CD45 is a more sensitive marker than CD3 to diagnose lymphocytic myocarditis in the endomyocardium


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ABSTRACT

To diagnose lymphocytic myocarditis (LM) immuno-histopathological examination of endomyocardial biopsies (EMB) is used with a cut-off value of ≥14 leukocytes/mm², composed of CD3 and CD68 positive cells. We hypothesized that a more common leukocyte marker, CD45, instead of CD3 could increase the diagnostic sensitivity. In hearts of mice with acute viral myocarditis (n=9), controls (n=7) and in the EMB sampling area of the left ventricular posterior wall (LVPW) obtained from autopsy hearts of patients diagnosed with LM (n=18) and controls (n=6) were stained with anti-CD68, -CD3 and -CD45. When applying the threshold of ≥14 leukocytes/mm², 33% of the mice would be diagnosed for LM with the use of CD3+CD68 and 89% with the use of CD45+CD68. In the EMB sampling area of autopsied hearts, using the cut-off value of ≥14 leukocytes/mm², CD3+CD68 could only confirm 17% of the diagnosis of LM, while CD45+CD68 could confirm 50% of the LM cases. Moreover, we compared inflammation in the EMB sampling area of the LVPW to the remaining myocardium of the LVPW and observed a significant increase of CD45+CD68 cells/mm² in LM patients. In conclusion, the use of the common leukocyte marker CD45 increases the sensitivity of the diagnosis of LM. Furthermore, the inflammatory infiltrate in the EMB sampling area is significantly increased compared to the remaining LVPW, indicating that the sampling area constitutes the highest chance for histological diagnosis of LM.
INTRODUCTION

Lymphocytic myocarditis (LM) is an important cause of cardiac failure and death in young patients. In part of the patients LM leads to chronic myocarditis, dilated cardiomyopathy and heart failure. The clinical presentation of LM is highly diverse and varies from shortness of breath and flu-like symptoms to chest pain, heart failure and sometimes sudden death. Due to this diversity in clinical presentation the diagnosis of LM is difficult.

The gold standard for diagnosing myocarditis includes (immuno)histological examination of endomyocardial biopsies (EMB). However, LM is often characterized by a patchy inflammatory infiltrate, which creates a significant risk of underdiagnosing LM in EMB because of sampling errors. In the past the diagnosis of LM in EMB was based on the so-called Dallas criteria as histological evidence of an increase of inflammatory cells within the myocardium, coinciding with cardiomyocyte necrosis of non-ischaemic origin. However, these criteria were found to be insufficiently specific and sensitive for interobserver variability. Therefore quantitative immunohistochemical analyses have been added to improve the pathological diagnosis of LM in EMB that are now recommended as guidelines by the European Society of Cardiology Working Group. A clear cut-off value is recommended of ≥14 leukocytes per mm², including up to four (CD68-positive) macrophages per mm² with the presence of ≥7 (CD3-positive) T-lymphocytes per mm². We hypothesized that the use of a more common leukocyte marker i.e. CD45 could increase the pathological diagnostic sensitivity for LM. CD45 is leukocyte marker expressed on hematopoietic cells, including lymphocytes, macrophages and monocytes. In this study we compared the efficacy of the common leukocyte antigen CD45 with a specific T-lymphocyte marker CD3 in the diagnosis of LM.

In addition, we aimed to investigate how the ventricular endocardium, from where EMB are usually taken, relates to the rest of the myocardium in the diagnosis of LM. This because late gadolinium enhanced cardiovascular magnetic resonance (CMR) primary detects myocardial injury related to LM in the subepicardial layer, often as a patchy distribution and almost never in the subendocardial compartment as seen with ischemia-mediated injury. Disturbances of the distribution volume of gadolinium chelate relate to fibrosis, inflammatory processes as well as cardiomyocyte damage, suggesting that the endocardium, albeit the only practically available site to collect biopsies, may in fact be a poor location to diagnose LM. Therefore in this study we also compared the inflammatory infiltrate in the endomyocardium of the left ventricular posterior wall (LVPW), a common EMB sampling area, to that of the remaining LVPW.
METHODS

Viral myocarditis in mice

Male C3H mice (Harlan, Horst, The Netherlands, 20–25 grams) were injected intraperitoneally with 1x10^5 plaque forming units of coxsakievirus B3 (CVB3; Nancy strain, ATCC, Manassas, Virginia, United States) to develop myocarditis (n=9) or with phosphate buffered saline as healthy control (n=7) at day zero. Mice were euthanized 14 days after infection. Hearts were fixed in 4% formaldehyde and embedded in paraffin. All virus injected animals developed acute myocarditis as confirmed by the presence of multiple inflammatory lesions throughout the entire heart. Inflammatory cells were investigated on cross sections in the middle of the heart. All animal procedures were approved by the animal ethics committee of the VU University Medical Center, and conforms to the Guide for care and use of laboratory animals published by the US National Institutes of Health.

Patient material

Transmural human heart tissue of the LVPW, the LV lateral wall (LVLW), the LV anterior wall (LVAW), the septum and the right ventricular anterior wall (RVAW) was obtained at autopsy. LM (n=18) in the LV was based on the Dallas criteria, namely the histological evidence of the aggregates of lymphocytes, adherent to cardiomyocytes and myocytolysis 1. One of these patient had borderline myocarditis, i.e. myocarditis without myocytolysis. In addition, control patients (n=6) were included that died of a cause not related to cardiac disease as confirmed by (immune)histological analysis of the LVPW, LVLW, LVAW, septum and RVAW. The characteristics of patients are depicted in Table 5.1. There were no significant differences in sex (p=0.871) or age between the groups (mean age of control 68.17 ± 10.91 and of LM 51.83 ± 22.81; p=0.137).

All heart tissue samples obtained from patients were fixed in 4% formaldehyde and embedded in paraffin. This study was approved by and performed according to the guidelines of the ethics committee of the VU University Medical Center, Amsterdam, and conforms to the principles of the Declaration of Helsinki. Use of the leftover material after the pathological examination has been completed is part of the patient contract in our hospital.

Immunohistochemical analysis

All immunohistochemical stainings were performed on paraffin sections of 4 µm. First, sections were deparaffinized, rehydrated and blocked for endogenous peroxidases (incu-
antigen retrieval was then performed by heat inactivation in 10 mM citrate buffer (pH 6.0; boiled for 10 minutes) for all stainings, except for slides stained with anti-mouse CD3 that were heated in a 10 mM Tris-EDTA buffer (pH 9.0; boiled for 10 minutes) and anti-human CD45 staining that required no antigen retrieval step. Subsequently, the sections of mouse heart tissue stained for CD3 and macrophages were pre-incubated with Normal Swine Serum (1:20, Monosan X10964) for 10 minutes. Then, the mouse sections were incubated with either CD3 (1:50, Abcam Ab16669) or Macrophage (1:1000, Gentake AIAD31240) for 60 minutes or with CD45 (1:50, B&D 550539) overnight. The human sections were incubated with CD45 (1:100, Dako M0701), CD3 (1:100, Dako A0452), CD68 (1:400, Dako M0814) or C3d (1:1000, Dako A0063) for 60 minutes. As secondary antibodies Envision (Dako K5007, 30 min)

Table 5.1: Patient characteristics LM group and of control

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<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Clinical cause of death</th>
<th>Primary cause of death after autopsy</th>
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<tr>
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<td></td>
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<tr>
<td>1</td>
<td>66</td>
<td>M</td>
<td>Sudden death</td>
<td>LM with pneumonia</td>
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<td>2</td>
<td>18</td>
<td>M</td>
<td>Sudden death</td>
<td>LM</td>
</tr>
<tr>
<td>3</td>
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<td>M</td>
<td>Sudden death</td>
<td>LM</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>F</td>
<td>Sudden death</td>
<td>LM</td>
</tr>
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<td>F</td>
<td>Sudden death</td>
<td>LM</td>
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<td>Sudden death</td>
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<td>LM with dilated cardiomyopathy</td>
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<td>18</td>
<td>86</td>
<td>F</td>
<td>Infection</td>
<td>LM</td>
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Control group (n=6)

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<td>Pneumonia</td>
</tr>
<tr>
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<td>F</td>
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<td>Hemorrhage after femoral angioplasty</td>
</tr>
<tr>
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<td>Euthanasia</td>
<td>Lung carcinoma</td>
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<td>M</td>
<td>Unknown</td>
<td>Bowel necrosis after perforation</td>
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<tr>
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<td>M</td>
<td>Hemorrhagic shock</td>
<td>Perforation in pulmonary artery</td>
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<td>78</td>
<td>F</td>
<td>Unknown</td>
<td>Pneumonia</td>
</tr>
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</table>

Sex: male (M) or female (F). Lymphocytic myocarditis (LM).
was used for the human sections and anti-rabbit/rat horseradish peroxidase (1:200, Dako P0217/1:50, Dako P0450) for the mouse sections. The staining was visualized using 3,3’-diaminobenzidine (0.1 mg/ml, Dako K3468) for 10 minutes. Finally, the slides were counterstained with haematoxylin, dehydrated and covered. With each staining slides were included incubated without a primary antibody as a negative control and all these controls showed no staining.

Moreover, of 10 randomly selected patients (LM n=5, control n=5) the heart tissue was also stained at the Academic Medical Center (AMC) with their staining protocol. Slides were stained in an immunostainer with CD3 marker (1:100, ThermoFisher RM-9107-S) or CD45 (1:400, Dako M0701) and subsequently an optiview DAB detection kit (06396500001, Roche) was used.

**Identification and quantification of inflammatory cells**

Inflammatory cells on serial cross sectional slides of the heart were counted under a light microscope (Zeiss, Germany, 250x magnification) and the number of inflammatory cells was calculated per mm². Extravascular inflammatory intact cells were included. Epitopes of activated degraded cells were not included as positive cells since the original number of cells could not be extrapolated. CD45-positive mononuclear leukocytes were counted based on their morphology. Only the CD45+ cells that were round with scant cytoplasm and showed a distinct peripheral reactivity for CD45 were counted and not the elongated cells with large cytoplasmatic compartments as these are most likely macrophages 19.

In the autopsied hearts the EMB sampling area was first defined as a 2.0 mm thick area of intact endocardium of the LVPW (representing a common biopsy area).

The slides of the human hearts were scanned using the Panoramic Desk scanner (3DHistech, Budapest, Hungary) and the surface areas of the EMB sampling area and the remaining LVPW were measured using Panoramic Viewer 1.15.2 software (3DHistech, Budapest, Hungary). The total cross-sectional surface area of the mouse hearts was determined on scanned slides using the Pathscan Enabler IV slide scanner (Meyer Instruments, Houston, Texas) and with QuickPhoto Micro analysis software (Windows version 3.0, Promicra, Prague, Czech Republic).

**Statistics**

Statistical analysis was performed with SPSS software (version 20). Statistical significance between groups was evaluated with a Mann-Whitney U test and if data was paired with
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a Wilcoxon signed rank test or with a Friedman test followed by a Wilcoxon posthoc test. Patients characteristics were analysed with the chi-square test. A p-value <0.05 was considered statistically significant. Numeral data in the text and figures is expressed as median with interquartile range.

RESULTS

Comparing CD45 and CD3 staining in the hearts of mice with viral myocarditis

We hypothesized that use of an antibody against CD45 rather than CD3 could improve the diagnostic sensitivity of myocarditis. This was first studied in mice with viral myocarditis. Both CD3+ T-lymphocytes and CD45+ leukocytes (Figure 5.1A) were found in inflammatory lesions as well as solitary cells scattered in the rest of the heart. As seen clearly in mice with viral myocarditis there were more positive stained inflammatory cells as compared to control (Figure 5.1A).

Moreover, in the myocardium of mice with viral myocarditis the number of CD45+ leukocytes (29.73; 22.08–71.21 per mm²) was a significant 3.8-fold higher (p=0.011) than the number of CD3+ T-lymphocytes (8.02; 4.10–12.62 per mm²) (Figure 5.1B).

Although not designed for mice, we applied the current guidelines of LM on EMB to the whole myocardium of mice with viral myocarditis by adding the numbers of either CD45+ leukocytes or CD3+ T-lymphocytes to a maximum of four macrophages (Figure 5.1C) 11. The combined score of CD45+ leukocytes and macrophages (33.73; 25.83–75.21 per mm²) was significantly higher compared to the combined score of CD3+ T-lymphocytes and macrophages (12.02; 7.86–16.62 per mm²) (p=0.008). Moreover, when using CD3, in only 3 of the 9 mice (33%) the threshold of ≥14 leukocytes per mm² was reached and would be diagnosed with myocarditis, whereas with CD45 in 8 of the 9 mice (89%) the threshold was reached (Figure 5.1C). None of the healthy control mice reached the threshold of ≥14 leukocytes per mm² when combining the numbers of either CD45 leukocytes or CD3+ T-lymphocytes with maximum four macrophages.

These data thus indicate that CD45 is a more robust marker for the diagnosis of viral myocarditis in mice than CD3, suggesting that the use of CD45 could increase the diagnostic sensitivity of LM in humans also.
Figure 5.1: Inflammatory cells in mice with viral myocarditis.
A) Example of staining of Hematoxylin-Eosin, CD45-positive leukocytes, CD3 positive T-cells and macrophages (MØ) in the heart of a mouse with viral myocarditis (VM) and a healthy control. The pictures of the immunological stainings of the mice with VM were stained on serial section and are of the same area. A black arrow indicates an example of a positive cell. The bar indicates 50 µm. B) Number of extravascular CD45, CD3 positive cells and macrophages (mø) per mm² in mice with VM (n=9) or control (n=7). C) Number of extravascular positive CD45 or CD3 cells in combination with up to four macrophages per mm² in mice with VM (n=9) or control (n=7). Each point in the graphs represents the value of one individual sample; the dotted line represents the number of 14 cells per mm²; * p<0.05; ** p<0.01; *** p<0.001. Data is expressed as median with interquartile range.
Comparing CD45 and CD3 staining in the EMB sampling area of autopsied LM patients

To compare the use of CD45+ leukocytes with CD3+ T-lymphocytes in diagnosing LM in humans, we quantified these cells and CD68+ macrophages in the EMB sampling area of the LVPW (Figure 5.2A) of autopsied hearts obtained from LM patients and controls (Figure 5.2B). The patient characteristics are summarized in Table 5.1.

First of all we observed in LM patients stained with CD45 residual epitopes of aggregated and activated leukocytes adherent on the cell surface of cardiomyocytes, but not with CD3 staining (Figure 5.2B). This indicates that activated deformed leukocytes are missed with CD3 staining, but are identified with CD45 staining.

Similar as in the mice, we found in the EMB sampling area of LM patients a significant 2.4-fold higher number of CD45+ leukocytes (11.19; 6.42–20.62 per mm²) compared to CD3+ T-lymphocytes (4.69; 1.72–6.70 per mm²) (p<0.001; Figure 5.2C).

We again applied the guidelines for the diagnosis of LM on EMB by adding the numbers of either CD45+ leukocytes or CD3+ T-lymphocytes to a maximum of four macrophages. When combining the score of CD45+ leukocytes with macrophages (15.19; 10.10–38.10 per mm²) this resulted in a significantly higher number of cells than combining CD3+ T-lymphocytes with macrophages (7.47; 5.28–10.05 per mm²) in the EMB area of LM patients (p<0.001; Figure 5.2D). When applying the cut-off criteria only 3 out of 18 cases (17%) were diagnosed with LM for the combined score of CD3+ T-lymphocytes and CD68+ macrophages in the LM group. However, LM was diagnosed in 9 out of 18 cases (50%) when applying the combined score of CD45+ leukocytes with macrophages. Importantly, none of the control patients exceeds the 14 leucocytes per mm² cut-off value when combining the numbers of either CD45 leukocytes or CD3+ T-lymphocytes with maximum four macrophages.

To determine whether the difference in cell numbers between CD45 and CD3 could also be extrapolated to other diagnostic pathology centers we compared cell numbers from a randomly selection of patients stained for CD45 and CD3 at the VU University Medical Center with slides from the same patients stained at the Academic Medical Center according to their staining protocols. No differences were found in cell numbers in the EMB sampling area of the patients for both CD45 (p=0.85, Figure 5.2E) and CD3 (p=0.43, Figure 5.2F) between sections stained at the different centers.

These results indicate that the quantification of CD45+ leukocytes in the LVPW biopsy area is a more potent diagnostic marker to identify LM than quantification of CD3+ T-lymphocytes.
Figure 5.2: Inflammatory cells in the EMB sampling area of patients with lymphocytic myocarditis.
A) Example of the heart tissue of the EMB sampling area of the endocardium of 2.0 mm thickness with endothelial cells at the endocardial side of the LVPW. Bar represents 5.0 mm. B) Example of CD45, CD3 and CD68 staining in the heart tissue of a post-mortem patient with LM and in controls. The pictures of heart tissue of the LM patient is of the same area and was stained on serial section. Blue arrows indicate example
CD45 is a more sensitive diagnostic marker than CD3 in myocarditis.

Increased inflammatory infiltrate in the EMB sampling area compared to LVPW

We demonstrated that use of CD45 rather than CD3 can improve the diagnosis of LM in the EMB sampling area of patients with LM. However, it is unknown how inflammation in the endocardium relates to the rest of the myocardium, especially since with CMR myocardial injury related to LM is primarily seen in the subepicardial compartment rather than the endomyocardial compartment. Therefore, we quantified the number of CD45+ leukocytes and a maximum of four macrophages of the EMB sampling area in the LVPW, a common biopsy site, and compared this with the remaining LVPW in autopsied hearts obtained from patients with LM (n=18) and controls (n=6).

Interestingly, we found a significant 2.9-fold increase in the number of CD45+ leukocytes combined to macrophages in the EMB sampling area (15.19; 10.10–24.62 per mm²) compared to the remaining LVPW (5.25; 2.69–8.04 per mm²) in patients with LM (p<0.001; Figure 5.3). Also in the control patients there were significant more cells in the EMB sampling area of LM patients compared to controls. Each point in the graphs represents the value of one individual sample; * p<0.05; *** p<0.001. Data is expressed as median with interquartile range.
sampling area (8.58; 4.99–10.72 per mm²) than in the remaining LVPW (4.49; 2.56–5.28 per mm²; p=0.036). In addition, the number of CD45+ leukocytes combined with macrophages in the EMB sampling area of LM patients was significantly higher than that in the EMB sampling area in control patients (p=0.032). Whereas there was no significant difference between the numbers of CD45+ leukocytes combined with macrophages in the remaining LVPW of LM patients and controls.

**DISCUSSION**

In this study we compared the sensitivity of the recommended pan T-lymphocyte marker CD3 with the leukocyte common antigen CD45 to diagnose LM in the hearts of mice with CVB3-induced myocarditis and in patients with LM. In mice using the recommended threshold of ≥14 leukocytes per mm² we found that CD45 increased the diagnostic sensitivity to 89% from only 33% with CD3. Also in the EMB sampling area of LM patients the diagnosis of LM could be made in only 17% of cases with CD3 when the recommended threshold was applied. This increased significantly to 50% with CD45. In addition, we compared diagnostic sensitivity of a common endocardial EMB sampling area with the rest of the ventricular wall. Interestingly, the number of CD45+ leukocytes with macrophages was almost 3-fold higher in the EMB sampling area of the LVPW than in the remaining of the LVPW, indicating that despite the risk of underdiagnoses in EMB, the endocardial sampling area constitutes the highest chance for positive histological diagnosis of LM.

Especially in the hearts of mice with viral myocarditis, but also in the hearts of LM patients, we found considerably more CD45+ cells than CD3+ cells. On average there were respectively, 3.8-fold and 2.4-fold more CD45+ cells than CD3+ cells indicating that the majority of the cells identified with the CD45 antigen were not T-lymphocytes. We deliberately excluded those CD45+ cells that, based on their morphology, were macrophages. Probably the other CD45+ non T-cells are other subtypes of lymphocytes. However, it is also known that not all T-lymphocytes express CD3 and T-lymphocytes are thus automatically underscored. Even more, T-lymphocytes can lose re-expression of CD3 on their surface when activated, however this is only reported with flow cytometry.

Currently, the mostly used criteria for LM in EMB is an inflammatory infiltrate of ≥14 leukocytes per mm², composed of activated T-lymphocytes and up to four macrophages per mm². It could be that the clear cut off value is too strict. Related to an autopsy study with LM patients based on the Dallas criteria, Nielsen et al. suggested that the cut-off value can range between 11 and 16 CD3 positive T-cells per mm² for the diagnosis of LM. They stained for CD3 cells in LM patients and evaluated the accuracy by combining
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the sensitivity and specificity between the cut-off value from 5 to 25 T-cells per mm². The highest accuracy was observed at a cut-off value of 13 T-cells per mm², however this combined level of sensitivity and specificity remained the same within the range between 11 and 16 T-cells per mm²²¹. Moreover, Maisch et al. suggested that LM could also be diagnosed in EMB even when the criteria of ≥14 leukocytes per mm² is not reached, if there are extravascular foci's of leukocytes (≥3 lymphocytes, preferably T-cells)¹². In this study we clearly show that foci's of leukocytes are revealed much more clearly with the CD45 staining, since CD45 is still expressed in degraded activated cells. Therefore, CD45 makes it easier to distinguish aggregates of lymphocytes and to find the distribution of the inflammatory infiltrates than with CD3. This is due to an increase of the number of positive CD45 intact cells, but also especially due to positively stained epitopes of degraded cells. Since these positively stained degraded cells were not included as lymphocytes the lymphocyte count is even underscored. Moreover, there is only a small interobserver discordance for identifying lymphocytes and diagnosing myocarditis in EMB with myocarditis stained for this common leukocyte marker¹⁹.

The Dallas criteria were suggested in 1987 and have provided a pathological categorization on which the diagnosis of myocarditis can be made in EMB¹. However, the Dallas criteria do not take in account sampling error of EMB, variation in expert interpretation, variation in stainings for inflammatory cells or the number of inflammatory cells⁸. Hauck et al. already demonstrated in 1989 that one EMB of the left ventricle could diagnose myocarditis only in 21% of the cases in post-mortem hearts of patients who died with myocarditis⁶. Additional EMB will increase the chance of diagnosing myocarditis, as Chow et al. have shown that myocarditis could be diagnosed in 79% of the cases when on average 17.2 EMB per patient were taken⁷. Unquestionably this is a clinically unrealistic number in living patients.

In conclusion, the use of the common leukocyte marker CD45 increases the sensitivity of the diagnosis of LM. In mice with viral myocarditis the diagnosis of myocarditis was increased from 33% to 89% when using a CD45 staining compared to a CD3 staining, in combination with a macrophage staining. Also, in the EMB sampling area of autopsy hearts with LM, diagnosis of LM increased from 17% to 50% with these stainings. In addition, the EMB sampling area of the LVPW has increased amount of leukocytes with macrophages compared to the rest of the myocardium of the LVPW. Even though there is a high probability of underdiagnoses in the EMB sampling area in patients with LM, EMBs are taken at a representative area.
REFERENCES
