Measurement of Autoimmune Response against Collagen Types I, III, and IV by Enzyme-Linked Immunosorbent Assay, and Its Application in Infective Endocarditis

Marc L. De Buyzere,1 Ian K. De Scheerder,1 Joris R. Delanghe,2 Johan H. Robbrecht,2 Denis L. Clement,1 and Roger J. Wieme2

In these enzyme-linked immunosorbent assays for determination of autoantibodies (IgG, IgM) against collagen type I (ACA I), III (ACA III), and IV (ACA IV), we use commercially available antigen preparations. Inhibition curves showed limited cross-reactivity between these different collagen preparations, the major interference being observed after addition of collagen type III in the ACA I procedures. Imprecision (CVs) for high- and low-titer samples ranged between 1.5% and 7.9% (within-run) and 5.5% and 12.7% (between-run) for ACA I, between 2.2–8.7% and 4.5–10.7% for ACA III, and between 1.3–5.9% and 7.4–11.6% for ACA IV. Significantly increased humoral immune response against collagen types I and III (P < 0.001) could be demonstrated during the first month of infective endocarditis. In contrast, only borderline increases, however constant, of autoimmune response against basement-membrane collagen (ACA IV) were noticed during 90 days of follow-up.

Additional Keyphrases: autoantibodies • autoimmune disease • heart disease • valve-replacement surgery

Autoantibodies to native or denatured human collagens (ACA) have been described earlier in several diseases in which autoimmune features were incriminated in their pathogenesis—e.g., rheumatoid and psoriatic arthritis (1), Buerger's disease (2), otosclerosis (3), and scleroderma (4). Humoral immune response to collagen types I and III (mainly interstitial), type II (cartilage), and type IV (mainly basement membrane) has been determined previously by ELISA (5) or RIA (6) techniques in which highly purified antigen preparations were used (7). Etiological or secondary involvement of ACA in endothelial and basement-membrane injury has been studied in several models, including arthritis (8) and poststreptococcal glomerulonephritis (9). However, humoral antihyaluronate immune response has not been studied hitherto in heart diseases.

Notwithstanding substantial improvements in echocardiography during the last decade, infective endocarditis (IE) remains a cumbersome diagnosis. Humoral immune response in IE has been evaluated by an indirect immunofluorescence technique. A good relationship between the prevalence of anti-heart antibodies and cardiac function could be demonstrated, suggesting that anti-heart antibodies reflect myocardial damage involved in IE (10). Two distinct types of immunofluorescence could be differentiated by indirect immunofluorescence: predominantly sarcolemmal or predominantly subsarcolemmal. Maisch et al. (11) found anti-endo-
cardial and anti-sarcolemmal antibodies in 60–100% of their patients with subacute or chronic endocarditis. However, autoantibodies were nearly absent in the acute phase of the disease. These authors suggested that antibody-mediated cytotoxicity plays a role in the pathogenesis of endocarditis.

Here we describe new ELISA techniques for determining ACA types I, III, and IV in microwells coated with commercial antigens, and our studies on cross-reactivity of the different antigens in the assays also are described. Autoantibodies against interstitial and basement-membrane collagen have been evaluated during a three-month follow-up of IE and during the same period after valve-replacement surgery for valvular heart disease.

Materials and Methods

Patients

Serum specimens were obtained from 13 consecutive patients (eight men, five women; 21–69 y, mean age 46 y) with diagnosis of infective endocarditis who survived at least three months after admission, and who did not undergo cardiac-surgery procedures. Final diagnostic criteria for infective endocarditis were heart murmurs, echocardiographically proven valve vegetations, inflammatory biology, and positive blood cultures.

Antibodies against collagen types I, III, and IV were determined in serum samples collected daily during the first week after admission and serially further on days 10, 15, 20, 30, 40, 45, 50, 60, 70, and 80, and finally on day 90. The same blood-sampling schedule was also used in a series of 35 consecutive cases (19 men, 16 women; 31–72 y, mean age 54 y) of valve-replacement surgery starting the day before surgery. Control serum samples were obtained from 180 apparently healthy volunteers (20–30 y, n = 90; 31–50 y, n = 50; 51–75 y, n = 40). All of them showed no abnormalities on clinical examination and electrocardiogram, and results of a cycloergometric test were within normal limits.

Serum samples were centrifuged (3000 x g, 15 min, 4 °C) immediately after collection, and aliquots were stored frozen for no longer than 30 days before analysis.

ELISA Tests

Reagents. Collagen types I (no. C 7774), III (no. C 4407), and IV (no. C 7521) from human placenta and alkaline phosphatase-conjugated anti-human antibodies (Fab) (anti-IgG no. A 5403, anti-IgM no. A 3914) were purchased from Sigma Chemical Co., St. Louis, MO 63178. Tween 20 surfactant and diethanolamine (respectively no. 822184 and no. 803116) were from Merck-Schuchardt, Darmstadt, F.R.G.; p-nitrophenyl phosphate, disodium salt (no. 3551) from J. T. Baker, Deventer, The Netherlands; and gelatin powder (no. 44045) from BDH Chemicals, Poole, U.K. All other reagents were of analytical grade, from Merck, Darmstadt, F.R.G.

Antigen-coated microtiter plates. Precleaned (with 3.5 mol/L HCl) polystyrene microtiter plates (no. 4.39454;
NUNC, Roskilde, Denmark) were washed with Na₂CO₃/NaHCO₃ buffer (30 mmol/L, pH 9.5) and phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.0, NaCl 150 mmol/L) and further incubated for 2 h with a 20 mL/L solution of glutaraldehyde. Each well was coated with collagen types I, III, or IV by incubating 200 μL of a solution of 100 ng of antigen per liter of PBS, for 12 h at 4 °C. The plates were then washed twice with PBS, and incubated with PBS containing 5 g of gelatin per liter for 12 h at 4 °C. After three final washing procedures with PBS containing 0.5 mL of Tween 20 per liter (PBS-Tween) the microtiter plates were sealed and stored at 4 °C.

**Assay procedure.** Before assay, serum samples were diluted 200-fold with PBS and centrifuged (10 min, 12000 × g). We incubated 100-μL aliquots of supernate for 12 h at 4 °C and then washed the wells three times with PBS containing 2.5 g of gelatin and 0.5 mL of Tween 20 per liter (PBS-gelatin-Tween), and then added 100-μL aliquots of alkaline phosphatase-conjugated anti-human IgG and IgM antisera (1000-fold dilutions in PBS). After another 12-h incubation at 4 °C the washing procedure included three cycles with PBS-gelatin-Tween and one with PBS-Tween. Alkaline phosphatase enzyme activity was measured spectrophotometrically at 405 nm (Model EL 309; Bio-Tek Instruments, Burlington, VT) after addition of 100 μL of a solution of p-nitrophenyl phosphate (disodium salt, 56 mmol/L) in a 100 mL/L solution of diethanolamine (pH 9.2) containing 1 g of MgCl₂ and 200 mg of NaH₂PO₄ per liter. The enzymatic reaction (60 min, 37 °C) was stopped by adding 100 μL of a 1.0 mol/L solution of NaOH. Each determination of ACA I, III, and IV in serum was performed in triplicate.

**Standard and inhibition Curves**

**Comparison methods.** To discuss results of the described ELISA assays, we compared net absorbances from the spectrophotometric determination of alkaline phosphatase activity with those from control samples. Mackel et al. (4) determined ACA types I, II, and IV in patients suffering from scleroderma, and calculated an ELISA ratio: mean net absorbance of the unknown sample/net absorbance of control samples from apparently healthy persons. In our study, aliquots of a pooled specimen of serum from 180 healthy volunteers were incubated in each microtiter plate used. Results of ACA I, III, and IV determinations in our patients are expressed as 100 × (net absorbance sample/net absorbance pool serum).

**Standard and competitive inhibition binding curves.** Specificity of the ELISA assay depends in part on the purity of the antigen preparation that is coated onto the solid phase, and it can be controlled by comparing titration curves for high-titer ACA samples obtained after incubation with the competitive collagen preparations. Nonspecific binding can be evaluated by testing dilutions of high-titer ACA samples after absorption with the corresponding collagen type. Serial dilutions (1:10, 1:20, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400) of a high-titer serum sample of respectively ACA I, III, and IV (IgG, IgM) in PBS (1.0 mol/L, pH 7.0) were applied to microtiter wells coated with antigen, 200 ng/L and the absorbance was determined after the assay procedure after a 15-min substrate incubation (standard dilution curves). Antigen specificity was evaluated by absorption of 100-μL aliquots of collagen antigen solutions (200 ng/L in PBS)—types I, III, and IV, respectively—with 100-μL aliquots of serial dilutions in PBS (same dilution factors as for the dilution curves) of high-titer ACA I, III, or IV samples (competitive inhibition binding curves). After incubation for 4 h at 37 °C, and centrifugation at 10 000 × g for 30 min, the supernatant fluid was removed and the dilution series was assayed for ACA I, III, and IV. High-titer ACA serum samples were obtained from patients with IE (ACA I and III) and from patients with primary heart disease (ACA IV).

**Statistical Analysis**

Using the Mann–Whitney U-test, we tested the statistical probability that the prevalence of autoantibodies against collagen type I, III, and IV in the patient groups was comparable with that in an age-matched control group.

**Results**

**Antigen coating concentration.** To evaluate appropriate antigen coating concentrations and serum dilution factors for the assays of ACA type I, III, and IV, we incubated high-titer control sera with coating concentrations of 2, 5, 10, 25, 50, 100, 250, 500, and 1000 ng of antigen per liter and serum dilutions of 10-, 100-, and 1000-fold in PBS. Net absorbances obtained after a substrate reaction time of 20 min are summarized in Figure 1. Whenever the antigen concentration exceeded 25 ng/L, ELISA test results were nearly independent of antigen coating concentrations. Use of higher serum dilutions (1:100, 1:1000) induced an even smaller dependency on antigen coating concentration. During the IE study, an antigen coating concentration of 100 ng/L was
was used for all further ACA I, III, and IV determinations, with serum diluted 200-fold.

**Precision.** Within-assay precision (as reflected by the CV) was determined from data on 60 low- and high-titer samples (ACA type I, III, and IV; IgG, IgM). Between-assay precision data were obtained from results for sixfold simultaneous application of high-titer samples on 10 different microtiter plates. The day-to-day CV was calculated from data on sixfold application of these samples on 10 consecutive days. Within-assay CVs ranged widely, from 1.3% (ACA IV, IgG, high) to 8.7% (ACA III, IgG, low), the low-titer samples always having higher CVs (Table 1). Between-assay CVs varied from 4.5% (ACA III, IgG) to 8.3% (ACA IV, IgM). The day-to-day CV was rather high (8.3–12.7%) in all cases.

**Standard and competitive inhibition binding curves.** Standard and inhibition curves of ACA types I, III, and IV high-titer samples (IgG, IgM) (Figure 2) are sigmoidal, with a quasi-linear range between dilutions of 50- and 800-fold. For serum samples we used a 200-fold dilution throughout the rest of the study. CVs for each point of the curves were within the within-assay precision limits.

Inhibition curves for ACA I (IgG, IgM), obtained after addition of appropriate competitive amounts of collagen type III and IV, are also shown in Figure 2. Similar experiments were performed for the inhibition curves of ACA type III and IV. Cross-reactions were observed to a limited extent in all assays, except for collagen type III in the ACA I procedures.

**Linearity.** Table 2 summarizes the linearity of response for ACA determinations in human sera samples for each method. Quantitative results of these determinations were expressed according to Mackel et al. (4). Nearly all (98%) values for serial ACA analysis of IE patients were within these limits; otherwise, sera were diluted twofold in PBS.

**Infective endocarditis.** Figure 3 compares the evolution of ACA type I, III, and IV (IgG, IgM) during a follow-up period of 90 days in the two model groups of endocardial and valvular injury (IE, valve surgery for settled valvular disease). In the control group of valve-surgery patients, humoral immune response against basement-membrane collagen did not differ significantly from preoperative values, except for a small dip in the curve during the first postoperative week, probably ascribable to hemodilution. For the IE group there was a slightly increased but constant immune response against type IV collagen. In contrast, we saw a significantly increased immune response (P < 0.001 vs the valve group) for ACA types I and III in IE during the first weeks after admission. For IgG curves (ACA I, III) maximal values were obtained between days 5 and 20. In contrast, for IgM curves values were highest immediately after entrance to the study. Comparing ACA values on admission for the IE group with those for age-matched controls (47.8 ± 14.0 y, n = 10), we noticed significantly higher titers of autoantibodies. All IE patients showed at least one episode of above-normal titers of ACA I and III (IgM) during the course of the disease, and other ACAs also were frequently positive. By the end of the study (day 90) important patient subgroups still tested positive for autoantibodies against several types of collagen, ranging from 39% for IgG ACA III and IgM ACA IV to 100% for IgM ACA III (Table 3). In the valve-surgery group, more than 90% of the patients were negative for ACA, both before surgery and by the end of the study. Only subgroups ranging from 6 to 29% of the surgical patients developed positive ACA titers—also significantly lower than in the endocarditis group.

**Discussion**

In earlier-described ELISA and RIA procedures for determination of ACA, the workers used highly purified antigen preparations (native as well as denaturated collagens and procollagens), e.g., collagen type IV from bovine anterior lens capsule (12) or from murine EHS sarcoma (7), and collagen types I and III from lathyritic rat or fetal bovine skin (13, 14). This substantial purification step was considered necessary because antibodies against unrelated contaminants have been detected in antisera against crude (pro)collagen preparations (15).

However, we were interested in determining whether a commercially available collagen preparation could be used to set up an ELISA test for anti-collagen antibodies. Thus we coated commercial antigen preparations on microtiter plate wells. When we used an appropriate coating concentration, 10–250 ng/L, standard dilution curves were quasi linear in a large interval (serum dilutions of 20- to 1600-fold). Analogous titration curves of high-titer ACA serum samples in PBS could be obtained when collagen antigen coating concentration was kept constant (Figure 2).

**Table 1.** Precision of ELISA Assays for Serum Anti-Collagen Antibodies Type I, III, and IV

<table>
<thead>
<tr>
<th>No. measurements</th>
<th>ACA I</th>
<th></th>
<th></th>
<th></th>
<th>ACA III</th>
<th></th>
<th></th>
<th></th>
<th>ACA IV</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
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<td></td>
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<tr>
<td>Within-run</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Low IgG</td>
<td>123</td>
<td>9.7</td>
<td>7.9</td>
<td>121</td>
<td>10.5</td>
<td>8.7</td>
<td>160</td>
<td>9.5</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 High IgG</td>
<td>114</td>
<td>8.5</td>
<td>7.5</td>
<td>113</td>
<td>6.9</td>
<td>6.1</td>
<td>123</td>
<td>10.1</td>
<td>1.3</td>
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<tr>
<td></td>
<td>759</td>
<td>16.6</td>
<td>2.2</td>
<td>1072</td>
<td>23.4</td>
<td>2.2</td>
<td>835</td>
<td>15.8</td>
<td>1.9</td>
<td></td>
<td></td>
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<tr>
<td>Between-run</td>
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<td></td>
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<tr>
<td>60 High IgG</td>
<td>424</td>
<td>25.0</td>
<td>5.9</td>
<td>861</td>
<td>38.7</td>
<td>4.5</td>
<td>1295</td>
<td>95.8</td>
<td>7.4</td>
<td></td>
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<tr>
<td>60 IgM</td>
<td>1263</td>
<td>69.5</td>
<td>5.5</td>
<td>1248</td>
<td>71.1</td>
<td>5.7</td>
<td>1003</td>
<td>83.2</td>
<td>8.3</td>
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<tr>
<td>Day-to-day</td>
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<tr>
<td>60 High IgG</td>
<td>417</td>
<td>34.6</td>
<td>8.3</td>
<td>842</td>
<td>77.5</td>
<td>9.2</td>
<td>1135</td>
<td>131.7</td>
<td>11.6</td>
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<tr>
<td>60 IgM</td>
<td>1237</td>
<td>158.0</td>
<td>12.7</td>
<td>1205</td>
<td>126.9</td>
<td>10.7</td>
<td>886</td>
<td>92.1</td>
<td>10.4</td>
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</table>

*Values expressed as 100 x (net absorbance serum sample/net absorbance pool serum) according to Mackel et al. (4).
Fig. 2. Effect of pre-absorption with collagen type I (A), III (B), and IV (*) on reactivity of anti-collagen antibodies type I, III, and IV in subsequent Elisa assays, using a series of serum dilution factors (1:10, 1:20, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400).

The top curve in each figure is a non-absorbed control (O). Net absorbance is that after serial dilution and absorption with 20 pg of antigen, 1000-fold diluted alkaline phosphatase-labeled anti-IgG (IgM) antibodies, and a substrate reaction time of 15 min. Each point is the mean of three determinations.

removal of the precipitate before the assay, gives evidence for the presence of immunoreactive material in the patients' sera. From the theoretical point of view, noncollagenous proteins or other (pro-)-collagen contaminations of commercial collagen preparations may evoke significant antibody titers by (cross-)reacation in the ACA assays. The extent of such adverse reactions in our study was evaluated by means of competitive inhibition binding curves obtained after absorption of diluted serum in the presence of constant antigen coating concentrations (Figure 2). Precise data concerning affinity constants of ACAs are lacking, but from earlier RIA experiments (16) it could be inferred that they are low. The highest apparent affinity constants (10^11 L/mol) are found on using type I and III propeptide collagens. Affinity of antibodies partly determines sensitivity of an Elisa assay (17), so detection limits are affected by this disadvantage.

In our study, immobilization of collagen antigens to the plastic wells was assured by means of incubation with a 20-mL/L glutardialdehyde solution under slightly alkaline conditions. Other investigators (18) prefer direct adsorption to the tubes. An alternative enzyme for the label introduced by the second antibody might have been peroxidase (19). Our choice of alkaline phosphatase-labeled anti-IgG (IgM) was mainly determined by the excellent results with this label in our previously described assays for anti-actin and

Table 2. Linearity of Response of Elisa Procedure for Determination of Anti-Collagen Antibodies

<table>
<thead>
<tr>
<th></th>
<th>ACA I</th>
<th>ACA III</th>
<th>ACA IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>44–889</td>
<td>58–1156</td>
<td>40–791</td>
</tr>
<tr>
<td>IgM</td>
<td>51–1025</td>
<td>46–926</td>
<td>50–990</td>
</tr>
</tbody>
</table>

*Linearity interval values are expressed according to Mackel et al. (4): 100 × (net absorbance serum sample/net absorbance pool serum).

Fig. 3. Evolution of anti-collagen antibodies type I, III, and IV (IgG, IgM) during a 90-day follow-up in 13 cases of acute infective endocarditis and in 35 patients with settled valvular heart disease who underwent valve-replacement surgery. The top curve in each figure shows infective endocarditis (IgG (O), IgM (*)); bottom curve shows valve surgery (IgG (*), IgM (A)). Results of anti-collagen antibodies determinations are expressed according to Mackel et al. (4).

Table 3. Evolution of Anti-Collagen Antibodies in Infective Endocarditis and Valve-Replacement Surgery

<table>
<thead>
<tr>
<th></th>
<th>Infective endocarditis</th>
<th>Valve-replacement surgery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 35)</td>
</tr>
<tr>
<td>ACA I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>85%</td>
<td>62%</td>
</tr>
<tr>
<td></td>
<td>Peak, %</td>
<td>d = 90, %</td>
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<tr>
<td>ACA III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>85%</td>
<td>69%</td>
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<tr>
<td></td>
<td>Peak, %</td>
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<tr>
<td>ACA IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>62%</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>Peak, %</td>
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</table>

*Percentage of patients presenting with significantly increased anti-collagen antibodies titers.

**Anti-collagen antibodies determinations on admission (d = 1, endocarditis), the day before valve surgery (d = 1, valve surgery), peak antibody concentrations (Peak), and at the end of the 90-day follow-up (d = 90).

P <0.001, probability that mean on admission anti-collagen autoimmune response is comparable with an age-matched control group.
anti-myosin antibodies (20, 21) in cardiac-surgery patients and those with acute myocardial infarct. The serum concentration of antibodies to collagen is usually not measured (4, 22). For serum samples, Mackel et al. (4) described an ELISA ratio of the absorbance value of test serum (10-fold dilution) to the mean absorbance value of 30 normal human sera with the same dilution factor for ACA type I, II, and IV in scleroderma patients. This ratio allowed comparison of individual results. In our IE study, an analogous formula was used for manipulation of ACA data. In each microtiter plate, besides the test sera, we also included aliquots from a pooled specimen of serum of apparently healthy persons, to minimize plate-to-plate errors in the calculated ELISA ratio.

Humoral and cellular immune response in infective endocarditis can be artificially subdivided in response to the causative microorganism and to the affected target organs, especially heart or valves. Secondary immunopathogenetic features owing to antigenic mimicry have been suspected in IE. Some evidence has been given for cross-reaction by cytolytic antibodies to streptococci (S. viridans, S. faecalis) in this disease (23). The remarkably high coincidence of anti-sarcolemmal, anti-endocardial, and anti-endothelial antibodies—antibodies with the highest incidence as determined by indirect immunofluorescence in IE patients (24)—induced speculations about a common antigen in this humoral immune response. Collagen or connective-tissue compounds could be major candidates for such antigens. On the other hand, heart-associated anti-myolemmal antibodies are seen less frequently, and it is also known that they do not cross-react with collagens (24).

In this study, new ELISA procedures for evaluation of anti-collagen autoimmune response (ACA I, III, and IV) have been introduced with good within- and between-assay CVs and only limited cross-reactivity of the different antigens. In an IE patient group, significantly higher (P <0.001) ACA I, III (IgG, IgM) autoimmune response was observed on hospital admission in most of the patients. By the end of the 90-day study period the antibody titer decreased in all patients, but in more than 40% of the patients the ACA titer remained significantly positive (Table 3). A borderline but constantly increased humoral autoimmune response against basement membrane (ACA IV) was noticed in more than 60% of the IE patients during the whole period of follow-up. These results suggest that ACA is involved in the pathophysiology of infective endocarditis. Whether this involvement is primary or secondary remains in question.

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References