Autoimmunohistochemistry: A New Method for the Histologic Diagnosis of Infective Endocarditis

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**Background.** Although the pathologic examination of cardiac valves remains the reference standard for the diagnosis of infective endocarditis, the detection of microorganisms often poses a challenge for pathologists. This can be done by use of nonspecific histochemical stains or by immunohistochemical analysis, but specific antibodies are often not available. We describe a novel method for the detection of microorganisms in valve specimens from patients with infective endocarditis.

**Methods.** Detection of microorganisms was performed in valve specimens from patients with endocarditis caused by gram-positive cocci (25 specimens), blood culture–negative endocarditis (15 specimens: 6 cases caused by Coxiella burnetii, 5 caused by Tropheryma whipplei, and 4 caused by Bartonella species), or noninfective degenerative damage (30 specimens, used as negative controls), using the patients’ own serum. This technique, called “autoimmunohistochemistry,” is an immunohistochemical peroxidase-based method that we compared with results of culture and polymerase chain reaction (PCR) assay.

**Results.** Bacteria were detected by autoimmunohistochemistry in 20 (80%) specimens from patients with endocarditis caused by gram-positive cocci and in 15 (100%) specimens from patients with blood culture–negative endocarditis but in no control specimens. The rate of detection of bacteria by autoimmunohistochemistry was significantly higher than that by culture but was similar to that by PCR.

**Conclusions.** Autoimmunohistochemistry may be useful for the detection of microorganisms in samples of valves from patients with infective endocarditis. This new diagnostic tool may be particularly useful in cases of blood culture–negative endocarditis.

Infective endocarditis—microbial infection of the endocardial surface of the heart—remains a life-threatening disease of worldwide importance, with very high mortality if it is not treated with antibiotics, either alone or in combination with surgery [1]. The current approach for patients with suspected infective endocarditis is determined by the highly sensitive Duke diagnostic criteria, among which transesophageal echocardiography is used to detect the presence of vegetations and blood cultures are used to isolate the causative organism [2, 3]. However, despite improved culture methods, the results of blood cultures may be negative if the patient has recently received antibiotics or if the organism is fastidious or requires special culture techniques [4]. Other diagnostic methods include serologic tests, molecular techniques, and pathologic examination [5]. Pathologic examination of resected valvular tissues, when these specimens are available, remains the reference standard for the diagnosis of infective endocarditis [6]. Histologic findings, which are included in the Duke criteria, are considered to be the criteria for definite infective endocarditis [2]. These include the histologic demonstration of microorganisms in a vegetation or intracardiac abscess and pathologic lesions (such as vegetations and active endocarditis) at the histologic level.

Visualization of microorganisms in valve tissues is a classic criterion for the histologic diagnosis of infective endocarditis [7, 8]. This is done for valve-tissue specimens using nonspecific histochemical stains or im-
munohistologic procedures with specific antibodies [6, 9–11]. However, many degenerative products can be confused with microorganisms in tissue sections when nonspecific special stains are used, and noncommercial specific antibodies directed against some bacteria are only available in specialized centers. In the present study, we developed a new method that is available commercially for the specific detection of microorganisms in valve-tissue specimens from patients with infective endocarditis. We call this immunohistological method “autoimmunohistochemistry.” The procedure uses a peroxidase-based method with the patient’s own serum as the source of antibodies directed against the etiologic microorganism of infective endocarditis. Using this methodology, we analyzed the rate of detection of microorganisms in a series of valves from patients with infective endocarditis.

PATIENTS, MATERIALS, AND METHODS

Case definition and patients. We retrospectively examined 70 native cardiac valve samples from patients at La Timone Hospital (Marseille, France) who underwent surgical valve removal and provided a serum sample. The patients were divided into 3 groups. The patients who presented with infective endocarditis caused by gram-positive cocci as detected by the presence of microorganisms in a histologic examination were assigned to group 1 (25 patients). These patients were considered to have confirmed infective endocarditis, because microorganisms were detected by standard blood cultures or culture of valvular material. Patients infected with agents of blood culture-negative endocarditis—such as Coxiella burnetii (6 patients), the agent of Q fever; Bartonella quintana (3 patients); Bartonella henselae (1 patient); and Tropheryma whippelii (5 patients), the agent of Whipple disease—who have been described elsewhere [9–11], were assigned to group 2. The patients who presented with noninfective degenerative valve damage (30 patients) were assigned to group 3 and served as control subjects.

Histologic and immunohistochemical analyses. All tissue samples that were excised during the course of the surgical removal of native cardiac valves were collected in a sterile container without fixative or culture medium. The entire sample was taken without delay to the diagnostic microbiology laboratory, for optimal recovery and identification of microorganisms. After the selection of valve-tissue samples for bacteriologic procedures such as valve-tissue culture and polymerase chain reaction (PCR) amplification, the remaining tissue samples were fixed in neutral buffered formalin, decalcified (if necessary), and embedded in paraffin [6].

Tissue specimens were then cut to 3-μm thickness and stained with hematoxylin-eosin-saffron by use of routine methods. Serial sections of each tissue specimen were also obtained for special staining or immunohistochemical examination. Special stains—including periodic acid–Schiff (PAS), Giemsa, Brown-Brenn Gram, Brown-Hopps Gram, Grocott-Gomori methenamine silver, and Warthin-Starry—were used for the detection of bacteria and fungi.

For each case, autoimmunohistochemical analysis was performed on paraffin-embedded cardiac valve sections, using the patient’s own serum as the primary source of antibodies, at working dilutions of 1:100 and 1:500. The immunohistologic procedure, in which a secondary antibody directed against human IgG (Dako) and an immunoperoxidase kit (Zymed) were used, has been described elsewhere [9]. For each tissue section, a negative control was performed by use of an irrelevant human serum sample from patients whose serum was sent to the laboratory for testing because of suspicion of rickettsial diseases. To avoid false-positive results for patients with autoantibodies to cardiac tissue, we excluded any patients with systemic lupus or other rheumatologic diseases.

Valve-tissue culture, PCR amplification of the 16S rRNA gene, and sequencing. Valve-tissue samples from patients in group 1 were inoculated onto 5% blood agar and chocolate agar (both from bioMérieux) and incubated for 10 days at 37°C in a 5% CO2 atmosphere. Valve samples were also cultured on buffered charcoal yeast extract (bioMérieux) for 15 days and on Colombia medium for 10 days under anaerobic conditions.

Heart- valve samples from patients in group 1 were stored at −80°C before DNA extraction, which was performed with the QIAmp kit (QIAGEN) or with the FastDNA kit (Bio 101). DNA was amplified using Taq DNA polymerase (Gibco BRL Life Technologies) as described elsewhere [12] but with the broad-range 16S rRNA gene primers 536f (5'-CAG CAG CCG CGG TAA TAC-3') and 1050r (5'-CAC GAG CTG ACA-3'). The sequences obtained from amplified DNA were compared with those available in GenBank by use of BLASTIN software (version 2.0; National Center for Biotechnology Information; available at: http://www.ncbi.nlm.gov/BLAST/) [13]. PCR was also conducted on negative control valve samples from patients without infective endocarditis. Only DNA corresponding to the bacterium responsible for the episode of infective endocarditis was considered to be reliable.

Statistical analysis. Using EpInfo (version 6; Centers for Disease Control and Prevention), we compared autoimmunohistochemical analysis, PCR, and culture for the detection of microorganisms in valve tissues. The quantitative data were compared using Fisher’s exact test. P<.05 was considered to be statistically significant.

RESULTS

The mean (±SD) ages of patients were 57.96 (±17.70), 53.26 (±11.66), and 65.24 (±12.73) years in groups 1, 2, and 3, respectively. The ratios of men to women were 1.77 (16 men and 9 women), 1 (15 men and 0 women), and 1.14 (16 men and 14...
women) in groups 1, 2, and 3, respectively. The mitral valve was involved in 14 (56%), 6 (40%), and 11 (36.66%) patients, whereas the aortic valve was involved 11 (44%), 9 (60%), and 19 (63.33%) patients, respectively, in groups 1, 2, and 3. No patient was immunocompromised or received immunosuppressive therapy. The causative organisms of infective endocarditis for group 1 are summarized in table 1.

**Pathologic findings, valve culture, and PCR amplification and gene sequencing.** In all cases, infective endocarditis was identified histologically by demonstration of an inflammatory reaction in valve tissues and vegetations. The pattern of inflammation and the size of vegetations varied in accordance with the type of microorganism. As described elsewhere [6], most cases of infective endocarditis caused by extracellular microorganisms are characterized by neutrophil-rich inflammation and vegetations of great size. In contrast, endocarditis caused by Q fever, Whipple disease, and infection with *Bartonella* species showed slight inflammation with a few inflammatory mononuclear cells and small vegetations [9–11]. On the other hand, the valve tissues used as negative controls showed fibrous degenerative changes, sometimes in association with calcifications, and were devoid of inflammation and vegetations.

Bacterial colonies in group 1 valve-tissue specimens were directly visualized in vegetations through the use of several special stains, including Giemsa, Grocott-Gomori methenamine silver, Brown-Brenn Gram, Warthin-Starry, and PAS. In group 2 valve-tissue samples, *Bartonella* species were detected by Warthin-Starry and Giemsa stains; PAS, Brown-Hopps Gram, Grocott-Gomori methenamine silver, and Warthin-Starry stains revealed the Whipple bacillus, as reported elsewhere [9–11]. *Bartonella* species, *T. whipplei*, and *C. burnetii* were also detected by immunohistochemical analysis by the use of specific antibodies, as described elsewhere [9–11]. No microorganisms were visualized through the use of special stains in group 3 valve-tissue specimens. Of a total of 25 valve samples from patients in group 1, valve culture was positive for 8 (32%); 18 (72%) PCR amplifications targeting the 16S rRNA gene were positive and were considered to reliably indicate the causative organism of infective endocarditis (table 1).

**Autoimmunohistochemistry.** Among the valve specimens

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**Table 1. Etiologic organisms in 25 confirmed cases of infective endocarditis caused by gram-positive cocci and results of polymerase chain reaction (PCR) amplification and autoimmunohistochemical examination.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Isolates, total no.</th>
<th>Positive PCR, no.</th>
<th>Positive autoimmunohistochemical detection, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococci</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Streptococci</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus bovis</em></td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>2</td>
<td>...</td>
<td>1</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>S. anginosus</em></td>
<td>1</td>
<td>...</td>
<td>1</td>
</tr>
<tr>
<td>Other <em>Streptococcus</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Other bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Abiotrophia defectiva</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

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**Table 2. Patterns of detection of microorganisms by autoimmunohistochemical examination.**

<table>
<thead>
<tr>
<th>Causative organism of endocarditis (no. of samples)</th>
<th>Location of bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracellular</td>
</tr>
<tr>
<td>Gram-positive cocci (25)</td>
<td>20 (80)</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em> (6)</td>
<td>0</td>
</tr>
<tr>
<td><em>Tropheryma whippeli</em> (5)</td>
<td>4 (80)</td>
</tr>
<tr>
<td><em>Bartonella</em> species (4)</td>
<td>4 (100)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of samples.
used for immunohistological staining to demonstrate the bacteria in valve tissues with patients’ own serum, microorganisms were identified in 20 (80%) of 25 in group 1 (table 1), 15 of 15 in group 2, and 0 of 30 in group 3. The false-negative results (20%) in the gram-positive group were randomly distributed and were not linked to any particular bacterial species. The microorganisms were visualized in valve-tissue samples either intracellularly or extracellularly, in accordance with their pattern of development (table 2). For intracellular bacteria, such as *C. burnetii* and *T. whipplei*, microorganisms were seen as immunopositive material in macrophage cytoplasm (figure 1). However, *T. whipplei* was also detected extracellularly. In contrast, for extracellular bacteria, such as *Bartonella* species, staphylococci, streptococci, and enterococci, organisms were seen extracellularly in dense immunopositive clusters that were mainly included in vegetations (figures 2 and 3A) and in neutrophil and macrophage cytoplasms (figure 3B). The microorganisms stained intensely with a granular aspect, and there was some light background staining. They were detected at a serum dilution of 1:500, except for Whipple endocarditis, for which a dilution of 1:100 was used. The location of bacteria could be superimposed on that in the special stains (figure 4A and 4B) or when specific antibodies were used (figure 5A and 5B). For the cases of endocarditis caused by Q fever, Whipple disease, and *Bartonella* infection, serum from each patient reacted with bacteria in valve-tissue samples from other patients who presented with infective endocarditis caused by the same microorganism (data not shown). No microorganisms were detected in valve-tissue samples when nonimmune human serum was used or in valve-tissue specimens from patients in group 3 when the patients’ own serum was used. Finally, the percentages of positive detection by autoimmunohistochemical analysis in valve specimens from patients in group 1 were similar to those when PCR was used (*P* = .5) but were significantly higher than those when culture was used (*P* < .001).

**DISCUSSION**

The detection of microorganisms in histologic sections may be of great importance, because it confirms a diagnosis of infectious disease and may guide the choice of antibiotic therapy when microorganisms are not identified by culture or non-culture laboratory methods. However, the detection of microorganisms often poses a challenge for pathologists, because some microorganisms are too small to be easily seen by light microscopy or are not clearly distinguishable on hematoxylin–eosin–stained sections. For this reason, numerous histochemical stains have been developed to visualize organisms in paraffin sections—such as Giemsa, Grocott-Gomori methenamine silver, Brown-Brenn Gram, Brown-Hopps Gram, Warthin-Starry, PAS, Ziehl-Neelsen, and Gimenez [14–16]. The application of several judiciously chosen special stains allows a skilled observer to make a rapid preliminary identification of many organisms on the basis of their morphologic characteristics [14]. However, these special stains are not specific, and the ability of a microorganism to be stained by a particular method does not mean that it will be identified accurately, because many other organisms may show the same staining reaction. Electron microscopy can be useful in recognizing and identifying microorganisms, but it is too laborious and time-consuming for routine use.

Immunohistochemistry is the other most commonly used ancillary technique for the detection of microorganisms in histologic sections. This technique uses monoclonal or polyclonal antibodies directed against specific microbial antigens. Im-
In the present study, for each valve specimen, immunohistochemical analysis was performed using the patients’ own serum as the primary source of antibodies for the detection of microorganisms on paraffin-embedded tissue sections. We have called this immunohistochemical examination “autoimmunohistochemistry.” The method was adapted from the centrifugation-shell vial technique, which is routinely and successfully used to isolate *Rickettsia* species [17], *Brucella melitensis* [18], *C. burnetii* [19], *Bartonella* species [20], *Legionella pneumophila* [21], *Chlamydia trachomatis* [22], *T. whipplei* [23], and *Mycobacteria* species [24] from blood or tissue-biopsy samples. Moreover, serum from convalescent patients who have had an infectious disease caused by a microorganism fully identified by microbial laboratory methods can be kept frozen and used

munoperoxidase methods are usually performed on formalin-fixed and paraffin-embedded tissues. This method is useful for the detection of fastidious or noncultivable microorganisms, and it may also be more sensitive than special stains for the detection of organisms that are difficult to locate in histologic sections. However, this method requires the commercial availability of antibodies directed against microorganisms or their local production in the laboratory. To date, such antibodies have been rare, especially those directed against bacteria. In our laboratory, we produced antibodies against *T. whipplei*, *C. burnetii*, and *Bartonella* species that we can use for immunohistochemical analysis of paraffin-embedded tissues [9–11]. However, these antibodies are not available commercially, which thus limits the use of this method to specialized laboratories.

Figure 3. Immunohistochemical detection of *Streptococcus bovis*, using the patient’s own serum, in a resected cardiac valve from a patient with a streptococcal endocarditis. Note the extracellular location of the bacteria (asterisk) in the vegetation (A) and intracellularly (arrows) mainly in the macrophage cytoplasm (B) (hematoxylin counterstain; original magnification, ×200 and ×400, respectively).

Figure 4. Darkly staining cocci in a valve-tissue specimen from a patient with infective endocarditis as revealed by Grocott-Gomori methenamine silver stain (A) and immunohistochemical detection of bacteria using the patient’s own serum (B). Note the extracellular (asterisk) and intracellular (arrows) locations of the bacteria revealed by the 2 histologic methods (original magnification, ×400 and ×200, respectively).
for specific antibodies to visualize the same microorganisms in tissue sections from other patients. This method may complete the histochemical stains usually used to image microorganisms by adding specificity and allowing more-accurate identification. In the field of infective endocarditis, this is particularly useful for culture-negative endocarditis, which constitutes ∼10% of cases [1, 4]. The causative organisms of culture-negative endocarditis are, for the most part, fastidious or nonculturable or are gram-positive cocci from patients who have received antibiotics [4, 25]. In the former group, the most common organisms are C. burnetii, Bartonella species, and T. whipplei [4, 20, 26, 27]. To detect these bacteria in tissue sections, we used serum from convalescent patients who presented with endocarditis caused by Q fever, Whipple disease, or Bartonella infection, similar to the method reported for lymph nodes from persons with cat-scratch disease [28].

Both extracellular and intracellular bacteria were noted in autoimmunohistochemical examination as causative organisms of infective endocarditis. In fact, obligate intracellular pathogens such as C. burnetii were detected only inside macrophages. However, extracellular bacteria (seen mainly in vegetations) were also seen intracellularly (e.g., in macrophages and polymorphonuclear leukocytes). The intracellular detection of bacteria with extracellular development, such as staphylococci and streptococci, is in keeping with phagocytosis and degradation by these scavenger cells. Taking all of the pathologic features and the results of the autoimmunohistochemical analysis into consideration, an algorithm is shown in figure 6 that demonstrates the pathologic aspects of cardiac valves with suspected blood culture-negative endocarditis; it does not consider the other diagnostic methods—such as echocardiography, serologic tests, valve culture, and PCR analysis—that were reviewed recently [4, 5]. Because the pathologic examination of resected valve tissues remains the reference standard for the diagnosis of infective endocarditis, pathologic features allow researchers to recognize or suspect infection, and the pattern of inflammation may be considered as a key to guide diagnosis [6, 29]. Endocarditis caused by Q fever, Whipple disease, and Bartonella infection is characterized by mononuclear leukocyte inflam-
mation [9–11]. By contrast, neutrophil-rich inflammatory infiltrates suggest infective endocarditis caused by gram-positive cocci—such as staphylococci, streptococci, and enterococci—which are responsible for >80% of all cases [1]. Autoimmunohistochemical examination allows the division of infective endocarditis into 2 main groups according to the intracellular and/or extracellular location of organisms. The specific immunohistologic method that we describe here allows the identification of organisms. Special stains, judiciously chosen, may aid pathologists in the identification of organisms when the patient’s serum is used for diagnostic procedures. In samples from patients with endocarditis caused by gram-positive cocci, autoimmunohistochemical examination was more sensitive than culture but was not more sensitive than PCR in identifying causative organisms. However, we did not consider the duration of antimicrobial therapy or prior cardiac valve surgery, which may have influenced the results of valve culture. On the other hand, although molecular-based diagnostic techniques (such as PCR) are highly sensitive, they may be less specific, depending on the procedures used and investigators, and they are at risk for DNA contamination.

In conclusion, we present a new method for the histologic diagnosis of infective endocarditis using the patient’s serum as a source of specific antibodies for the accurate detection of microorganisms in paraffin-embedded valve-tissue specimens. Autoimmunohistochemistry may be particularly useful in cases of blood culture–negative endocarditis. Although autoimmunohistochemistry alone cannot identify microorganisms as to genus or species, it can aid in the identification of new organisms that cause infective endocarditis when it is used in association with other biological methods.

References