

EXTENDED REPORT

High titres of serum antinuclear antibodies, mostly directed against nucleolar antigens, are associated with the presence of coronary atherosclerosis

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Background: Inappropriate inflammation is a key mechanism in the development of atherosclerosis. Antibodies against components of the atherosclerotic lesion, in particular, oxidised low density lipoprotein, have been described.

Objective: To determine whether a systemic autoimmune response, characterised by the presence of high titres of antinuclear antibodies, is associated with the presence of coronary atherosclerosis.

Methods: Serum was prepared from 40 subjects (aged 53–76) with at least 50% stenoses of three main coronary arteries (TVD subjects), and 30 subjects (aged 48–74) with no evidence of coronary atherosclerosis (NCA subjects) determined by coronary angiography.

Results: Antinuclear antibodies (ANA), characterised by immunofluorescent detection of human antibodies bound to HEp-2000 cells, were detected at a titre of at least 1/40 in 28 (70%) of the TVD subjects, but only five (17%) of the NCA patients (odds ratio 11.67 (95% confidence interval (CI) 3.91 to 17.82; $p < 0.001$)). Most ANA positive TVD subjects had a pattern typical of antibodies directed against nucleolar antigens. The antigen has not yet been identified, but several common extractable antigens were excluded. The presence of ANA was not associated with incidence of prior myocardial infarction among the TVD group.

Conclusion: The presence of ANA, commonly associated with autoimmune diseases, is substantially more prevalent among subjects with severe coronary atherosclerosis than those with normal coronary arteries. This association merits further assessment as a potentially useful indicator of increased risk of coronary heart disease.

The importance of inflammation in the pathogenesis of atherosclerosis has recently been highlighted by studies of mouse models, where genetic deletion of factors necessary for vascular inflammation markedly reduces vascular lipid lesion development. For example, deletion of the gene encoding either monocyte chemoattractant protein-1 or its receptor (CCR2) reduces the development of vascular lipid lesions in apolipoprotein E null mice.^{1,2} A role for vascular inflammation in the development of atherosclerosis in man is harder to establish formally, but a number of inflammatory markers have been reported to be increased in subjects with proven coronary artery disease. For example, raised levels of C reactive protein have been associated with the presence of atherosclerosis in cross sectional studies.^{3,4}

Inappropriate inflammatory responses may be associated with both increased chronic development of atherosclerotic plaques and with increased plaque rupture related to acute myocardial infarction. Secretion of proteases by macrophages recruited to the plaque may have an important role in determining the likelihood of plaque rupture and its clinical sequelae.^{5,6} During chronic plaque development, however, humoral immunity may also play an important part.^{7–13} For example, in the 1970s endothelial damage through the formation of autoimmune complexes was first suggested to be an important step in the development of atherosclerosis.^{14,15} More recently, evidence has been gathered for a causative role of immunoglobulins in chronic vascular lipid lesion development in mice: apoE knockout mice given immunoglobulin treatment develop less severe vascular lipid lesions.^{16–19}

In man, however, evidence is limited to reports of increased levels of specific autoantibodies in subjects with atherosclerosis. These include cytoskeletal proteins normally found in the vessel wall,²⁰ cardiolipin^{21,22} and, in particular, oxidised, or

otherwise modified, low density lipoprotein (LDL).^{23–25} An autoimmune response to modified LDL is one of the mechanisms by which LDL oxidation has been proposed to promote atherogenesis in man.²⁶

Despite these reports indicating an association between autoimmunity and atherosclerosis, there are no definitive reports of the prevalence of a systemic autoimmune reaction, characterised by the presence of high titres of antinuclear antibodies (ANA) in patients with advanced atherosclerosis. We therefore analysed serum from 40 patients with angiographically defined coronary artery disease resulting in stenosis in three major coronary arteries (TVD patients) and compared this with serum from 30 patients with no evidence of coronary artery disease on angiography (NCA patients) for the presence of ANA. None of the subjects studied, or their first degree relatives, had been diagnosed with an autoimmune disorder. The ANA detected were then further characterised for titre, antibody isotype, and antigen specificity.

PATIENTS AND METHODS

Patients

Patients were recruited to the TVD group who had significant coronary artery disease (defined as a reduction of more than 50% in the intraluminal diameter) of all three coronary arteries (left anterior descending, circumflex, and right coronary

Abbreviations: ENA, extractable nuclear antigens; LDL, low density lipoprotein; NCA subjects, subjects with no evidence of coronary atherosclerosis; PBS, phosphate buffered saline; TVD subjects, subjects with at least 50% stenoses of three main coronary arteries

Table 1 Characteristics of the study groups. Values are reported as mean (SEM) except for triglyceride (median and interquartile range).

	NCA	TVD	p Value
Number	30	40	
Age	57 (1.6)	63 (1.2)	0.003
Sex (M/F)	9/21	37/3	<0.0001
Total cholesterol (mmol/l)	5.95 (0.20)	6.17 (0.14)	0.35
LDL-cholesterol (mmol/l)	4.24 (0.20)	4.49 (0.13)	0.28
HDL-cholesterol (mmol/l)	1.08 (0.05)	0.78 (0.03)	<0.0001
Triglyceride (mmol/l)	1.2 (0.9–1.7)	1.8 (1.4–2.7)	0.001
Systolic BP (mm Hg)	141 (4)	138 (3)	0.44
Diastolic BP (mm Hg)	78 (2)	76 (2)	0.39
Current smokers (No (%))	2 (7)	2 (5)	0.99
Ex-smokers (No (%))	10 (33)	21 (53)	0.003
Previous MI (No (%))	0 (0)	22 (55)	<0.0001

The groups were compared using Student's *t* test for continuous variables (except for triglyceride where the Mann Whitney U test was applied) and χ^2 test for categorical variables.

arteries). The symptoms of angina had been stable for at least one month and no patient had had a myocardial infarction in the preceding three months. Patients were recruited to the NCA group who had chest pain and a positive exercise electrocardiogram (the Bruce protocol was used, where the presence of at least 1 mm of horizontal or downward sloping ST segment depression at 80 ms after the J point is considered positive) but normal coronary angiograms (judged by two independent observers). NCA patients with hypertension, diabetes mellitus, valvular heart disease, or left ventricular hypertrophy were excluded. Consecutive patients presenting at Papworth Hospital who met the above criteria for either the TVD or NCA group were recruited to the study. None of the subjects studied, or their first degree relatives, had been diagnosed with an autoimmune disease. Table 1 shows the clinical data for the patient groups.

Blood was drawn from each patient, allowed to clot in plastic tubes for two hours at room temperature, and the serum was collected by centrifugation. Aliquots of serum were stored at -80°C until assayed.

Detection of antinuclear antibodies

The presence of antinuclear antibodies was determined by the HEp-2000 indirect fluorescent antibody test system (ImmunoConcepts) in accordance with the manufacturer's instructions. Briefly, HEp-2000 cells were exposed to patient sera, diluted in phosphate buffered saline (PBS) for 30 minutes, and unbound antibody was washed away in PBS for 10 minutes. Bound antibody was detected using FITC labelled antihuman IgG antibody provided with the kit. Specific detection of IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, and IgM was achieved using highly specific monoclonal antibodies to each subclass of human immunoglobulin (Skybio Ltd) followed by FITC labelled antimouse IgG. Each HEp-2000 slide was scored for the presence of ANA and for the pattern of ANA binding by two independent observers unaware of the clinical details of the patients.

The presence of antibodies to extractable nuclear antigens (ENA) was determined with a commercially available enzyme linked immunosorbent assay (ELISA) (ENA Screen; Sigma Diagnostics) in accordance with the manufacturer's instructions.

Statistical analysis

The statistical significance of differences between the TVD and NCA groups was assessed with the χ^2 test (for categorical variables with more than two levels), Fisher's exact test (for categorical variables with two levels), Mann-Whitney U test (for continuous variables that were non-normally distributed), or Student's unpaired *t* test (for normally distributed continuous variables). In all cases, $p < 0.05$ was taken to indicate significance.

RESULTS

Serum was prepared from 30 NCA subjects and 40 TVD subjects. The TVD subjects were slightly, but significantly, older than the NCA subjects, and there were significantly more men among the TVD group (table 1).

The presence of ANA was determined at a titre of 1/40 for all 70 subjects in the study, using HEp-2000 cells (ImmunoConcepts Inc) as the substrate.²⁷ This methodology has been widely adopted for screening for ANA in subjects with autoimmune diseases.^{27–30} ANA positivity was scored and graded by two separate observers unaware of the artery status of the subjects. The two observers agreed in 63/70 cases, and the remaining seven discrepancies were resolved by discussion before unblinding the samples. Among the NCA group, 5/30 (17%) were ANA positive. This is consistent with the range of values previously reported for subjects in this age range (50–70 years), although it lies in the upper range of earlier values, reflecting the high sensitivity of the immunofluorescent detection system we routinely use.^{31 32}

In marked contrast, among the TVD subjects 28/40 (70%) were ANA positive ($p < 0.001$; Fisher's exact test). The higher incidence of ANA positivity among the TVD group was unlikely to be due the influence of age, because there was no difference in the incidence of ANA positivity across tertiles of age in either the NCA group ($p = 0.48$) or TVD group ($p = 0.91$; χ^2 test). Similarly, the incidence of ANA positivity was similar for men and women in the NCA group ($p = 0.90$) and TVD group ($p = 0.53$). We conclude that there is a higher incidence of ANA positivity among subjects with angiographically proven coronary artery disease than among those with angiographically normal coronary arteries, with an odds ratio of 11.67 (95% CI 3.91 to 17.82; $p < 0.001$).

An estimate of the titre for the ANA was made for each subject scored as ANA positive by a semiquantitative method, in which serial twofold dilutions of serum were exposed to HEp-2000 cells and the lowest dilution at which both observers scored the slide as ANA positive was recorded as the titre. The median titre was 1/160 among the ANA positive subjects in the NCA group, compared with 1/320 for the TVD group (table 2). However, this difference was not significant ($p = 0.52$; χ^2 test), possibly owing to the small number of subjects who were ANA positive in the NCA group. Nevertheless,

Table 2 Semiquantitative titres of ANA antibodies among NCA and TVD subjects.

Group	Titre							Total
	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	
NCA	0	2	1	0	1	1	0	5
TVD	1	4	6	7	8	1	1	28

The titre of ANA antibodies for each individual scored as ANA positive was determined by exposing twofold dilutions of serum to HEp-2000 cells, recording the lowest dilution at which both observers scored the slide as ANA positive as the titre. The number of subjects from each group for each titre is shown. The distribution of titres between the two groups is not significantly different ($p = 0.52$; χ^2 test).

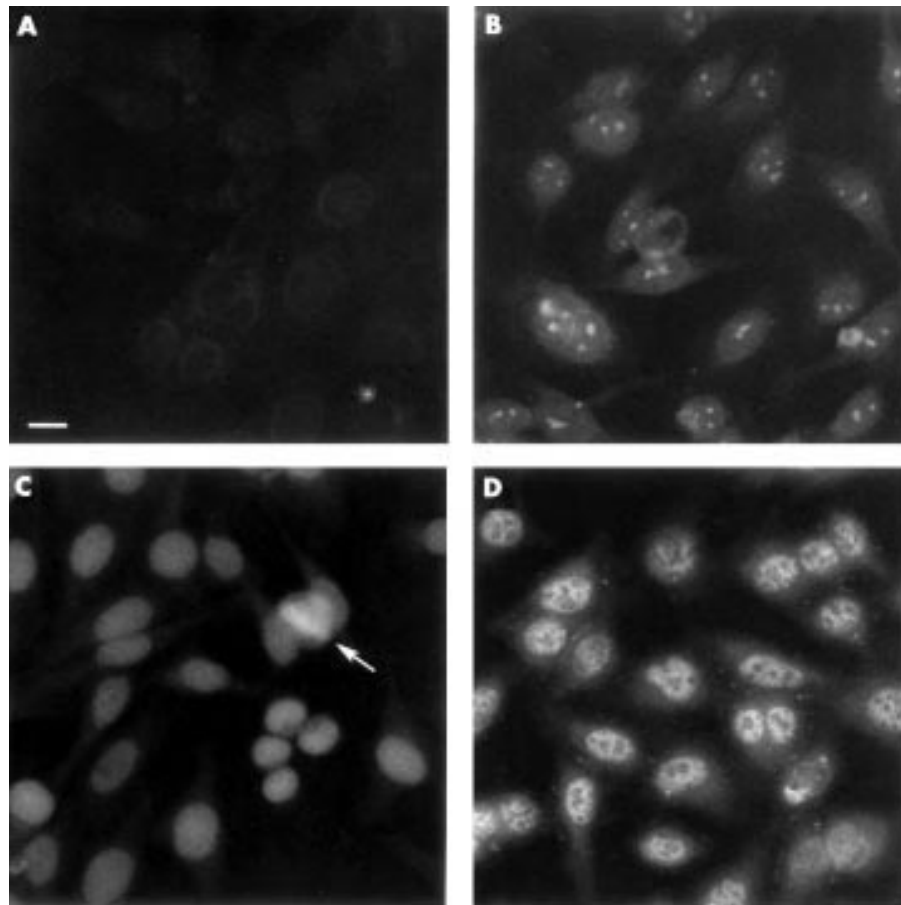


Figure 1 Immunofluorescent detection of ANA binding to HEp-2000 cells. In each case the serum was from a subject in the TVD group and was assayed at a dilution of 1/40 in accordance with the manufacturer's instructions. Bar=5 μ m. (A) Cells stained with serum containing no detectable ANA. (B) Cells stained with serum containing ANA that give an atypical speckled pattern, characteristic of detection of nucleolar antigens. (C) Cells stained with serum containing ANA that react with DNA (later confirmed using *Crithidia luciliae*). Intense staining of the metaphase chromosomes is indicated by the arrow. (D) Cells stained with serum containing ANA that yield a typical speckled pattern, possibly owing to reaction with nuclear matrix components.

the ANA titres among the subjects in the TVD group were similar to those reported previously for subjects with classical autoimmune conditions.²⁸

Most ANA positive subjects (3/5 in the NCA group and 17/28 in the TVD group) had a speckled pattern, typical of nucleolar staining (fig 1B). Only one subject (in the TVD group) had a homogeneous staining pattern (fig 1C), with strong staining of the metaphase chromosomes, suggesting the presence of anti-DNA antibodies. This was confirmed using *Crithidia luciliae* in place of HEp-2000 as the substrate. Several subjects exhibited a speckled pattern that was not localised to the nucleoli (fig 1D), possibly as a result of ANA reactivity against the nuclear matrix.

Four subjects (one in the NCA group and three in the TVD group) were positive for anticentrosome antibodies, but this incidence of anticentrosome antibodies was too low to determine whether there is likely to be a difference between the two groups. Similarly, 9/40 (23%) of the TVD subjects and 3/30 (10%) of the NCA subjects were positive for anticytoplasmic antibodies, which strongly stained cytoskeletal elements. This is consistent with a previous report of a higher incidence of autoantibodies against cytoskeletal proteins in subjects with vascular disease,²⁰ although in our study the difference did not reach significance ($p=0.16$; Fisher's exact test).

Next, we performed an ENA screen for antibodies against extractable nuclear antigens. When a mixture of Smith, RNP, SS-A(Ro), and SS-B(La) antigens was used only one subject (in the TVD group) was ENA positive, suggesting that none of these four common nuclear autoantigens was responsible for

the majority of ANA positive reactions among subjects with atherosclerosis, consistent with our observation that most ANA positive subjects among the TVD group had autoantibodies directed against the nucleolus (the majority of nucleolar autoantigens are not extractable).

The isotype of the ANA response was also investigated in the five ANA positive subjects in the NCA group and five ANA positive subjects selected at random from the TVD group. ANA of each of the isotypes were detected in serum from at least one subject, with a substantially more diverse range of ANA isotypes in the TVD group. Of the five subjects in the NCA group, 4/5 had ANA of only the IgG1 and/or IgG2 isotypes, while the fifth subject had IgG1, IgG2, and IgD class ANA antibodies. In contrast, one subject in the TVD group had strong ANA staining for IgG1, IgG2, IgG3, and IgD with weaker, but detectable staining for IgM, IgA, IgG4, and IgE. The remaining four subjects all had IgG1, IgG2, and IgD class ANA, and three of the four additionally had IgM, IgG3, and IgA class ANA. On average, the ANA positive subjects in the NCA group had 1.8 different classes of ANA, compared with 5.8 different classes of ANA in the TVD group ($p<0.01$; Mann-Whitney U test).

Finally, we investigated whether ANA positivity was associated with the occurrence of myocardial infarction before the serum sample was prepared. Because plaque rupture involves cell death, it may result in exposure of the immune system to intracellular autoantigens which are normally cryptic. Among

the TVD group, just over half the subjects had had a myocardial infarction before serum preparation (22/40). The incidence of ANA positivity was no higher among this subgroup than among those who had not had a documented myocardial infarction (16/22 (73%) ANA positive with previous myocardial infarction v 12/18 (67%) without prior myocardial infarction; $p=0.74$ Fisher's exact test). A documented prior myocardial infarction is not associated with increased incidence of ANA positivity, although it remains possible that subclinical infarctions occurring in many of the TVD subjects were responsible for ANA seroconversion.

DISCUSSION

We conclude that the incidence of ANA positivity is higher among subjects with severe coronary atherosclerosis, defined by angiography, than in subjects with angiographically normal coronary arteries. The incidence of ANA positivity in our NCA control group was similar to that reported for unselected groups of similar ages.³¹ This suggests that ANA positivity is associated with the presence of coronary atherosclerosis. The odds ratio for being in the TVD group when found to be ANA positive was 11.67 (95% CI 3.91 to 17.82). Despite the magnitude of this association and the robust confidence intervals, it is important to note that while the use of a cohort of consecutive patients such as ours minimises the risk of selection bias it does not allow adequate matching of age and sex between the two groups. Although there is no evidence to suggest that the prevalence of ANA positivity was associated with age or sex in our group, nevertheless our findings should be used to provide the impetus for further investigation of the role of humoral autoimmunity in the development of atherosclerosis using a much larger cohort.

This association may result from either a correlative association or a causative association in either direction. For example, because a proinflammatory phenotype has been repeatedly implicated in the development of vascular diseases it is possible that some misregulation of inflammatory responses independently leads to increased development of atherosclerosis and to increased incidence of ANA positivity. Alternatively, the presence of existing coronary artery disease may lead to ANA seroconversion. Although we have not shown any association between the presence of myocardial infarction and ANA positivity, subclinical infarctions among the TVD group may lead to the increased incidence of ANA positivity. For example, recent studies of serum troponin levels suggest that subjects with coronary atherosclerosis undergo numerous small infarctions associated with myocardial ischaemia.³³⁻³⁵ Repeated myocardial necrosis, even on a small scale, may be sufficient to lead to leakage of intracellular antigens that can induce ANA production.

Alternatively, the presence of ANA in the circulation may contribute to the pathogenesis of atherosclerosis. Although there is no direct evidence for this, at least one observation suggests that it merits further investigation: vasectomy can lead to rapid seroconversion to ANA positivity, presumably because of the resorption of sperm (rich in nuclear antigens) which then occurs in the epididymis.³⁶ As a result, vasectomy has been described as an experimental autoimmune disorder in humans.³⁷ Two well conducted studies then went on to show increased development of atherosclerosis in monkeys after vasectomy, both in those receiving a cholesterol-rich Western diet³⁸ and also a cholesterol-free diet when lesions took 9–14 years to develop³⁹; a more recent study failed to reproduce these findings.⁴⁰ The presence of ANA may potentiate the development of atherosclerosis by assisting the local inflammatory response at sites of lipid deposition into the vascular wall where cellular necrosis is known to occur.

Irrespective of the mechanisms which underlie the association, the strength of the association between ANA positivity (in particular, antibodies directed against nucleolar antigens)

and angiographically defined coronary atherosclerosis in the groups we have studied suggests that the ANA titre may be useful as an additional non-invasive diagnostic tool for identifying subjects at risk of coronary artery disease in the whole population. Adopting a titre of 1/320 as the cut off for ANA positivity in our study groups would have identified two false positives (7%), while correctly identifying 17/40 subjects in the TVD group (43%). Although this represents poor performance for a diagnostic kit in many clinical arenas, it must be considered in the context of existing serum markers for atherosclerosis (such as LDL-cholesterol, fibrinogen, or PAI-1) which, despite being strongly associated with coronary heart disease in cross sectional epidemiological studies, have little practical application in diagnosing the presence of atherosclerosis in subjects.⁴¹ If our results are replicated in larger cohorts then ANA analysis may be a useful addition to the battery of tests presently used to diagnose the presence of coronary artery disease.

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