). The results obtained by ELISA used in this study were corrected for non-specific serum binding. Schwimmbeck *et al*^{20 70 71} had not reported such correction. Women were excluded from the studies by Tsuchiya *et al* because they found increased titres against the B27 derived peptide were present in seven of 34 (21%) B27 negative female controls and also in two of nine (22%) B27 positive female controls who had previously been pregnant. Most of their female patients had been pregnant, and the titres in these patients were

similar to the titres in the B27 positive or B27 negative female controls with previous pregnancies. The group of female patients without previous pregnancies was too small to study. Cavender *et al*⁷⁶ found neither antibodies against B27 in any of six male AS or RS patients, nor in sex and age matched B27 negative controls. They used B27 positive lymphoblastoid cells as targets in a microlymphocytotoxicity assay.

Ewing *et al*⁷⁷ used a variety of synthetic peptides derived from HLA-B27 as substrates in ELISA systems. Analysing the specificity of a number of patient sera recognising peptides from B*2705, they found two different reactive sites in the B27 peptides. Using octamers they observed that reactivity to the sequence 72-QTDREDLR-79 was absent, and that reactivity was mainly present to two peptides, 68-KAKAQTDNR-75 and 76-REDLRITLL-83. At a dilution of 1:2000, sera from 50 B27 positive AS patients, and 22 B27 positive and 22 B27 negative healthy first degree relatives as controls were studied for antibodies to 68-KAKAQTDNR-75. The mean absorbance values in the ELISA of the sera from the patients were significantly higher than the mean absorbance of either groups of control sera. However, this difference was mainly caused by three of the 50 patient sera, which showed extremely high reactivity against B27. No significant differences between the mean absorbance values of the B27 positive and B27 negative controls were found. No sex or age matches were performed.

De Vries *et al*⁷⁸ used the same technique and peptide substrate (69–84 B*2705 peptide) as Schwimmbeck *et al*.^{20 70} They studied 81 B27 positive AS patients, 38 B27 positive RS patients, and 32 B27 positive and 49 non-typed healthy controls. Like Tsuchiya *et al*^{74 75} they could not reproduce the results reported by Schwimmbeck *et al*, although their first findings confirmed the results of Schwimmbeck *et al*. Sera from AS patients bound the B27 peptide significantly better than the control serum samples. However, differences were only significant if the results from all patients were compared with those from non-HLA typed controls. The results from B27 positive patients and the B27 positive controls were not significantly different. Patients with RS showed a higher, although not significantly, binding than non-HLA typed controls. Binding of sera from 32 B27 positive healthy controls was relatively high, but this was caused by nine serum samples of which seven were obtained from women of whom no data of possible previous pregnancies were available. Control experiments carried out with uncoated wells or wells coated with an irrelevant peptide, showed that there was a highly significant correlation between binding to both the B27 peptide and an irrelevant peptide, and even to uncoated wells. Apparently the degree of a-specific binding was high. All groups were matched for sex (79% men), but not for age.

Hermann *et al*⁷⁹ studied proliferation of peripheral blood lymphocytes (PBL) using 13 short synthetic peptides of B27. The only con-

clusion that could be drawn from the results of this study was that B27 positive AS patients as well as B27 positive healthy controls recognise a peptide corresponding to residues 60–72 from B*2705. A peptide corresponding to residues 151–165 was recognised by almost all groups studied (B27 positive AS, B27 positive other SpA, B27 positive healthy, B27 negative healthy). These groups were only matched for B27 and not for sex or age.

All the above studies make use of short synthetic peptides. There are only very few reports studying the possible recognition of conformational epitopes on whole B27 molecules by antibodies or T cells.

Kellner *et al*⁸⁰ used flow microfluorocytometry analysis to measure antibodies reacting with B27 positive cells transfected with the *Mycobacterium tuberculosis* HSP60 gene. Sera of 11 B27 positive AS patients, 13 B27 positive RS patients, six B27 positive healthy persons and 12 non-HLA typed healthy controls were studied. No sex or age matches were performed. They found that the mean fluorescence intensities for the RS patient sera and for the AS patient sera were significantly higher than for either control group sera. Experiments were done that indicated that this was mediated by serum antibodies that were recognising HLA-B27 complexed with peptides derived from the *Mycobacterium tuberculosis* HSP60.

Gao *et al*⁸⁸ reported on cytotoxic T cell (CTL) recognition of homocysteine modified B27 molecules. CTL in PBL cultures of two of three B27 positive AS patients, one of one B27 positive ReA patient and none of two healthy controls of which one was B27 positive, specifically recognised homocysteine modified B27 molecules on autologous B cell lines. No sex or age matches were performed. HLA-A68 restricted homocysteine specific CTL (Hom-CTL) from a B27 negative patient also recognised *Salmonella* infected target cells, suggesting that also *Salmonella* infection can lead to the modification of HLA antigens that is recognised by Hom-CTL.

Hermann *et al*⁸¹ found in synovial fluid from four (one B27 negative, three B27 positive) of six SpA patients (four ReA, two AS) a significant frequency of autoreactive CTL. Also B27 restricted autoreactive CTL clones specific for an as yet unidentified peptide were isolated. No studies were done on synovial fluid from controls.

CROSSREACTIVITY BETWEEN BACTERIA AND HLA-B27

After the sequence of the B27 molecule had been unravelled, more precise data about possible crossreactive epitopes on B27 and bacteria could be obtained than from experiments with antisera raised in animals. Using DNA hybridisation techniques, some sequence homologies between *Klebsiella pneumoniae* and *Klebsiella otica* and B27 were thought to be present, especially between these bacteria and the 66–74 region located within the B*2705 hypervariable region.^{82 83}

Schwimmbeck *et al*^{20 70 71} used computer searches to identify sequence homologies

Table 3 Amino acid sequences of HLA-B27 compared with the sequences of bacterial proteins

Antigens	Residues	Amino acid (residues in bold are homologous with B*2705)																				
B*2705 B*2703 B*2707 B*2709	67–84				C	K	A	K	A	Q	T	D	R	E	D	L	R	T	L	L	R	Y
B*2701	—				-	-	-	-	-	-	-	Y	-	-	N	-	-	-	A	-	-	-
B*2702	—				-	-	-	-	-	-	-	-	-	-	N	-	-	I	A	-	-	-
B*2704	—				-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-
B*2706	—				-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-
B*2708	—				-	-	-	-	-	-	-	-	-	-	S	-	-	N	-	R	G	-
<i>Klebsiella</i> Nitrogenase	183–200				I	C	N	S	R	Q	T	D	R	E	D	E	L	I	I	A	L	-
<i>Klebsiella</i> PulD	590–605					R	P	T	V	I	R	D	R	D	E	Y	R	Q	A	S	S	-
<i>Shigella</i> pHs-2					G	T	V	C	A	Q	T	D	R	H	S	L	S	C	I	A	M	Q
<i>Yersinia</i> (O:3) Yop 1	171–188				A	I	G	D	R	S	K	T	D	R	E	N	S	V	S	I	G	C
<i>Salmonella</i> OMP-H	50–68	Q	R	L	Q	S	M	K	A	G	S	D	R	T	K	L	E	K	D	V		

between the B*2705 sequence and known sequences from bacteria. They found a homology of six consecutive amino acids (QTDRED) shared by B*2705 residues 72–77 located in the hypervariable region of the B27 molecule and *Klebsiella pneumoniae* nitrogenase 188–193. This is a hydrophilic sequence, and is thus predicted to be located at the outside of the protein possibly accessible to antibodies. Later, sequence similarities between B*2705 and proteins of other bacteria than *Klebsiella* were also identified. Table 3 shows the sequence similarities between some bacterial proteins and the various B27 subtypes.

Schwimmbeck *et al.*^{20 70 71} raised antibodies in rabbits reacting both with peptides from B*2705 and peptides from *Klebsiella pneumoniae* nitrogenase. If the rabbits were immunised with B27 or *Klebsiella* nitrogenase synthetic peptides containing the homologous sequence, it was shown that affinity purified antibodies raised against either peptide also reacted with the other peptide. Although Tsuchiya *et al.*⁶⁴ found the same results, De Vries *et al.*⁷⁸ were not able to reproduce these findings.

Schwimmbeck *et al.*^{20 70 71} also tested sera from AS and RS patients for reactivity against the peptide derived from *Klebsiella* nitrogenase 184-CNSRQTDREDEL-196 containing the homologous 72-QTDRED-77 B27 sequence.

As shown in table 4, they found that the frequency of antibodies against this peptide in sera from patients was higher than in sera from controls. This is in agreement with their finding that the frequency of antibodies against a B27 derived peptide containing residues 69–84 is increased.^{20 70 71} No cross inhibition studies were performed to study whether these antibodies were indeed crossreactive antibodies, and no age or sex matches were performed.

Tsuchiya *et al.*⁷⁴ found that the reactivity to *Klebsiella* nitrogenase peptide containing residues 184–196, in sera from B27 positive male patients and B27 positive male controls, either from Norway or New Mexico was not different. The mean age of Norwegian patients and controls was 38 years ranging from 20 to 69, and 39 years ranging from 21 to 62, respectively. The mean age of the New Mexican patients and controls was 43 years ranging from 16 to 76, and 40 years ranging from 25 to 66 respectively. Although sera from some patients were reactive with the *Klebsiella* peptide, there was no positive correlation between a positive reaction with the *Klebsiella* nitrogenase peptide and a positive reaction with the B27 peptide 69–84. Cross inhibition studies were negative.

A very elegant study was carried out by Ewing *et al.*⁷⁷ Using overlapping peptides of eight residues from the B*2705 molecule, they

Table 4 Frequencies of presence of anti-bacterial antibodies in sera from SpA patients and controls

Antigens	Reference	AS patients		RS patients		Healthy controls		Titre	Match
		B27 ⁺	B27 ?	B27 ⁺	B27 ?	B27 ⁺	B27 ?		
<i>Klebsiella</i> Nitrogenase	Schwimmbeck <i>et al.</i> ^{20 70 71}	11/24*		15/34*		0/22	1/90	1:10	B27
<i>Klebsiella</i> Nitrogenase	Tsuchiya <i>et al.</i> ⁶⁴	5/50	5/60	1/5	1/8	1/11	1/25	1:50	B27, Age, Sex
								1:50	100% male
				0/6	0/8	0/6	2/38	1:2000	N. Mexico
<i>Klebsiella</i> Nitrogenase	Ewing <i>et al.</i> ⁷⁷	↑*				n=22	n=44		B27
<i>Klebsiella</i> PulD	Fielder <i>et al.</i> ⁷³	↑*						1:50	—
<i>Klebsiella</i> PulD	Tani <i>et al.</i> ⁷²	↑*					n=25	1:50	—
		n=52					n=50		
		90% male					50% male		
<i>Yersinia</i> Yop 1	Tsuchiya <i>et al.</i> ⁷⁵	17/50	19/60			1/11	3/25	1:50	B27
									Sex
<i>Yersinia</i> Yop 1	Lahesmaa <i>et al.</i> ^{85 87}		28/84		5/12			1:20	100% male
			78% male		92% male				B27
				11/30			8/29	1:20	Age
				(ReA, 97%B27+)			(yers.B27-)		Sex
<i>Shigella</i> pHS-2	Tsuchiya <i>et al.</i> ⁶⁴	7/50	7/60			1/11	2/25	1:50	B27
								1:50	Sex
		2/26	2/26			0/6	1/38	1:50	100% male
									N. Mexico
<i>Salmonella</i> Omp-H	Lahesmaa <i>et al.</i> ⁸⁵		21/82		5/12			1:20	B27

↑ = Increased mean absorbance in ELISA. Bold type* = significant (p<0.05) if compared with healthy controls. yers = uncomplicated yersiniosis. Other abbreviations as in table 2.

showed that in fact the antibody response against the part of B*2705 containing the *Klebsiella* homologous sequence was not directed to the complete homologous 72-QTDRED-77 sequence, but rather to flanking regions that included only a few of the homologous residues—that is, 68-KAKAQTD-75 and 76-REDLRTLL-83 of HLA-B27. Interestingly, they found that sera reacting with peptides derived from *Klebsiella* nitrogenase also did not recognise octapeptides containing the complete homologous sequence, but an epitope containing QTDR and residues to the left of this sequence that is not homologous to B*2705—that is, 185-NSRQTDR-191. Fifty B27 positive AS patient sera and sera from 22 B27 positive and 22 B27 negative first degree relatives were tested for reactivity to the 185-NSRQTDR-191 *Klebsiella* peptide with ELISA assays. In patient sera the binding as measured by the mean absorbance value in the ELISA was significantly ($p < 0.002$) higher than in sera from the B27 negative control group, and also (but less significant, $p < 0.02$) than that in sera from the B27 positive control group. No age matches were performed. The overall conclusion by Ewing *et al* from their experiments was that sera from AS patients contained antibodies that reacted with overlapping epitopes of 5 to 8 amino acids within the sequence 183-ICNSRQTDR-191 on the *Klebsiella* nitrogenase molecule. Sera from AS patients showed a positive correlation between binding to the B27 peptide 68-KAKAQTD-75 and binding to the *Klebsiella* peptide 185-NSRQTDR-191. However, no cross inhibition studies, and studies with monoclonal or affinity purified antibodies were carried out, to show that antibodies were indeed crossreactive. Although QTDR seems to be an important sequence for antibody binding, the 4 amino acid peptide QTDR alone or QTDRED showed little reactivity with AS patient sera.

Tsuchiya *et al*⁸⁴ found that only three of 15 affinity purified anti-B27 peptide antibodies from AS patients showed a weak crossreactivity with the *Klebsiella* nitrogenase peptide 184-CNSRQTDRED-197. The experiments in which different peptides were used, showed that the ELISA used was specific for peptides containing certain amino acid residues. Lahesmaa *et al*, however, found that sera reacting with CNSRQTDREDELI also reacted with peptide CNSRRQDDTEELI containing the same amino acids but in which the crossreactive sequence was scrambled.⁸⁵

A computer search by Fielder *et al* revealed another sequence homology between a bacterial protein and HLA-B27.⁷³ The PulD secretion protein of the starch inducible starch debranching enzyme pullulanase contains a sequence DRDE, which shows some similarity with DRED of B*2705. Peptides 67-CKAKAQTDREDLRTLL-82 from B*2705 and 590-RPTVIRDRDEYRQASS-605 from PulD were used as substrates to study the reactivity of patient and control sera. They found significantly increased antibody levels in sera from AS patients compared with controls or

RA patients. No experiments were done to confirm the presence of crossreactive antibodies in positive sera. Patients and controls were not matched for HLA, sex or age. Control peptides from the scrambled PulD sequence were used to confirm sequence specificity of the tests.

Tani *et al*⁷² found significantly increased titres of IgG and IgA against the PulD peptide in sera from active AS patients compared with sera from controls. No HLA, sex or age matches were performed, and controls were only 50% men compared with 90% men in the AS group. No differences were found in antibody levels against a control peptide, consisting of a scrambled sequence of the pulD peptide, between the different groups. However, the absorbance values obtained with the control peptides were much higher (± 0.7) for all groups studied than those obtained with *Klebsiella* peptides and sera with negative (± 0.15) and positive (± 0.45) reactions.

Sequence similarities between B*2705 and proteins of other Gram negative bacteria than *Klebsiella* were also studied.^{75–84–86} It was found that *Shigella flexneri* strains that are isolated from ReA patients carry a plasmid (pHS-2).⁸⁶ DNA sequence analyses of this plasmid revealed that it contains an open reading frame (ORF) encoding a 22 amino acid peptide with sequence homology to B27, again involving the sequence QTDR—that is, GTVCAQTDRHSLSCIAMQ. Affinity purified rabbit antibodies against B*2705 showed a strong reactivity with the pHS-2 peptide. Of 15 affinity purified anti-B*2705 peptide antibodies from B27 positive patients only three recognised the *Klebsiella* nitrogenase 184–196 peptide, while 12 showed strong crossreactivity with the pHS-2 peptide. Affinity purified anti-B*2705 peptide antibodies from these patients reacted with whole *Shigella* bacteria that contained the pHS-2 plasmid, but also with plasmid negative *Shigella* bacteria. This finding suggests that these antibodies were not directed to the peptide encoded by the pHS-2 plasmid, and that the tests used were not specific for antibodies against sequences encoded by this plasmid. When large numbers of AS and RS patient sera from Norway or New Mexico were studied, only a relatively low level of reactivity to the pHS-2 peptide was demonstrated.⁸⁴ A higher proportion of AS patients compared with controls recognised the pHS-2 peptide and patients had increased titres against this peptide, although the differences were not significant.

The sera from the same Norwegian patients were used to study reactivity to a peptide from Yop1 of *Yersinia enterocolitica*, as Tsuchiya had identified sequence homology between B*2705 and the plasmid encoded Yop1 from *Yersinia enterocolitica* O:3 and *Yersinia pseudotuberculosis*, in contrast with Yop1 of the non-ReA associated *Yersinia enterocolitica* O:8.⁷⁵ The 171-AIGDRSKTDRENSVSGIC-188 peptide used in the ELISA contained the homologous sequence. A larger but not significant higher proportion of Norwegian AS patients had increased anti-Yop1 antibody levels than

Norwegian controls, especially IgA antibodies. However, anti-Yop1 antibody levels were not correlated with anti-B27 peptide antibody levels, although in one patient cross inhibition of the anti-Yop1 peptide antibodies with the B27 peptide was found. The mean age of the patients was 38 years, ranging from 20 to 69. Controls used were matched and had a mean age of 40 years, ranging from 21 to 62.

Another sequence homology exists between B*2705 and OMP-H of *Salmonella typhimurium*, again within the hypervariable region of B27. Lahesmaa *et al.*⁸⁵ tested for antibodies against the *Yersinia enterocolitica* O:3 Yop1, as well as for antibodies against the peptide from OMP-H containing the homologous sequence—that is, 50-QRLQSMKAGSDRTKLEKDV-68, in sera from AS, RS and ReA patients and healthy controls without performing HLA, sex or age matching. Thirty three per cent of AS patients and 42% of RS patients had antibodies against the Yop1 peptide, and 25% of AS patients and 40% of RS patients had antibodies against OMP-H. No data about healthy controls were included. The antibodies against Yop 1 were not directed against a part of the peptides with B27 homology, but to a flanking region left from this sequence. In this study it was also found that anti-*Klebsiella pneumoniae* nitroge-nase antibodies were not directed against the B*2705 homologous sequence, but against a flanking region.

In 1992 a study from Lahesmaa⁸⁷ was published in which 30 yersiniosis patients with ReA (one B27 negative, 30% female), 29 uncomplicated yersiniosis patients (all B27 negative, 34 % female) and 79 controls with various rheumatic diseases other than SpA or with infections by bacteria other than *Yersinia*. They tested sera of these subjects for antibody reactivity against the 171–188 Yop 1 peptide. Thirty two per cent of the yersiniosis patients had antibodies against this peptide compared with only 4% of controls (two had RA, one had a *Klebsiella* infection). The antibodies were mostly IgA, and only two patients (without arthritis) had IgG antibodies reactive with the Yop 1 peptide. No difference in frequencies was found between the yersiniosis patients with arthritis and those without arthritis. Experiments were also performed to determine more precisely the sequence specificity of the antibody reactivity. These showed that the antibodies were not directed against the tetrapeptide shared by Yop 1 and B27, but against a left flanking sequence of Yop 1. While proliferation against whole *Yersinia* lysates was present, no lymphocyte proliferation against the Yop 1 peptide was found when 15 yersiniosis patients (eight with ReA) and 16 healthy controls (50% B27 positive) were tested. None of the yersiniosis patients had IgG or IgA against the 184–197 *Klebsiella* nitrogenase peptide.

Conclusions

From the data available in the literature it can be concluded that there are no consistent results confirming the presence of increased

autoreactivity in SpA patients compared with controls. When reviewing the literature on this subject, some problems are encountered.

The results reported by the different groups are obtained by various methods, different dilutions of sera are tested, and different antigen preparations have been used. In addition, another problem is the specificity of the ELISA assays used. Tsuchiya⁷⁵ and Lahesmaa^{85–87} showed that experiments with appropriate control peptides should be carried out to confirm the sequence specificity of the ELISA assays used. De Vries *et al.*⁷⁸ clearly showed that a correction for non-specific binding of sera used in the ELISA assays is required. In none of the studies with positive findings, using sex and HLA matched controls, such corrections were done. The main problem, however, in determining the presence of autoreactivity in B27 associated SpA is that only in a few studies HLA, sex and age matched controls were included, as shown in table 1, table 2, and table 4.

The first studies showing an increased reactivity against B27 in the sera of AS and RS patients were done by Schwimmbeck *et al.*^{20–70–71} However, their findings could only be confirmed in those studies lacking both HLA and sex matched controls.^{72–73–77–78} Studies from groups using HLA and sex matches were not able to reproduce the results. Tsuchiya *et al.*,^{74–75} who found very high anti-B27 activity in some healthy B27 negative or even B27 positive women with previous pregnancies, illustrated the importance of using sex matches.^{74–75}

The above mentioned problems also apply for the studies determining the presence of antibodies crossreacting with B27 and bacterial proteins. It is clear that sequence similarities between HLA-B27 and some bacterial proteins exist, and that at least in rabbits antibodies reacting with both bacterial proteins and B27 can be raised. The relevance of this finding for the pathogenetic mechanisms in B27 associated SpA is not evident, and inconsistent data about the presence of these antibodies in patients are obtained. When the frequencies of the presence of antibodies against bacterial proteins and B27 in sera from AS and RS patients were compared with those in sera from healthy controls, the latter were almost never properly matched for age, while only three studies performed sex and HLA matches. Most studies did not perform any experiments to confirm the crossreactivity of antibodies. Tsuchiya⁷⁵ and Lahesmaa⁸⁵ clearly showed that experiments with appropriate control peptides should be carried out to determine the specificity of affinity purified antibodies.

Of the studies on crossreactive antibodies, in which controls were matched both for HLA-B27 and sex, one study found increased reactivity to *Yersinia* Yop 1 in patients.⁷⁵ However, although in one patient cross inhibition of the anti-Yop 1 peptide antibodies with the B27 peptide was found, there was no correlation with anti-B27 peptide antibody levels. Another study produced negative results with *Klebsiella* nitrogenase.⁷⁴ A positive correlation was found between recognition of B27 and

pHS-2 peptides, and crossreaction of affinity purified anti-B27 antibodies with pHS-2 was observed in 13 of 15 patients who recognised B27. However, a statistically non-significant higher proportion of AS patients recognised the *Shigella* pHS-2 peptide.⁸⁴

Some results suggest that in people with an anti-B27 response, the antibodies are not directed to parts of the B27 molecule showing sequence similarities with bacterial proteins, but rather to flanking regions.^{77 85} This is in agreement with findings that mice transgenic for HLA-B*2705 and human β_2 -microglobulin are tolerant to the B27 and bacterial crossreactive epitopes,⁵⁵ and that peptides from regions that are non-homologous to self proteins are more readily recognised by the immune system than peptides from parts that are more homologous to self.⁸⁸

The sequence similarities between B27 and the bacterial proteins nitrogenase, pullulanase, Yop1, pHS-2, and OMP-H, are located in a region of B27 where different B27 subtypes differ in one or more amino acids. The crossreactive sequences of the B27 molecule are completely identical in B27 subtypes associated with disease (B*2705 and B*2707) and a subtype that is probably not associated with disease (B*2709). The sequence similarities with bacterial proteins in B*2704 (which is associated with disease), and B*2706 (which is not associated with disease) are also identical, but differ both from B*2705 by a D to S substitution at residue 77. Schwimmbeck *et al*⁷¹ reported a strong influence of the sequence differences as found in parts of the various B27 subtypes containing the reported sequence similarities with bacterial proteins on anti-B27 antibody binding. They found that a single amino acid change of D to N (as found at residue 77 in B*2701 and B*2702) significantly reduced recognition by affinity purified antibodies raised against a B*2705 peptide, while a change of D to S (as found at residue 77 in B*2704, B*2706 and B*2708) completely abolishes this reactivity. This suggests that antibodies against these parts of the B27 molecule, containing the reported sequence similarities with the above mentioned bacterial proteins, are not likely to be involved in B27 associated disease.

HLA-B27 is not the only HLA molecule sharing sequence similarities with bacterial proteins.¹⁶ After the first studies had been published in which the role of molecular mimicry in B27 associated SpA was investigated, it has been suggested that the small chance that a sequence of 6 amino acids occurs in two different proteins, being only $1:64\,000\,000$ ($1:20^6$), supported the idea that molecular mimicry plays a part in the onset of B27 associated diseases.^{20 70} However, if a protein of x amino acids is screened for a perfect 6 amino acid match in a database of n amino acids the expected number of perfect matches is $0.05^6 \times n \times x$. Searching the SWISS-PROT database of 1996 that contained 1.5×10^7 residues for a perfect six amino acid match with a 360 amino acid protein like B27, one would expect $0.05^6 \times 1.5 \times 10^7 \times 360 = 84$ matches.⁸⁹ Therefore,

it is actually very possible to find 5, 6 and 7 amino acid matches between unrelated proteins,⁸⁸ and indeed this is found for HLA-B27 as well as for other HLA-B alleles.¹⁶

Only very few experiments have been carried out to determine T cell reactivity against B27 in patients and controls. Interesting results have been obtained that suggest that the three dimensional structure of B27 can be modified by peptides or by homocysteine, thereby creating new conformational epitopes that are recognised by antibodies as well as T cells of some SpA patients.^{38 80} However, too few data are yet available about the presence of B27 specific, MHC class I or MHC class II restricted, T cell responses in patients and controls.

From this review it is concluded that there is no evidence that B27 associated diseases are autoimmune diseases as a consequence of autoimmune reactions. Although the B27 molecule shares some immunological determinants with bacterial constituents, there is no proof that these determinants are important for the pathogenesis of B27 associated SpA. Although some patients and healthy people produce antibodies to B27 and crossreactive antibodies to both B27 and bacterial proteins, there is no evidence that the frequency of increased titres of such antibodies in sera from patients is significantly higher than in sera from matched controls.

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