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Relation of antivimentin antibodies to anticardiolipin antibodies in systemic lupus erythematosus

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SUMMARY Tests for antivimentin antibodies (AVA) were performed on 50 systemic lupus erythematosus (SLE) and 63 control sera by indirect immunofluorescence and enzyme linked immunosorbent assay (ELISA). The prevalence was significantly raised in SLE (38% and 50% of sera positive for IgM-AVA and IgG-AVA, respectively, by immunofluorescence; 36% and 64% of sera positive for IgM-AVA and IgG-AVA, respectively, by ELISA) in comparison with the control sera. A significant correlation existed between IgM-AVA, on the one hand, and anticardiolipin antibodies (ACA) and anti-single-stranded DNA (ssDNA), on the other. A stepwise principal component analysis demonstrated that IgM-AVA and IgG-AVA accounted for 71% of the total variance in SLE (50 patients × 5 parameters=total variance). Twenty ACA positive serum samples from patients with syphilis were therefore tested for the presence of AVA, but hardly any were found to be positive. IgM-AVA from patients with SLE were inhibited by cardiolipin and absorbed with ssDNA. An association between AVA positivity and arthralgia was also shown in SLE.

Key words: anti-single-stranded DNA antibody.

Intermediate sized filaments are polymeric fibrous structures.[1] Together with microfilaments and microtubules they form part of the extensive cytoplasmic network called the cytoskeleton. The collective term intermediate sized filaments refers to those filaments whose diameter is approximately 10 nm, and therefore intermediate between that of microfilaments (7 nm) and microtubules (25 nm). Most cells contain only one type of intermediate sized filament. For example, epithelial cells contain keratin, whereas cells of mesenchymal origin, e.g., fibroblasts, contain only vimentin.

Vimentin has been shown to be a target for an autoimmune reaction, not only in bacterial,[2] parasitic,[3] and viral[4] infections, but also in rheumatic diseases,[5–8] especially systemic lupus erythematosus (SLE).

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As part of our studies on anticytoskeletal antibodies[9 10] we looked for the presence of antivimentin antibodies (AVA) in SLE sera, using an indirect immunofluorescence technique and an enzyme linked immunosorbent assay (ELISA). The second objective of the present work was to elicit information concerning the cross reacting specificities and the interrelationship between AVA, on the one hand, and anticardiolipin antibodies (ACA), anti-double-stranded (ds) and anti-single-stranded (ss) DNA antibodies, on the other.

Patients and methods

PATIENTS

Fifty serum samples from patients with SLE were obtained from the Brest University Hospital. The group included eight men and 42 women, ranging in age from 10 to 71 years (mean 40·8), and meeting the Amerian Rheumatism Association preliminary[11] and revised[12] criteria for SLE. Patients were selected because they were new patients at their first

encounter with us in the hospital (department of rheumatology and internal medicine). The serum was drawn at the same time that the clinical evaluation was performed. Stored sera from 20 patients with syphilis, as demonstrated by strongly positive Venereal Disease Research Laboratory slide flocculation and *Treponema pallidum* immobilisation tests, were also examined. The control sera were from 25 men and 38 women with ages ranging from 23 to 79 years (mean 34·2). They were members of the medical staff or residents of a home for the elderly. All the serum samples were stored at −70°C until tested.

CELL LINE

IMR-33, derived from a gerbil fibroma, was originally obtained from the American Type Culture Collection (Rockville, MD), and cultured in minimal essential medium 199 supplemented with 20% heat inactivated fetal calf serum and antibiotics. Cells were seeded onto multispot slides after brief trypsinisation of stock cultures. Some of them were treated with colchicine (20 μg/ml) for 18 hours before use. After washing the slides with phosphate buffered saline (PBS), pH 7·4, the cells were fixed in methanol at −20°C for 10 minutes, rinsed for 15 seconds in acetone prechilled to −20°C, and thoroughly washed in PBS before serum samples were applied.

INDIRECT IMMUNOFLUORESCENCE

The slides were incubated for 45 minutes at 20°C in a moisture chamber with the sera diluted 1:10 in PBS containing 0·1% NaN_3 to prevent contamination, washed three times with PBS, and further incubated for 45 minutes at 20°C with a second layer of two antibodies mixed 50:50 together. These were an affinity purified, fluorescein conjugated goat $F(ab')_2$ antihuman IgM, μ chain specific, and an affinity purified, Texas red conjugated goat $F(ab')_2$ antihuman IgG, γ chain specific (Jackson Immunoresearch Laboratories, Avondale, PA). After extensive washing with PBS the final preparation was covered with a glycerol mounting solution containing 25 g/l diazobicyclo-octane to prevent fading.[15] Cells were examined under a Leitz Dialux 22 fluorescence microscope equipped with epifluorescence illumination and the appropriate filters for fluorescein and Texas red. Pictures were taken on Fujichrome film with an oil immersion objective Leitz Neofluar (50×). Cytoskeletal structures were identified by using a panel of commercially available (Boehringer, Mannheim, West Germany) monoclonal antibodies as markers. Positive sera stained cytoplasmic arrays of filaments in untreated cells (Fig. 1a) and perinuclear filamentous coils in colchicine treated cells (Fig. 1b). A titre of at least 1/10 was considered positive.

ISOLATION AND PURIFICATION OF VIMENTIN

Vimentin intermediate sized filaments were isolated from human skin fibroblasts and purified by the method of Steinert *et al.*[14] Briefly, the cells were lysed with PBS containing 0·6 M KCl, 1% Triton X-100, 10 mM $MgCl_2$, 0·5 mM phenylmethylsulphonyl fluoride, 1 mg/ml *N-p*-tosyl-L-arginine methyl ester HCl (TAME), and 0·5 mg/ml DNAse 1 (Sigma Chemical Co, St Louis, MO). The pelleted filaments were resuspended (1 mg/ml total protein) in 5 mM trometamol (TRIS) HCl, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis (*N,N'*-tetra-acetic acid), and 1 mg/ml TAME, homogenised and dialysed against 1000 volumes of PBS for 16 hours at 4°C. Centrifugations at 40 000 *g* for 15 minutes and 250 000 *g* for one hour were performed to clarify the solution. Vimentin reassembled within six hours at 4°C upon addition of 2·5 M KCl to a final concentration of 0·17 mol/l. It was then purified by two further cycles of disassembly-reassembly.

ENZYME LINKED IMMUNOSORBENT ASSAY

An ELISA[15] was performed by a modification of the microtitre technique of Voller *et al,*[16] which will be described in detail elsewhere (Boehme and Blaschek, manuscript in preparation). Aliquots of vimentin intermediate sized filaments were put into polyvinylchloride microtitre plates (10 μg/ml, 300 μl/well), and the plates were incubated at 37°C for three hours. After three washes with PBS containing 0·05% Tween 20 (PBS-Tween) the plates were incubated with PBS containing 0·3% gelatin for five minutes to allow blocking of any free binding sites. Test sera, diluted 1:500 in PBS-Tween, were then incubated in triplicate wells for three hours at room temperature. After washing the wells three times with PBS-Tween a mixture 50:50 of peroxidase conjugated, rabbit antihuman IgM (Medac, Hamburg, West Germany) and alkaline phosphatase-conjugated, goat antihuman IgG (Dako, Glostrup, Denmark), both 1:500 in the same diluent as used for the sera, was added, then after a three hour incubation the plates were washed three times in PBS-Tween. *o*-Phenylenediamine and *p*-nitrophenyl phosphate were used as substrates for anti-IgM and anti-IgG antibodies respectively. The absorbance at 492 and at 405 nm was read in a Titertek multiscanner (Flow Laboratories, McLean, VA). Standard curves were prepared with a reference serum, previously tested on several cell line preparations for AVA by indirect immunofluorescence assay and found to be strongly positive.

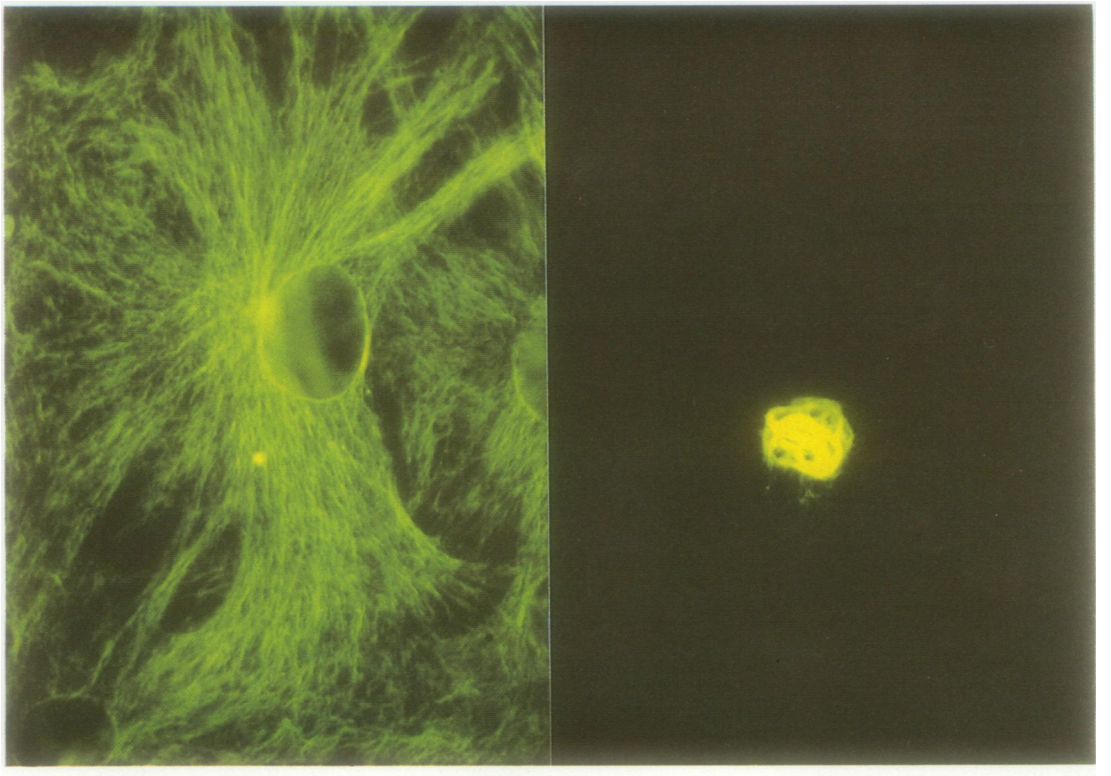

Fig. 1a Fig. 1b

Fig. 1 *Identification of IgM-antivimentin antibody (AVA) by indirect immunofluorescence. (a) Fluorescence pattern due to a systemic lupus erythematosus serum on IMR-33 cell line. (b) The coiling of AVA around the nucleus after treatment with colchicine.*

AVA INHIBITION BY CARDIOLIPIN

Ethanol was evaporated from the cardiolipin stock solution (Sigma Chemical Co) under a nitrogen stream, and the dried phospholipid was then dissolved in PBS-Tween to a concentration of 800 µg/ml under sonication. Three AVA positive and ACA positive sera from patients with SLE and three AVA-negative/ACA-positive sera from patients with syphilis were diluted 1:250 in PBS-Tween. Aliquots were incubated in a 1:1 ratio with varying amounts of the cardiolipin inhibitor (100, 200, 400, and 800 µg) for one hour at 20°C and one hour at 4°C. Sera were then centrifuged at 3000 *g* for 15 minutes at 22°C, and supernatants were tested for AVA by ELISA according to the method outlined above.

AVA ABSORPTION WITH SINGLE STRANDED DNA

Salmon sperm DNA (Sigma Chemical Co) was denatured by heating at 90°C for 10 mintues and fast cooling to 0°C, and ssDNA was fixed to cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia, Upsala, Sweden) by means of poly-L-lysine at 20°C by the method of Kubota *et al.*[17]

Sepharose human Cohn fraction II (CFII) and Sepharose bovine serum albumin (BSA) were prepared by coupling CFII and BSA (Sigma Chemical Co) respectively, to a concentration of 5 mg protein/ml Sepharose. Serum samples from five patients with SLE were diluted 1:2, passed through the ssDNA, CFII, or BSA column and reconcentrated until the original sample volume was obtained. The effluents were diluted 1:500 and tested for AVA.

OTHER SEROLOGICAL TESTS

ACA were determined by the ELISA technique described by Harris *et al.*[18] Alkaline phosphatase conjugated, goat antihuman immunoglobulins (IgG, IgM, and IgA; Sigma Chemical Co) diluted 1:500 were used. Fifty microlitres of 1 mg/ml *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9·8, was added to each well. After 45 minutes the

reaction was stopped by adding 50 µl aliquots of 3 M sodium hydroxide to each well. Absorbance was read at 405 nm. Standard positive sera were supplied by Dr E N Harris, St Thomas's Hospital, London.

Anti-dsDNA antibodies were tested by a commercial ELISA kit purchased from Cordis Laboratories (Miami, Flor) and the results reported in international units/ml (IU) traceable to the World Health Organisation antinuclear antibody serum.

To set up the ELISA test for anti-ssDNA antibodies, dsDNA (Sigma Chemical Co) was denatured as previously described, coated to discs (Cordis Laboratories Inc), and processed as above.[19]

STATISTICAL ANALYSIS

All tests were performed in triplicate and the data were averaged. In the five ELISA tests the upper limit of normality was taken as two standard deviations above mean normal control levels. The sensitivity and the specificity of the tests for SLE were calculated using the formulas established by Youden.[20] Specificity is the number of subjects without presumed SLE with a negative assay divided by the number of subjects without presumed SLE, i.e., the true negative rate. Sensitivity is the number of patients with SLE with a positive assay divided by the number of patients with SLE, i.e., the true positive rate.

The analysis of these data was carried out using the Statistical Package for the Biological Sciences on the Person 1600 IBM compatible at the French National Institute of Marine Biology computing centre. The relation between autoantibodies and certain clinical features was examined using χ^2 analysis with Yates's correction for small numbers. Correlations between the different autoantibodies were established by Spearman's correlation coefficient. A stepwise principal component analysis was performed. This method of analysis allows the evaluation of the statistical weight of each of the five quantitative variables (ACA, IgM-AVA, IgG-AVA, anti-dsDNA, and anti-ssDNA antibodies) in a system of five orthogonal axes (one for each of the variables). The percentage of the total variance (the total variance is the variance of the five variables multiplied by the number of individuals, i.e., 50) is accounted for by each of the axes.

Results

Analysis of the 50 serum samples from patients with SLE and 63 from normal controls by indirect immunofluorescence assay (Table 1) showed that 19 (38%) and 11 (17%), respectively, had significantly raised titres of IgM-AVA whereas eight (16%) and four (6%), respectively, had significantly raised titres of IgG-AVA. With the ELISA (Fig. 2), of the

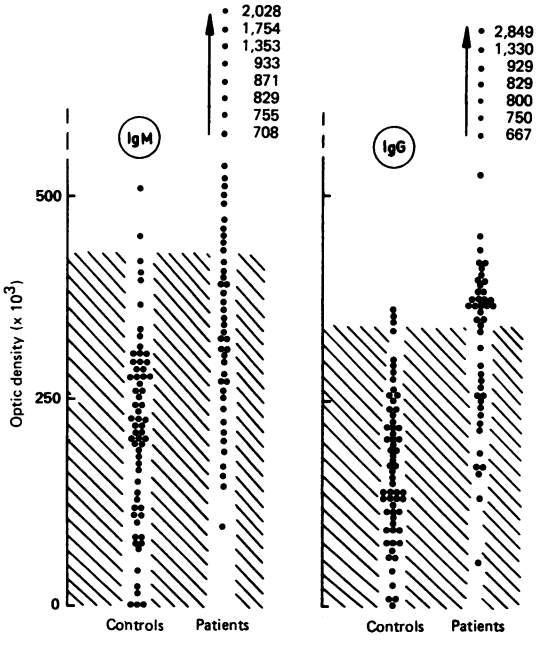

Fig. 2 *Optical densities obtained with antivimentin antibody by an enzyme linked immunosorbent assay in serum samples from 50 patients with systemic lupus erythematosus and from 63 normal controls.*

Table 1 *Prevalence of IgM- and IgG-antivimentin antibodies by indirect immunofluorescence and enzyme linked immunosorbent assay in serum samples from 50 patients with systemic lupus erythematosus and from 63 normal controls*

*IIF**	*ELISA**	*IgM-AVA**		*IgG-AVA*	
		No of patients	*No of controls*	*No of patients*	*No of controls*
+	+	13	1	6	0
+	−	6	10	2	4
−	+	5	1	26	3
−	−	26	51	16	56

*IIF=indirect immunofluorescence; ELISA=enzyme linked immunosorbent assay; AVA=antivimentin antibody.

63 serum samples from normal controls, the range of absorbance at 492 nm, representing the results for IgM-AVA, was 0·010–0·507 (mean (SD) 0·214 (0·113)), and the upper limit of normal was set at 0·440 optical density (OD) units (mean + 2SD). The range of absorbance at 405 nm, representing the results for IgG-AVA, was 0·009–0·375 (mean (SD) 0·172 (0·087)), and the upper limit of normal was set at 0·346 OD units (mean + 2SD). Eighteen (36%) of the 50 SLE sera contained raised levels of IgM-AVA and 32 (64%) raised levels of IgG-AVA.

Comparison of the indirect immunofluorescence assay and ELISA methods of measuring AVA (Table 1) showed that the latter was more specific and more sensitive than the former technique (specificity: 97% *v* 83% for IgM-AVA, and 95% *v* 94% for IgG-AVA; sensitivity: 38% *v* 38% for IgM-AVA, and 52% *v* 16% for IgG-AVA). Therefore, for convenience, only the ELISA results will be referred to in the following part.

Table 2 shows the number of serum samples from patients and controls in which increased activity against vimentin, cardiolipin, dsDNA, and ssDNA was detected.

Table 2 *Results of various serological investigations in 50 patients with systemic lupus erythematous and in 63 normal controls*

	No (%) positive	
	Patients (n=50)	*Controls (n=63)*
AVA*	33 (66)	4 (6)
ACA*	27 (54)	1 (2)
Anti-dsDNA Ab*	44 (88)	2 (3)
Anti-ssDNA Ab*	43 (86)	3 (5)

*AVA=antivimentin antibody; ACA=anticardiolipin antibody; anti-dsDNA Ab=anti-double-stranded DNA antibody; anti-ssDNA Ab=anti-single-stranded DNA antibody.

Table 3 *Correlation of antivimentin, anticardiolipin, and anti-DNA antibodies*

	ACA	*IgM-AVA*	*IgG-AVA*	*dsDNA*
IgM-AVA	r=0·52 p<0·001	—	—	—
IgG-AVA	r=0·09 p>0·10	r=0·29 p<0·05	—	—
dsDNA	r=0·42 p<0·01	r=0·27 p>0·05	r=0·11 p>0·10	—
ssDNA	r=0·77 p<0·001	r=0·46 p<0·001	r=0·12 p>0·10	r=0·25 p>0·05

Correlations established using Spearman's correlation coefficient. For abbreviations see Tables 1 and 2.

Correlations were sought between IgM-AVA, IgG-AVA, ACA, anti-dsDNA antibodies, and anti-ssDNA antibodies (Table 3). Statistically significant correlations were found between both isotypes of AVA, between IgM-AVA and ACA/anti-ssDNA antibodies, and between ACA and both anti-DNA antibodies. Because this study was devoted to AVA we focused on the relation of IgM-AVA to ACA (r=0·52) and to anti-ssDNA antibodies (r=0·46). Twenty ACA positive sera from patients with

Table 4 *Results of the stepwise principal component analysis*

Axis	*Variables associated*	*% Of the total variance* accounted for by each of the axes*
1	IgM-AVA	48·551
2	IgG-AVA	22·583
3	ACA	14·449
4	anti-dsDNA Ab	7·854
5	anti-ssDNA Ab	6·563

*Total variance=50 patients × 5 variables.
For abbreviations see Tables 1 and 2.

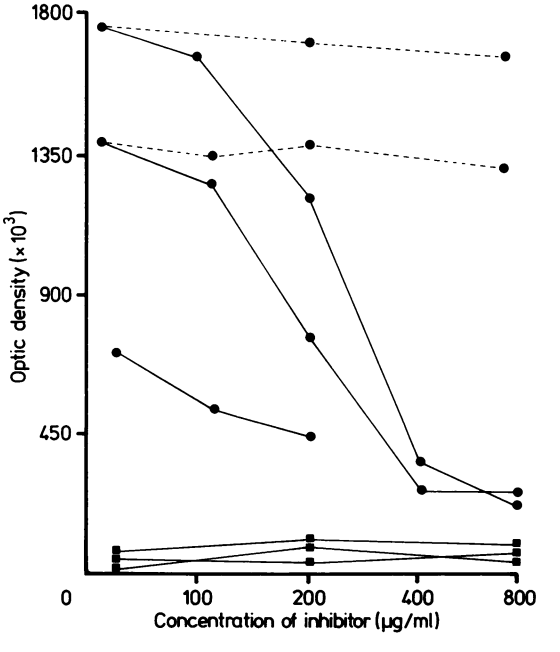

Fig. 3 *Inhibition of IgM-antivimentin antibody by cardiolipin (CL). Systemic lupus erythematosus serum inhibited by CL=●——●, or by bovine serum albumin (BSA)=●– –●. Syphilis serum inhibited by CL or BSA=■——■.*

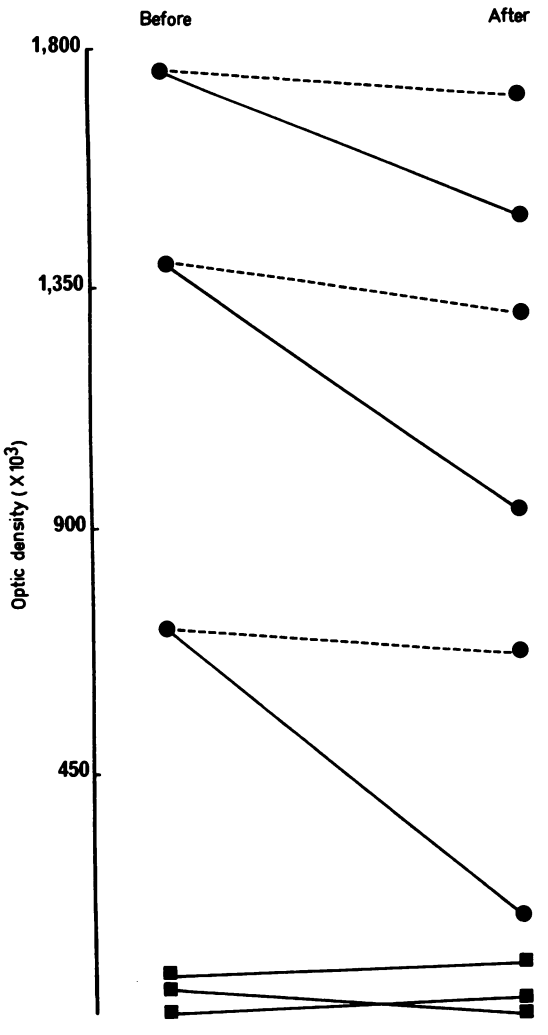

Fig. 4 *Absorption of IgM-antivimentin antibody with single stranded DNA (ssDNA). Systemic lupus erythematosus serum absorbed with ssDNA=●——●, or by bovine serum albumin (BSA)=●– –●. Syphilis serum absorbed with ssDNA or BSA=■——■.*

syphilis were examined for the presence of IgM-AVA and IgG-AVA. Three (15%) and two (10%), respectively, were found to be positive.

Inhibition experiments with cardiolipin liposomes were carried out for three SLE and three syphilis sera (Fig. 3), and the same sera were extensively absorbed with ssDNA (Fig. 4). The binding was readily inhibited by cardiolipin liposomes, and to a lesser degree absorbed with ssDNA, whereas BSA and human CFII were inefficient.

Table 4 and Fig. 5 show the results of the stepwise principal component analysis. It can be seen that axis 1 (IgM-AVA) accounts for 48·6% and axis 2 (IgG-AVA) for 22·6% of the total variance (total variance=the variance of five variables multiplied by 50 patients). Thus axes 1 and 2 appear to be the most important axes as together they account for 71·2% of the total variance. This is correct when considering the patients group as a whole, but is incorrect when considering any given individual.

Careful review of the charts of these 50 patients with SLE (Table 5) showed that IgG-AVA were significantly associated with arthralgia in SLE.

Discussion

In the present study serum samples from patients with SLE were shown to react with cytoskeletal proteins. There is a six year difference in the average age of the patients with SLE and the normal controls, and the range is much wider in the former than in the latter group. There are no data on the variability of these autoantibodies with age, however. Despite the close association in cells between vimentin and tubulin we can exclude the latter protein. Indeed, the pattern of indirect immunofluorescence assay obtained in cells treated with colchicine, an inhibitor of microtubule polymerisation, indicates that the fibrous pattern is due to vimentin,[21] and the molecular weight of the protein used to coat the wells in the ELISA test (Boehme and Blaschek, manuscript in preparation) has been

Table 5 *Clinical features associated with IgM and IgG antivimentin antibodies (AVA). The number of patients suffering from a given clinical symptom is in parentheses on the left. Of these, some have IgM-AVA or IgG-AVA, or both*

Clinical features	*IgM-AVA*				*IgG-AVA*			
	Positive	*Negative*	χ^2†	*p Value*	*Positive*	*Negative*	χ^2	*p Value*
Arthralgia (n=40)	16	24	0·66	0·42	30	10	8·25	0·01
Renal disease (n=22)	6	16	1·41	0·24	15	7	0·35	0·55
CNS* disease (n=9)	5	4	0·93	0·34	7	2	0·32	0·57
Rash (n=44)	17	27	0·66	0·42	30	14	1·38	0·24

*CNS=central nervous system.
†χ^2 with Yates's correction for small numbers.

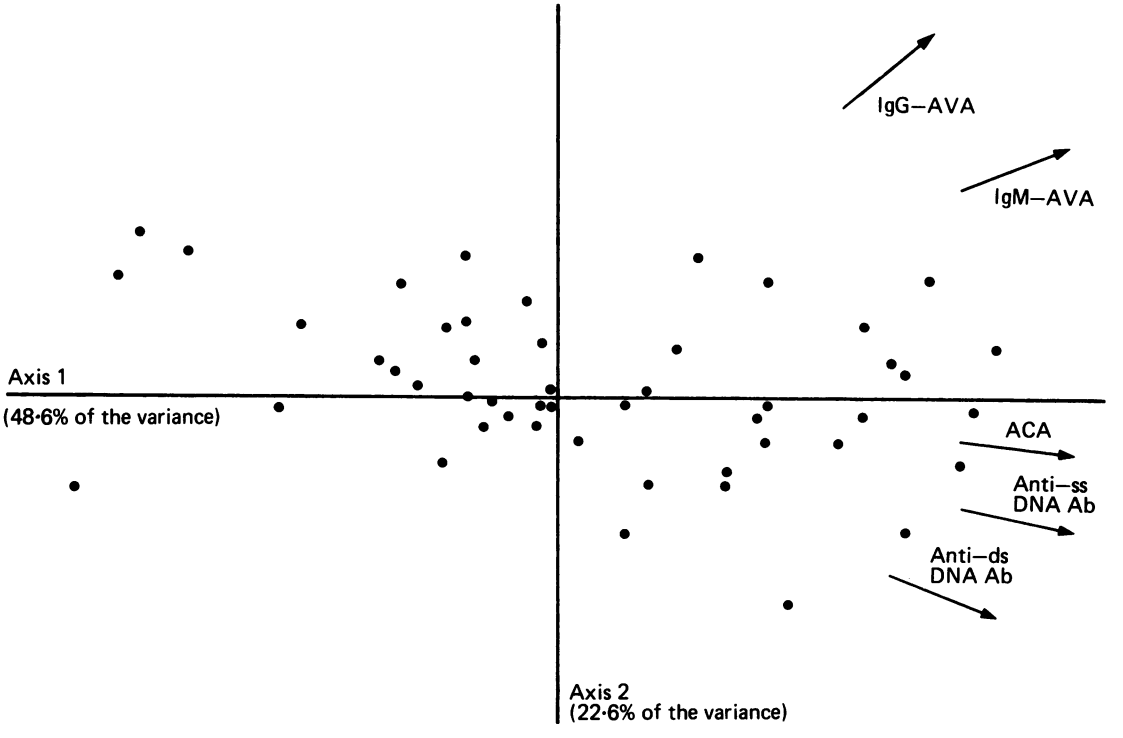

Fig. 5 *Stepwise principal component analysis. Distribution of 50 patients with systemic lupus erythematosus according to axis 1 (IgM-AVA) and axis 2 (IgG-AVA). This combination of axes accounts for 48·6+22·6=71·2% of the total variance (see text).*

shown to be 54 000 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.[22] Detection of AVA with class specific antisera to human IgG or IgM suggests that AVA are composed of both classes of immunoglobulins in the sera from patients with SLE. An effect of detergent in PBS-Tween on the cardiolipin liposome is possible, but as a difference was shown between SLE and syphilis, this is probably not significant.

Osung *et al* found that the prevalence of AVA was 10% in sera from patients with SLE,[23] whereas Alcover *et al*,[5] Kurki *et al*,[6] and Senecal *et al*[7] reported that 38–53% of SLE sera contained AVA. Our immunofluorescence results agree with these findings. In an attempt to resolve the discrepancies between reports we performed further experiments with an ELISA, a technique claimed to be more sensitive than indirect immunfluorescence assay. The prevalence of IgM-AVA was 36%, and that of IgG-AVA 64%, in SLE sera.

Statistical analysis of the serological results from 50 patients with SLE showed correlations between AVA and other non-organ-specific autoantibodies. We showed a significant association between AVA, ACA, and anti-DNA antibodies, especially between IgM-AVA, on the one hand, and ACA and anti-ssDNA antibodies, on the other. Further, the stepwise principal component analysis showed that IgM-AVA and IgG-AVA accounted for 71% of the total variance (variance of 50 patients × 5 parameters). Although the statistical correlations do not prove that a given antibody may necessarily bind to two different antigens, they make it more likely. Prompted by this observation, we sought AVA in 20 ACA positive sera from patients with syphilis, but none of them reacted with cytoskeletal proteins. IgM-AVA from patients with SLE were inhibited by cardiolipin and absorbed with ssDNA.

These results are not surprising, given the previously described cross reactions,[24–26] Murine[27] and human[28] monoclonal anti-DNA antibodies can bind to cardiolipin, and human monoclonal anti-DNA antibodies from patients with SLE[29] and from normals[30] can bind to cytoskeleton proteins. Any relation between ACA and AVA has never been unequivocally shown. Several indirect arguments

may be put forward. For example, patients with Behçet's disease have been shown to be capable of making antibodies to cardiolipin[31] and antibodies to the cytoskeleton.[32] ACA[31] and AVA[5-7 23] exist in normal sera. We showed hardly any AVA positive serum in syphilis despite the presence of ACA in this disease. This may be due to differences in epitope specificity. ACA from patients with SLE are more likely to bind to phosphatidylserine than to phosphatidylethanolamine, and vice versa with syphilis serum.[33] The binding of syphilitic antibodies to cardiolipin was readily inhibited by cardiolipin liposomes, whereas this pattern of reactivity was noted in only one of four sera from patients with acute infection by Vaarala *et al.*[34]

The successful inhibition of AVA from SLE with cardiolipin may mean that the epitope shared by vimentin and phospholipids is related to phosphatidylserine.[27] Some of the ACA, however, may react with the cytoskeletal epitopes that are shared with vimentin.[35]

Analysis of the clinical details showed an association between AVA positivity and arthralgia. One explanation of this finding is that proliferating synovial lining cells contain vimentin as a prominent constituent.[36] Thus a vicious circle may be set off once joints are involved.

The cross reaction between vimentin, cardiolipin, and DNA raises the important question of polyreactive autoantibodies. The likelihood that DNA is the immunogen in SLE is extremely questionable.[24-26 37]

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