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

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**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 antirejection 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.

**Autofluorescence**
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
their 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.

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.
by use of a modified International Society for Heart and Lung Transplantation (ISHLT) grading system. The graders were blinded as to the precise nature of the heart (native, isograft, or allograft), time interval from transplant to euthanasia, treatment group, and spectroscopic results. The modified ISHLT grading system included scores as follows: 0, no rejection; I, IA, and IB, mild; II, moderate; and IIIA, IIIB, and IV, severe. ISHLT grade IA and IB spectra were amalgamated into the single grade I and grade IIIA and IIIB spectra were similarly combined because the A and B designations refer to focal (or multifocal) and diffuse inflammation, respectively, and not to a difference in severity at a given numerical grade. In utilizing these slight modifications of the ISHLT grading system, we also recognize the difference in evaluating human heart biopsy samples versus whole rat hearts.

**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, would maximize the between-group differences and minimize within-group variations. This method, unlike other common classification methods, was not constrained by a specific model of the data, and therefore does not require any a priori knowledge of the shape of the class distributions.

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.

**Results**

Photomicrographs from a control heart and representative transplant hearts with modified ISHLT grades 0, I, II, and III rejection and correspondent autofluorescence spectra are shown in Figure 3. Rejection caused a shift of the main peak to longer wavelengths, as well as an increase in spectral intensity of longer wavelengths relative to the intensity of the main peak. In addition, the distinct “bump” at 600 nm in the control spectrum disappeared in rejecting hearts.

There was no statistical difference between the average spectra of nontransplant controls treated or not treated with cyclosporine (Figure 4). Thus, cyclosporine itself did not contribute to heart tissue autofluorescence and had no apparent effect that could obscure spectral changes of interest, those associated with inflammation or injury due to rejection.

To correlate autofluorescence spectra with tissue rejection severity, the spectra were separated into groups corresponding to modified ISHLT grades 0 (n=180), I (n=223), II (n=69), and III (n=29). DF analysis was performed with grade 0 and grade III used as the training set, and a DF with wavelength values of 480, 496, 504, 518, and 522 nm was generated. These wavelengths encompass the visually apparent position and shape changes in the main peak of the autofluorescence spectra. This DF was then used to score spectra from native (n=618) and control hearts (n=236), as well as those grafts with a full range of rejection grades (Figure 5). Increasing tissue rejection severity was accompanied by a clear progression of decreasing DF scores. Each successive modified ISHLT grade (0, I, and II) is different (Student-Newman-Keuls [SNK] test, P<0.01). The mean DF score for modified ISHLT grade II rejection was higher than that of grade III (SNK test, P=0.06). To test the strength of correlation between the modified ISHLT grade and DF score, we calculated the Spearman rank-order correlation coefficient, R_s, based on all grade 0 spectra (including both allograft and isograft) being assigned a value of 0 and grades I, II, and III being assigned values of 1, 2, and 3, respectively. The result was R_s=−0.6, reflecting a highly significant correlation (P<0.001). It should be emphasised that the DF was trained only on hearts with ISHLT grade 0 and grade III. Thus, control hearts, native hearts, and grade I and II groups provided genuine tests of the DF performance.

**Discussion**

The differences between DF scores derived from autofluorescence spectra of tissue with different grades of rejection are very encouraging. If a mean rejection score of 0.5 is used as the dividing line between grade 0 and grade III, 95% of the grade III spectra can be classified correctly, while only 20% of the grade 0 scores will be misclassified. Subtle differences in DF scores for the various nonrejecting tissue groups are also interesting and have implications for interpretation of the
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 bioptome-acquired tissue are evaluated microscopically, whereas the likelihood rises to 98% with 4 pieces of tissue. 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 bioptome. 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 bioptome 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.

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