New Method for Detection of Heart Allograft Rejection
Validation of Sensitivity and Reliability in a Rat Heterotopic Allograft Model

David C. Morgan, PhD; Janet E. Wilson, BSc, MT(ASCP); Calum E. MacAulay, PhD; Nicholas B. MacKinnon, BSc, AScT; Jennifer A. Kenyon, BSc; Paul S. Gerla, BSc; Chunming Dong, MD, PhD; Haishan Zeng, PhD; Peter D. Whitehead; Christopher R. Thompson, MD, CM; Bruce M. McManus, MD, PhD

Background—Patients with inflammatory heart muscle diseases would benefit from a safe, convenient, rapidly performed diagnostic technique with real-time results not involving tissue removal. We have performed a detailed evaluation of detection of heart allograft rejection by autofluorescence in a heterotopic abdominal rat heart allograft model ex vivo.

Methods and Results—Recipient rats with allograft (Lewis to Fisher 344; n = 71) and isograft (Lewis to Lewis; n = 33) hearts, treated with cyclosporine or untreated, were killed at days 2, 4, 7, 14, 21, 28, and 56 after transplant. Nontransplant controls with (n = 24) or without (n = 24) immunosuppressive therapy were also studied. When the rats were killed, autofluorescence spectra were acquired under blue-light excitation from midtransverse ventricular sections of native and transplanted hearts. Corresponding sections were then evaluated pathologically by a modified International Society for Heart and Lung Transplantation (ISHLT) grading schema. The spectral differences between rejecting and nonrejecting hearts were quantified by linear discriminant functions, producing scores that decreased progressively with increasing severity of tissue rejection. Mean ± SD discriminant function scores were 2.9 ± 1.6, 1.8 ± 2.2, −0.1 ± 2.8, −1.2 ± 2.3, and −2.3 ± 3.0 for isografts and allograft ISHLT grades 0, I, II, and III, respectively (Spearman rank-order correlation −0.6; P < 0.001, test for trend). Cyclosporine had no detectable effect on the spectra.

Conclusions—The correlation between changes in autofluorescence spectra and ISHLT rejection grade strongly supports the possibility of catheter-based, fluorescence-guided surveillance of rejection. (Circulation. 1999;100:1236-1241.)

Key Words: transplantation ■ rejection ■ biopsy ■ spectroscopy

A major challenge in cardiac transplantation is prevention and surveillance of acute graft rejection.1 Maintenance of immunosuppression at the appropriate level to protect an allograft from the alloimmune response requires a reliable method for allograft surveillance and early recognition of acute rejection. Histological grading of the rejection process in endomyocardial biopsy specimens2-3 currently guides anti-rejection therapy. Many centers perform routine surveillance biopsies up to 18 times in the first year and annually thereafter, in addition to biopsies for clinically suspected rejection. Methods that provide immediate, accurate results with limited invasiveness, reduced risks, and less inconvenience to the patient would be attractive alternatives to the endomyocardial biopsy.4-5 To date, surface and intracardiac ECGs,6,7 echocardiographic parameters including 2D and Doppler measures of systolic and diastolic function,8-11 and radionuclide imaging including ventriculography and anti-myosin imaging,12-14 MRI,15 and cytoimmunological monitoring16-18 have demonstrated limited predictive accuracy or practicability for monitoring transplant rejection.

Auto fluorescence
Spectroscopy typically involves placing a probe in contact with or near a tissue surface and obtaining fluorescence spectra for that single location. Typical biological fluorophores (molecules, cells, or tissue structures that give off fluorescent light when appropriately excited), with specific fluorescence signatures, include tryptophan, NADH, flavins (FAD), collagens, collagen cross-links, elastins, and porphyrins. Autofluorescence spectra obtained from tissue reflect both the spectra of the individual fluorophores present and

Received October 19, 1998; revision received May 4, 1999; accepted May 5, 1999.
The prototype device and analysis system used in this study was developed by Biomax Technologies, Inc. Drs McManus and MacAulay are consultants to Biomax Technologies, Inc, which also provided financial support for this study.
Correspondence to Bruce M. McManus, MD, PhD, St. Paul’s Hospital–University of British Columbia, 1081 Burrard St, Vancouver, BC V6Z 1Y6. E-mail mcmanus@interchange.ubc.ca
© 1999 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org

1236
The modification by adsorptive and dispersive tissue factors.19,20

Excitation wavelength (λ) selection depends both on the efficacy achievable for an intended purpose and on safety. Autofluorescence spectroscopy has been applied in characterization of pathological changes in the lung, gastrointestinal tract, larynx, cervix, skin, and eye,21,22 and tissue autofluorescence induced by blue-light excitation has been effective in the early diagnosis of a variety of cancers.23–27 Characterization of heart and vascular tissue has been attempted mainly with UV excitation, including identification of the sinoatrial and atrioventricular nodal conduction tissue,28 detection of myocardial ischemia,29 monitoring of myocardial redox status,30 and localization of atherosclerotic plaque.31–33 Nilsson et al34 used both near-infrared spectroscopy and laser-induced fluorescence to identify various types of cardiac tissue, and Perk et al35 studied fibrosis of the endocardium and myocardium. Two important changes that occur in acutely rejecting tissue are edema and the infiltration of leukocytes. Increased protein-rich interstitial fluid could cause changes in the autofluorescence response of heart tissue. The autofluorescence properties of most leukocytes under blue-light excitation are not well understood. Eosinophils have a characteristic autofluorescence signature when excited by blue light, primarily due to FAD,36,37 although their role in tissue rejection remains unclear. The absorptive and light-scattering properties of any infiltrating cells should be different from in situ myocytes and would probably affect the autofluorescence signature of heart tissue. Similarly, necrotic or markedly damaged myocytes will likely have different absorptive and scattering properties than viable myocytes.

To the best of our knowledge, a systematic investigation of the autofluorescence properties of tissue inflammation in heart rejection or of the diagnostic potential of autofluorescence spectra in this regard has not been reported. We now present evidence that autofluorescence under blue-light excitation may be useful in grading acute rejection based on studies ex vivo in a heterotopic abdominal rat heart allograft model.38,39

Methods

Rat Heart Transplant Model

With Lewis-to–Fisher 344 allografts and Lewis-to-Lewis isografts, the native heart of the rat receiving the transplant serves as an internal control for the systemic immune environment. Allograft recipients and isograft recipients, as well as nontransplant controls, were treated with cyclosporine (2.5 mg·kg⁻¹·d⁻¹) via subcutaneous injection for 7 days or left untreated (Table). All animal experiments were approved by the University of British Columbia Committee on Animal Care (protocol number A96-0322) in accordance with the Canadian Council on Animal Care. All rats were acclimatized for 1 week and weighed 200 to 225 g at the time of surgery. The technical details of the transplant procedure are well described elsewhere.38,39

Euthanasia and Tissue Triage Protocol

Rats were killed by carbon dioxide narcosis, and the native and transplanted hearts were removed, sectioned, and processed. A first
most-basal ventricular bread-loaf section (2 mm thick) was frozen in OCT embedding medium. A second ventricular bread-loaf section (4 to 6 mm thick) was removed, the ventricles were opened anteriorly, and autofluorescence spectra were acquired every 5 mm along all endocardial and epicardial surfaces. The autofluorescence probe was placed perpendicular to the endocardial or epicardial surface to mimic the way tissue is taken with an endomyocardial bioptome. Once all autofluorescence measurements were completed on a given bread-loaf section, it was processed for histopathological review.

**Spectral Measurements**

The optical probe and spectroscopy systems used to acquire the autofluorescence spectra are represented in Figure 1. The 200-μm-diameter fiber in the center of the probe tip illuminated the tissue with 442-nm excitation light from an HeCd laser. Autofluorescence light emitted by the tissue was collected by six 200-μm-diameter optical fibers surrounding the central excitation fiber. Signals from the collection fibers were first filtered (475-nm long-pass filter) to remove any reflected excitation light and then relayed to a spectrometer. The resulting autofluorescence spectra were calibrated for the light response of the system, normalized to 1 at their maximum intensity, and then binned into 2-nm-wavelength intervals in the range of 480 to 800 nm.

A contour plot based on a Monte Carlo simulation of the sensitivity of the probe to the tissue area (1000 μm diameter, 650 μm depth) near its tip is shown in Figure 2A; the illumination fiber is at the origin. The optical properties used for these simulations were estimated on the basis of dog myocardium values. The contour plot shows that the largest contribution comes from an area centered at ~200 μm in depth and extending radially, ~75 μm from the center of the probe. The dashed boxes represent the tissue regions that account for 50% and 90% of the signal. The 90% box is roughly 700 μm in diameter and extends ~550 μm below the tissue surface. A breakdown of the contribution to the total signal from various depths in 50-μm increments demonstrates that the largest contributions come from the 150-to-200 μm and 200-to-250-μm-deep regions (Figure 2B).

**Histopathological Evaluation**

For each rat, 5-μm sections from the native and transplant hearts were cut perpendicular to the endocardium/epicardium, placed on a single glass slide, stained with hematoxylin and eosin, marked to identify the sites of optical measurements, and scored at these sites.
The first step was to remove from the analysis set in an unbiased fashion those spectra (n=118) that had clearly identifiable spectral features related to artifacts or blood absorption by use of a linear DF analysis. The remaining spectra (n=1354), largely free of blood absorption effects, were then grouped and analyzed by linear DF analysis to assess the correlation between the histopathological grades and spectral changes.

Data Analysis

To discriminate between 2 groups of autofluorescence spectra on the basis of spectral shape, a full, forward, and backward stepwise linear discriminant function (DF) analysis was performed. This analysis was designed to pick an optimal set of points along the spectral curve that, when combined as a linear weighted sum, generate a score that distinguishes between the groups:

\[ \text{Score} = c_0 + c_1l(\lambda_1) + c_2l(\lambda_2) + c_3l(\lambda_3) + c_4l(\lambda_4) + c_5l(\lambda_5) \]

In the above equation, \( c_i \) is a constant, \( c \) values are coefficients, and \( l(\lambda) \) is the autofluorescence intensity at wavelength \( \lambda \). In the present report, we will refer to the foregoing process as “training” a linear DF. The DF is then used to score (or test) any other spectra.
results. None of the sites probed for fluorescence or examined histologically were confounded by perioperative infarcts.

As expected, the DF does not discriminate between controls (unoperated) and native hearts of animals with an isograft (n=88), wherein there is little or no immune response. We categorized native hearts of allograft animals as those from animals whose transplanted hearts had ISHLT grade 0 or only a few grade I sites (n=194) and those whose allografts had sites with ISHLT grades II and III (n=336). The significant difference between the spectra of the native hearts of animals with an isograft and the native hearts of animals with an allograft suggests that the immune response to the allograft has a detectable remote effect on the autofluorescence of the native heart. Moreover, the difference in the native heart DF scores of the 2 allograft groups implies that this immune response is progressive and linked to increasing severity of tissue rejection in the transplanted heart. The spectral difference between ISHLT grade 0 in the isografts (n=85) and native heart of the isograft group is arguably a direct measure of the effect of the unnatural position and surgical manipulation of the abdominal heart. However, there is little or no visible immune response in animals in either of these groups.

The mean DF score of the allograft grade 0 group is slightly below that of the isograft grade 0 group, which is also consistent with a systemic response that affects tissue beyond the rejecting heart. In addition, any imprecision in matching the tissue sampled by the optical probe with that graded by the pathologist could allow tissue samples in the allograft grade 0 group to be influenced by neighboring tissue with slight rejection. Such “neighborhood” effects would slightly alter the average fluorescence score.

It is widely appreciated that rejection, as defined histopathologically, is a very diffuse process. Thus, the likelihood of detecting rejection, when present, has been shown to be 95% when 3 pieces of bioprobe-acquired tissue are evaluated microscopically, whereas the likelihood rises to 98% with 4 pieces of tissue.44,45 The grade of rejection in a given biopsy specimen may not reflect the average rejection grade for an entire heart or chamber; however, the potential virtue of the optical interrogative approach is the ability to sample perhaps 4 times as many sites as conventionally sampled by endomyocardial biopsy in a small fraction of the time. Such a larger representation of ventricular myocardium offers a potentially significant advantage in acquiring an accurate mean score of ongoing rejection in a particular heart.

To date, no particular information exists on the discrimination of different types of inflammation in the heart with the optical bioprobe. The differentiation of rejection from infection and the injury or inflammation associated with either process also requires more evaluation. Myocardial infections are relatively rare in contemporary patients; however, we will need to know their differentiating optical features. The large number of sampling sites envisaged with the optical bioprobe will help to differentiate a more focal inflammatory infectious process from diffuse organ allogenesis. We intend to address these issues in the future in a swine allograft model using dual tissue and optical biopsy sampling. The swine study has already shown the feasibility of obtaining meaningful spectra in a beating heart.

In conclusion, the autofluorescence spectra from rat heart tissue excited by blue light changes consistently as the hearts undergo transplant rejection, and linear DF analysis can distinguish spectra corresponding to the degrees of tissue rejection. ISHLT grade 0 is readily distinguishable by autofluorescence from grade III and is different from grades I and II. Coupled with its capability of extensive and rapid interrogation of the ventricular endomyocardium, the optical method may facilitate a better understanding of the rejection process and may allow targeting of biopsy sites to increase the diagnostic efficiency of endomyocardial biopsy. Identification of human heart allograft rejection currently remains bound to histopathological evaluation; however, the strong relationship between autofluorescence spectra and modified ISHLT rejection grades we have observed thus far in rat allografts suggests a role for fluorescence-based surveillance of rejection. No insurmountable challenges are anticipated with this technique in orthotopic allografts; indeed, fewer difficulties are likely than in the heterotopic swine model.

Acknowledgments
The authors gratefully acknowledge Biomax Technologies, Inc., for support for this work and recognize grant support from the Heart and Stroke Foundation of British Columbia and Yukon (Dr McManus) and the British Columbia Transplant Society (Dr McManus). The authors thank Dean English, Diane Minshall, and Dr Shuxin Zheng (technical assistance), Anson Chang (database), and Shelley Wood and Josh King (editorial assistance).

References


New Method for Detection of Heart Allograft Rejection: Validation of Sensitivity and Reliability in a Rat Heterotopic Allograft Model

David C. Morgan, Janet E. Wilson, Calum E. MacAulay, Nicholas B. MacKinnon, Jennifer A. Kenyon, Paul S. Gerla, Chunming Dong, Haishan Zeng, Peter D. Whitehead, Christopher R. Thompson and Bruce M. McManus

_Circulation_. 1999;100:1236-1241
doi: 10.1161/01.CIR.100.11.1236

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/11/1236

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/