Occurrence of Circulating Heart-reactive Antibodies in a Population of Cardiac Transplant Recipients

Correlation with Cardiac Rejection and Subsequent Course

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SUMMARY To determine to what extent cardiac allograft transplantation induces the production of heart-reactive antibody or antibodies (HRA), we assayed pre- and postoperative sera from 68 cardiac transplant recipients. During the first postoperative month, HRA was detected in 63% of transplant patients, but in only 25% of 40 cardiac surgical controls (p < 0.01). The incidence of detectable preoperative HRA did not differ in the two groups (13% transplant vs 10% nontransplant patients).

To evaluate whether HRA may serve as a monitor of cardiac rejection, we further analyzed sera during 90 episodes of rejection in 65 patients, as diagnosed by endomyocardial biopsy. HRA was present in 65% of first rejection episodes, 62% of all episodes, and in at least one episode in 69% of patients rejecting. HRA generally rose before initial rejection, peaked near rejection, and decreased gradually with rejection therapy. In many patients, HRA appeared to be an early signal of posttransplant immune activation. A relatively neutral role for circulating HRA with respect to clinical outcome was suggested.

We conclude that HRA appears after cardiac transplantation, despite immunosuppression, in a frequency and intensity too great to be explained on the basis of pericardiectomy alone. Because HRA does not appear in all transplant patients during rejection episodes, a rising HRA titer cannot be used as a sole clinical indicator of impending rejection.

IMMUNOFLUORESCENCE ASSAY of circulating heart-reactive antibody or antibodies (HRA) was proposed more than 8 years ago by Ellis, Zabriskie and colleagues as a noninvasive method to monitor cardiac rejection.\(^1\)\(^\text{a}\) Data supporting this proposal came from animal studies as well as from a few patients with limited survival, showing the appearance of HRA with cardiac allograft rejection and the disappearance of HRA with rejection therapy.\(^2\)\(^\text{a}\)\(^\text{b}\) Even though the assay is noninvasive and was claimed to be capable of detecting rejection earlier than other methods, the occurrence of HRA after cardiac transplantation and its value in monitoring early signs of rejection have not yet been clearly defined.

HRA has been found in varying incidence in association with the postpericardiectomy syndrome, the postmyocardial infarction syndrome, after blunt or penetrating chest trauma, in rheumatic fever, recurrent idiopathic pericarditis, cardiomyopathies, and various other disease states, in addition to cardiac graft rejection.\(^5\)\(^\text{a}\)\(^\text{b}\)\(^\text{c}\)\(^\text{d}\)\(^\text{e}\) The presence and role of HRA in these various cardiac disease states have also been the subject of extensive reviews.\(^10\)\(^\text{a}\)\(^\text{b}\)

HRA has been best described in the postpericardiectomy syndrome, and has been shown to be non-species specific and partially organ-specific.\(^8\)\(^\text{a}\)\(^\text{b}\) Absorption studies have shown that HRA binds to heterologous as well as autologous heart tissue, to skeletal muscle as well as to cardiac muscle, and, to a lesser degree, to smooth muscle. Absorption with muscle but not with other organs neutralized serum containing HRA.

The role of HRA in cardiac rejection, as in other
diseases, is unknown. Definite roles for humoral immunity in rejection have been suggested by work in renal transplantation, but such roles have been less well defined in experimental and clinical cardiac transplantation. The Stanford University program had accomplished cardiac transplantation in 143 patients as of May 1, 1978, with 61 current survivors, approximately two-thirds of the total world-wide survivors. Since early 1974, when immunosuppressive therapy began to approach current standards, 1-year survival has been 67%, and 4-year survival 57%.

The availability of this large patient series with close follow-up and frequent serum sampling allowed us to formulate the following study goals: 1) to confirm the occurrence of HRA in recipients of cardiac allografts and document its incidence; 2) to compare this incidence with that in a postoperative control group; 3) to observe the temporal behavior of HRA close to the time of biopsy-proved graft rejection; and 4) to observe any effect of HRA on long-term survival and any association of HRA with pretransplant lymphocytotoxic antibodies. We have completed analysis of serum for HRA on nearly all patients transplanted since early 1974, and present the results below.

Methods

The current status of cardiac transplantation at Stanford, including criteria for patient selection, surgical technique, and immunosuppressive therapy, has been reviewed elsewhere. Unrelated donor hearts are ABO and sex-matched to recipients, but no attempt to match HLA tissue types is made. Preoperative immunologic testing includes a screen for lymphocytotoxic antibody in the recipient against a panel of random lymphocyte donors and also against specific donor lymphocytes.

Serum Sampling of Patients

The study included patients transplanted between March 1, 1974 and May 1, 1978. All patients surviving the immediate postoperative period for whom adequate serum was available for testing were included. Serum, sampled preoperatively or perioperatively (within 1–2 days of surgery) and frequently during the postoperative period of hospitalization (2–3 months) and also during subsequent clinic visits and hospitalizations, was separated into individual 100-μl aliquots and stored at −20°C. Serum stored for at least 4 years retained HRA activity.

Diagnosis of Cardiac Rejection

Criteria for the clinical diagnosis of cardiac rejection have been presented elsewhere. Cardiac rejection is most often suggested by a progressive fall in the electrocardiographic (ECG) voltage. The development of a new early or late diastolic gallop appears during a minority of episodes, and usually indicates severe rejection. Recently, thymic-derived ("T") lymphocytes have been assayed by spontaneous rosette formation with sheep erythrocytes, and used as an ancillary predictive index of rejection. The definitive diagnosis of cardiac rejection is made histologically on samples of endomyocardium obtained by transvenous biopsy. The method of biopsy of the transplanted heart, together with criteria for pathologic interpretation of rejection (mononuclear infiltrate, myocellular edema, etc.), have been the subject of a previous communication. Biopsies are graded as mild, moderate or severe. Endomyocardial biopsies are obtained routinely at 7–10-day intervals during the initial hospitalization, upon suspicion of rejection, 3–5 days after augmentation of immunosuppressant therapy for diagnosed acute rejection, and at 3 months, 8 months, 12 months, and annually thereafter.

Immunosuppressive Therapy

Immunosuppressive therapy includes antithymocyte globulin (ATG) of rabbit origin, corticosteroids, and azathioprine. ATG therapy consists of six initial intramuscular doses administered on postoperative days 0, 1, 2, 4, 6 and 8. Courses of ATG (three to six doses) are reinstituted for acute rejection. Intravenous methylprednisolone is given intraoperatively, continued for 48 hours after operation, and reinstituted during rejection. Oral prednisone is begun postoperatively at 1.5 mg/kg daily, tapered to 1 mg/kg/day by 2 months, to 0.5 mg/kg/day by 6 months, and eventually to 0.25–0.35 mg/kg/day maintenance therapy. Oral azathioprine is given in a loading dose preoperatively (5 mg/kg), then daily in maintenance doses (2–3 mg/kg), as limited by peripheral leukocyte count.

Immunofluorescence Staining Technique

No advantage of human heart over animal heart substrate for HRA assay has been described by others. The latter has the advantage of convenience and availability, which is important, since fresh, properly preserved heart tissue from a young animal is crucial to the reliability of the assay. Accordingly, care was taken in obtaining and preserving fresh myocardium for use as tissue substrate. Samples of canine left ventricular myocardium were obtained from experimental animals within minutes of death, cut into small (3–5 mm) individual sections, snap frozen, and stored at −70°C. Fresh rat myocardium, similarly obtained and preserved, yielded results similar to those of canine myocardium. The assay for HRA consisted of an indirect immunofluorescence method similar to that of Zabriskie et al. (Zabriskie J: personal communication). Representative 4-μm-thick sections were cut at −20°C in a cryostat, placed in multislot (9 mm) glass slides for immunofluorescence (Shandon Southern, Sewickley, Pennsylvania), acetone-fixed for 1 minute, and stored in a desiccator until used (within 24 hours). Each section was overlaid with 50 μl of the patient's stored serum (diluted 1:5 in phosphate-buffered saline [PBS, pH 7.0]), incubated at 37°C for 25 minutes in a
humidified chamber, and washed in PBS for 5 minutes. Fluorescein-conjugated goat anti-human IgG from a single lot (Meloy, Springfield, Virginia) was diluted 1:20 in PBS, 50 μl was applied to the section and incubated as above for 25 minutes. After a 5-minute wash in PBS, slides were counterstained in trypan blue (0.05%) for 4 minutes, rinsed in PBS for 5 minutes, and mounted in tris-buffered glycerol (pH 9.5). Serial samples from one patient were prepared on the same day, and each run included positive and negative control sera. Individual samples were also run on multiple occasions to check for consistency.

Fluorescence Microscopy

Slides were examined for sarcolemmal immunofluorescence with a fluorescence microscope (Fluoval, Zeiss aus Jena, German Democratic Republic) using both transmitted and incident ultraviolet light from an HBO-200 mercury lamp source with red light-excluding primary filters. For visualization of fluorescent staining, OG4 and OG1 secondary filters were used. Slides were viewed directly and then photographed on 35-mm color film (Ektrachrome 200, Kodak) using standard settings (i.e., 160 × magnification for 15 seconds). The patterns of immunofluorescent staining were similar to those described by others:1-5 sarcolemmal immunofluorescence was characteristic of a positive assay, and occasionally also interstitial, subsarcolemmal, or intermyofibrillar staining. Nonspecific (diffuse) myocellular staining did not contribute to a positive test. The intensity of sarcolemmal-subsarcolemmal staining for each section was graded 0-4+ in a blinded fashion, without reference to clinical information. Zero was defined as negative staining; 1+, borderline fluorescence; and 2-4+, various degrees of definite positive staining. Control sera (from healthy persons) were usually 0, occasionally 1+, but never 2+ in intensity. Serial serum samples for each patient (i.e., preoperative, 3-4 days prerejection, rejection [obtained within 24 hours of biopsy]) were assayed in parallel on the same day and compared with known negative and positive (postpericardiotomy syndrome) control sera. Selected positive sera were serially fourfold diluted until a “titer” equal to the dilution at which 2+ staining was just seen, was obtained. In order to estimate intraobserver error, slides were submitted for repeat examination by one observer. The repeated grade was the same as the original for 70% (130 of 185) of the slides, and differed by one grade in the remaining 30%. For purposes of the study, the results of blinded reading were always used. The reproducibility of the assay was estimated by reprocessing 66 serum aliquots. For 76% (50 of 66) of the samples, the repeated assay result was the same as the original and differed by one HRA grade in the remaining 24%. Differences were evenly distributed among the grades.

Postoperative and Other Control Sera

Serum from healthy controls and from patients with a postpericardiotomy syndrome and with Dressler’s syndrome served as negative and positive controls for the HRA assay. In addition, serum samples were obtained from 40 consecutive patients who underwent cardiac surgery involving entry through the pericardium, and who were subsequently followed for at least 1 month. Valvular surgery was performed in 11 patients, coronary surgery in 31, ventricular aneurysmectomy in two, and atrial septal defect repair in one. Five patients underwent two of these operations. Twenty-five were male, 15 female. Serum from these nontransplant, postpericardiotomy controls was obtained preoperatively, at 1 week, 2 weeks, and 1 month postoperatively.

Statistical Analysis

Group differences were compared using chi-squared analysis, with Yates correction where indicated. Serial changes were analyzed for variance with the t test for paired data.

Results

Patient Characteristics

Seventy-four patients received cardiac transplants between March 1, 1974 and May 1, 1978, and 72 survived the immediate postoperative period. Adequate serum for HRA assay was available on 68 (94%) of these patients, providing a nearly complete survey of this large population of cardiac transplant recipients. Selected characteristics of these 68 patients are presented in table 1.

Comparison of HRA in Transplant Patients vs Postpericardiotomy Controls

Cardiac transplantation was associated with a much higher incidence of postoperative HRA than non-transplant cardiac surgery (table 2). Limiting analysis of HRA to the first postoperative month, HRA (≥ 2+) was found in 40 (63%) of 63 transplant patients vs 10 (25%) of 40 consecutive nontransplant, postpericardiotomy controls (p < 0.01). HRA became strongly positive (≥ 3+) postoperatively in only 7.5% (three of 40) of surgical controls vs 38% (24 of 63) of cardiac transplant recipients during the first postoperative month (p < 0.01). Two of the three strongly positive surgical controls had symptoms of postpericardiotomy syndrome.

The incidence of detectable preoperative HRA, in contrast, did not differ in the two groups. Preoperatively, sera from 13% (eight of 63) of transplant patients and 10% (four of 40) of nontransplant patients were positive (p = NS).

Prevalence of HRA During Rejection

Sixty-five patients (96%) experienced at least one clinical episode of cardiac rejection, and three (4%) did not. Serum during one or more episodes of rejection was available along with nonrejection control serum for each of these 65 patients. In all, 90 episodes
of rejection were monitored, with a range of one to four episodes per patient.

Definite HRA staining (≥ 2+) was observed during at least one episode of rejection in 69% (45 of 65) of transplant patients, and was strongly positive (≥ 3+) in 43% (28 of 65) of patients. The sera of an additional 10 patients (15%) displayed borderline (weak) staining (1+) during rejection. If these potentially weakly positive sera are included, then 85% (55 of 65) of cardiac transplant recipients who rejected displayed HRA.

The occurrence of HRA during rejection appeared to be largely independent of patient characteristics, including HLA mismatches (table 1). Only the trend toward more negative assays in females reached statistical significance (p < 0.05).

Definite HRA (≥ 2 + staining) was detected in 62% (56 of 90) of all episodes of cardiac rejection assayed (table 3), including 65% (41 of 63) of first episodes and 56% (15 of 27) of second or subsequent episodes of rejection. The average HRA immunofluorescence grade for all patients was 0.6 ± 0.2 (SEM) preoperatively, 2.0 ± 0.3 with the first rejection episode and 1.9 ± 0.3 with any rejection episode (p < 0.001, paired t test, preoperative vs rejection). When we considered only patients who were positive at rejection, HRA rose from 0.8 ± 0.3 preoperatively to 2.7 ± 0.4 with rejection. First rejection episodes occurred at a mean of 22 days after transplantation (range 6-119 days). Second rejections occurred at a mean of 55 days after transplantation (23-196 days), and third or fourth episodes at a mean of 83 days (range 51-116 days). HRA positivity or negativity with one rejection episode usually predicted positivity or negativity with a subsequent episode (70%). Almost all patients who rejected (97%) were monitored during the first rejection episode; sera for many subsequent rejection episodes occurring within 6 months of transplantation were available for assay.

Incidence of Increased HRA Grade During Rejection

In addition to analysis of the prevalence of HRA positivity during rejection, the incidence of an increase in HRA grade during rejection was noted. The HRA grade rose by two or more in 65% (41 of 63) of all initial rejection episodes. Of 52 rejecting patients who had a pretransplant HRA grade of 0-1, 71% (37 of 52)

### Table 1. Patient Characteristics and HRA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Distribution</th>
<th>HRA positivity with rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>87% (59)</td>
<td>79% (44/56) p &lt; 0.05</td>
</tr>
<tr>
<td>Female</td>
<td>14% (9)</td>
<td>33% (3/9)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean 39.3 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range 14-55 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD = 53% (36)</td>
<td></td>
<td>82% (28/34)</td>
</tr>
<tr>
<td>CM = 46% (31)</td>
<td></td>
<td>63% (19/30)</td>
</tr>
<tr>
<td>CHD = 1% (1)</td>
<td></td>
<td>0% (0/1)</td>
</tr>
<tr>
<td>Blood group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A = 46% (31)</td>
<td></td>
<td>77% (23/30)</td>
</tr>
<tr>
<td>O = 40% (27)</td>
<td></td>
<td>73% (19/26)</td>
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<tr>
<td>B = 7% (5)</td>
<td></td>
<td>40% (2/5)</td>
</tr>
<tr>
<td>AB = 7% (5)</td>
<td></td>
<td>75% (3/4)</td>
</tr>
<tr>
<td>HLA mismatches</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or 2 = 23% (15)</td>
<td></td>
<td>64% (9/14)</td>
</tr>
<tr>
<td>3 or 4 = 76% (49)</td>
<td></td>
<td>69% (33/48)</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of HRA Positivity in Posttransplant vs Postpericardiotomy Patients

<table>
<thead>
<tr>
<th></th>
<th>Preoperative</th>
<th>Postoperative (first month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant patients</td>
<td>13% (8/63)</td>
<td>63% (40/63)</td>
</tr>
<tr>
<td>Nontransplant surgical patients</td>
<td>10% (4/40)</td>
<td>25% (10/40) (p &lt; 0.01)</td>
</tr>
</tbody>
</table>

### Table 3. Occurrence of HRA at Rejection

<table>
<thead>
<tr>
<th>Episodes of rejection</th>
<th>Positive (&gt;2+)</th>
<th>Borderline (1+)</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>90 62% (56)</td>
<td>11% (10)</td>
<td>27% (24)</td>
</tr>
<tr>
<td>First</td>
<td>63 65% (41)</td>
<td>11% (7)</td>
<td>24% (15)</td>
</tr>
<tr>
<td>Subsequent</td>
<td>27 56% (15)</td>
<td>11% (3)</td>
<td>33% (9)</td>
</tr>
</tbody>
</table>
underwent an increase in HRA grade of two or more during initial rejection.

**Pretransplant HRA and Lymphocytotoxic Antibody**

The incidence of pretransplant HRA was compared with that of lymphocytotoxic antibodies detected against any of a panel of 50 random lymphocyte donors as well as against donor cells. Of 56 patients receiving both tests, eight had detectable pretransplant lymphocytotoxic antibodies, six had pretransplant HRA, and 44 were negative for both. However, none were positive in both assays, indicating a distinction between lymphocytotoxic antibodies and HRA, at least preoperatively, as determined by the assay used. These results indicate a distinction between HRA and lymphocytotoxic antibodies, which are felt to be potentially deleterious to graft survival.

**Time Course of HRA Appearance and Disappearance**

To determine whether HRA appeared frequently in advance of histologically documented rejection, available sera drawn 4 days (range 2–7 days) before rejection were assayed in parallel with preoperative and rejection sera for first rejection episodes (n = 43). In this set, five of 43 (12%) were positive (≥2+) preoperatively, 17 of 43 (40%) at a mean of 3.9 days (range 2–7 days) prerejection, and 22 of 43 (51%) at biopsy-confirmed rejection (±1 day). The mean HRA grade of all preoperative sera was 0.6 ± 0.2; of all prerejection sera, 1.4 ± 0.3; and of all rejection sera, 1.9 ± 0.4. When we limited the analysis to patients with a positive HRA assay at rejection (≥2+), the mean HRA grade rose from 0.9 ± 0.2 preoperatively to 2.2 ± 0.2 prerejection (at a mean of 4.0 days prerejection) and to 3.0 ± 0.2 at rejection. The changes from preoperative to prerejection samples, and from prerejection to rejection samples, were highly significant by paired *t* test (*p* < 0.001, n = 22).

To determine the kinetics of the disappearance of HRA, sera were tested after complete clinical and biopsy resolution of rejection (usually 1–3 weeks after onset of rejection). For patients with positive HRA at rejection, the mean HRA grade fell from 3.2 ± 0.2 at first rejection to 2.3 ± 0.3 at a mean of 18 days postrejection (*p* < 0.01, n = 21). However, in three cases, HRA continued to rise (≥1 grade) on follow-up sampling, despite biopsy clearing. Thus, HRA generally decreased with rejection therapy, but frequently persisted beyond biopsy clearing of rejection.

Serial parallel changes in mean HRA grade from preoperative to prerejection, rejection and postrejection are displayed in figure 1 for patients who had *all* samples tested during these intervals and who showed HRA positivity at rejection (n = 15). Incremental increases between preoperative and “4-day” prerejection and between prerejection and rejection were highly significant (*p* < 0.005). A small mean decrease occurred between rejection and postrejection samples (*p* < 0.05). Thus, in general, HRA rose significantly over prerejection levels by 4 days before biopsy-diagnosed rejection, increased further to rejection, then gradually fell with therapy.

The kinetics of appearance and disappearance of HRA in one case are given in table 4 and figures 2 and 3. HRA, undetectable in pretransplant and early posttransplant serum, appeared 3 days before a biopsy read as equivocal for early rejection, and 6 days before a repeat biopsy that showed histology definite for rejection. HRA titers peaked at 1:1280 at the time of histologically evident rejection, plateaued at 1:320 during ongoing rejection under treatment, and gradually fell over 3 weeks during and after histologic clearing of rejection. The appearance of HRA in this case preceded not only histologic evidence of rejection, but also (by 6 days) the rise in rosette fraction that began on postoperative day 13, concurrent with the appearance of a lymphocytic infiltrate on biopsy. ECG criteria were falsely negative during this rejection episode; a fall in voltage did not occur. Temporal clearing of rejection was indicated more distinctly by biopsy, which showed resolution by postoperative day 25, than by HRA, which gradually declined over 3 weeks. In summary, HRA in this case provided the earliest indication of immune activation, and preceded eventual histologic rejection by 3–6 days. The changes in HRA titer in this case (fig. 2) parallel the group changes in mean HRA grade shown in figure 1.

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** Average serial change in HRA grade with rejection. Figure indicates mean ± SEM for HRA grade in patients with all four samples who show HRA positivity at rejection (see text). Abscissa indicates time in days. Tx = transplantation; R = rejection.
TABLE 4. Correlation of HRA with Various Parameters of Rejection

<table>
<thead>
<tr>
<th>POD</th>
<th>HRA titer*</th>
<th>Biopsy†</th>
<th>Percent‡ rosettes</th>
<th>V-ECG§</th>
<th>Immunosuppressive therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;1:5</td>
<td></td>
<td></td>
<td>1000</td>
<td>+ 150</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1:5</td>
<td></td>
<td></td>
<td>85</td>
<td>+ 125</td>
</tr>
<tr>
<td>7</td>
<td>1:5-1:20</td>
<td>neg</td>
<td>3.5</td>
<td>59.5</td>
<td>75 + 125</td>
</tr>
<tr>
<td>10</td>
<td>1:80</td>
<td>possible early R</td>
<td>4.4</td>
<td>65.5</td>
<td>60 + 125</td>
</tr>
<tr>
<td>12</td>
<td>1:1280</td>
<td>acute R</td>
<td>5.1</td>
<td>79</td>
<td>60 – 125</td>
</tr>
<tr>
<td>13</td>
<td>1:320</td>
<td>ongoing R</td>
<td>15.7</td>
<td>81.5</td>
<td>60 – 125</td>
</tr>
<tr>
<td>14</td>
<td>1:320</td>
<td>resolving R</td>
<td>17.0</td>
<td>85</td>
<td>1100 + 125</td>
</tr>
<tr>
<td>18</td>
<td>1:320</td>
<td>resolved R</td>
<td>7.1</td>
<td>85</td>
<td>85 + 125</td>
</tr>
<tr>
<td>21</td>
<td>1:80-1:320</td>
<td>resolving R</td>
<td>4.6</td>
<td>81</td>
<td>65 + 125</td>
</tr>
<tr>
<td>25</td>
<td>1:20-1:80</td>
<td>resolved R</td>
<td>2.6</td>
<td>80</td>
<td>60 – 0</td>
</tr>
<tr>
<td>28</td>
<td>neg</td>
<td>neg</td>
<td>2.2</td>
<td>69.5</td>
<td>60 – 0</td>
</tr>
<tr>
<td>31</td>
<td>1:20-1:80</td>
<td>neg</td>
<td>70</td>
<td>64</td>
<td>60 – 0</td>
</tr>
<tr>
<td>34</td>
<td>1:1-1:5</td>
<td>neg</td>
<td>75</td>
<td>72.5</td>
<td>60 – 50</td>
</tr>
<tr>
<td>41</td>
<td>1:5</td>
<td>neg</td>
<td>69</td>
<td>82.5</td>
<td>60 – 100</td>
</tr>
</tbody>
</table>

*HRA titer indicates greatest dilution at which 2+ HRA staining was obtained.
†Percutaneous endomyocardial biopsy.
‡Percent of peripheral lymphocytes forming rosettes (see text).
§V-ECG = summed electrocardiographic voltage (Leads I, II, III, V1 and V6).
Abbreviations: POD = postoperative day; neg = negative; R = rejection; CS = corticosteroid, dosage equivalent to mg-prednisone/day; ATG = antithymocyte globulin injection; Aza = azathioprine, dosage in mg/day.

HRA vs ECG Diagnosis of Rejection

Retrospectively, HRA was approximately as sensitive an indicator of rejection (as determined by endomyocardial biopsy) as was fall in ECG voltage. HRA was positive during 62% of all rejections. Of the 43 episodes of rejection with paired sera analyzed both 4 days before rejection on biopsy and also simultaneously with rejection, ECG voltage fell by 10% or more in 52%. However, at least one of the two tests was positive in 83% at rejection.

HRA in Transplant Patients Not Rejecting

Two of three patients who did not undergo clinical rejection postoperatively remained negative for HRA
on serial testing during the first postoperative month, while one converted to a persistently positive reaction at 2 weeks. There was no evidence of clinical postpericardiotomy syndrome in this latter patient, and routine biopsies remained negative for rejection.

**Long-term Survival and HRA**

Sera from nine long-term survivors without clinical or histologic evidence of cardiac rejection were assayed for HRA. These patients were clinically well at 34–97 months after transplantation (mean 55 months). No consistent results on HRA assay were found. Two patients were mildly positive (2+), one was borderline (1+), and six were negative for HRA.

**Discussion**

We screened a large population of cardiac transplant recipients for the development of post-transplantation HRA and demonstrated its postoperative occurrence in over two-thirds of these patients. HRA could be readily assayed and had excellent reproducibility. HRA appeared after cardiac transplantation in a frequency and intensity too great to be explained on the basis of cardiac surgery (and postpericardiotomy syndrome) alone. The fact that all transplant patients were on high-dose immunosuppressive therapy postoperatively further strengthens this observation.

Individual production of HRA was quite variable, and did not relate readily to other clinical parameters in general, except rejection. Allowing for individual variability, HRA typically rose several days before initial rejection, showed a further rise with histologic rejection, and then diminished slowly (but did not necessarily disappear) as rejection resolved during augmented immunosuppression. Because HRA did not appear in all transplant patients during rejection episodes, and in one case appeared without subsequent rejection, it may be concluded that a rising HRA titer alone cannot be used as the sole indicator of impending rejection.

Earlier reported experience differs from our own in terms of the small patient group analyzed (total five patients) and the lack of simultaneous histologic confirmation and timing of rejection by endomyocardial biopsy. More severe overall rejection in this earlier report is suggested by the occurrence of arrhythmias, congestive heart failure, and early death (at less than 3 months) with postmortem evidence of cardiac rejection. Serial HRA assays in two of these patients showed the appearance of HRA with suspected rejection, then disappearance with augmented immunosuppressive therapy. HRA was positive postoperatively at

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**Figure 3.** Serial immunofluorescent assays for HRA in patient JM (see Methods). All photos were taken at the same serum dilution (1:20) and the same photomicrographic settings. A) Postoperative day (POD) 0, HRA = trace; B) POD 7, HRA = 2+; C) POD 10, HRA = 3+; D) POD 12, HRA = 4+; E) POD 21, HRA = 3+; F) POD 41, HRA = 1+. Rejection was diagnosed on POD 12. See Table 4 and figure 2 for serial correlations with rejection.
some time in four of five patients; all four with positive staining during their course showed cardiac rejection at postmortem examination. One patient not developing HRA was negative at postmortem for rejection; however, rejection during the clinical course with subsequent resolution may have occurred. No postpericardiotomy control patients were included in this older study to assess the question of antibody development resulting from cardiac operations with or without a postpericardiotomy syndrome.

Improvements in postoperative care after cardiac transplantation, including endomyocardial biopsy to increase diagnostic certainty and potent antithymocyte globulin of rabbit origin to increase therapeutic ability, have allowed much earlier diagnosis and more successful therapy of rejection. The current 67% 1-year survival rate of cardiac transplant patients at Stanford reflects these and other advances. Our data thus define the development of HRA after cardiac transplantation given current standards of care that allow much earlier detection and termination of cardiac rejection. Despite these considerations, the occurrence of HRA was roughly similar to the results of earlier work noted above. Thus, 68% of all patients assayed (46 of 68) were found to have 2+ or greater HRA at some time postoperatively, and 84% (57 of 68) developed at least a 1+ reaction. This compares with a positivity of 80% of patients (four of five) in the group of Ellis et al. When the more stringent criterion of substantial (grade 2+ or more) immunofluorescent staining is applied, 69% of rejecting patients were positive in our assay.

Detection of HRA by indirect immunofluorescence is a sensitive but semiquantitative method. In the present study, intraobserver variation was quite acceptable, as was assay reproducibility. Grading bias was eliminated by blinding the interpreter to clinical information. A wholly quantitative assay system would be more objective, but would not necessarily circumvent problems with sensitivity and specificity. A more quantitative method is preferable for precisely describing the kinetics in individual cases, but the data derived did provide a statistically significant description of the general kinetic trend. A more quantitative, somewhat arduous titering method, applied selectively (fig. 2), was further supportive. Purification of antigen(s) against which HRA is directed is a desirable objective that should lead to a more quantitative approach. Specificity for rejection could not be accurately judged in our study because large numbers of random biopsies with which to correlate simultaneous serum HRA staining were not performed.

Our data provide substantial perspective on HRA as an assay for monitoring cardiac rejection. We have confirmed its usefulness and defined its limitations. HRA may indeed provide an early signal of immune activation after transplantation, frequently presaging overt initial graft rejection. Because false-negative and occasionally false-positive results occur, however, HRA should be used in conjunction with other noninvasive and invasive tests. The temporal appearance and disappearance of HRA is less distinct with respect to cellular rejection than previous investigators have suggested. HRA may precede the appearance of histologic evidence of rejection, and the disappearance of HRA may lag behind histologic resolution. These observations diminish the value of HRA in monitoring the resolution of initial rejection or close, subsequent rejection. Thus, HRA appears to reflect a different level of immune activation than endomyocardial biopsy. The earlier suggestion that immunosuppressive therapy might be regulated by HRA levels, i.e., titering dosage to keep patients just free of circulating HRA, is not confirmed by our correlations with endomyocardial biopsy, since HRA may persist after histologic clearing of rejection and, conversely, may fail to appear, despite histologic rejection.

The nature and significance of circulating HRA have not been clearly defined to date, but some insight is provided by this study. Present evidence suggests that HRA is not the same as lymphocytotoxic antibody. Patients in our series showing detectable pretransplant HRA did not have lymphocytotoxic antibody on pretransplant screening. Moreover, serum containing HRA or HRA plus complement has been shown to lack cytotoxic effect on cultured myocardial cells. Long-term survival did not depend on the absence or presence of HRA; some patients surviving longer than 3 years had detectable antibody, but others did not. McCabe et al. questioned whether HRA might augment or enhance the sensitized lymphocyte in its attack on sensitizing (myocardial) antigens; however, HRA might also serve to block this lymphocytic attack. The short-term and long-term clinical course of our patients with and without HRA does not support either of these opposing views in the transplant population. Rather, a relatively neutral overall role for circulating HRA with respect to clinical outcome in these patients is suggested. Further clarification of the physiologic and pathologic roles of HRA is needed.

The detection of circulating HRA raises the question of in vivo binding of these antibodies to the allografted heart. Bound immunoglobulins (IgG, IgM, IgA) have been found by immunofluorescent staining in some allograft samples obtained by means of endomyocardial biopsy at Stanford, and in individual cases such staining has been present in association with circulating HRA and rejection (Billingham M, Lee E: unpublished data). However, insufficient data have been obtained to allow correlation between circulating HRA and classes of bound immunoglobulins or between bound immunoglobulins and the rejection process. Immunoglobulin deposits have been noted in dog hearts after cardiac transplantation in association with serum antibodies. Rossen et al. found readily detectable bound immunoglobulins in the hearts of transplant patients dying of rejection soon after transplantation. In contrast, little bound immunoglobulin was found in original recipient hearts before transplantation.

Rejection is currently detected at a stage when only a minority of patients have evidence of cardiac failure.
on clinical examination. Nonetheless, diagnosis by ECG or biopsy criteria depends on a degree of immunologic insult to the graft. In patients in whom HRA rise precedes histologic rejection, the assay may permit interruption of immune activation before substantial immunologically mediated myocardial injury has occurred. Assay of HRA also may be used advantageously in combination with ECG voltage data and measurement of peripheral T-lymphocyte levels (rosettes) to yield a superior noninvasive index for surveillance of rejection. Further, prospective studies to test the usefulness of HRA, perhaps in such a combined index, appear to be indicated.

Increased understanding of the immune system has revealed a much greater interaction between the humoral and cellular branches in many forms of immune response than previously suspected. However, relatively little is known about the specific interactions of these two branches and their response to allografted organs in man. Our data suggest that humoral as well as cellular mechanisms are usually activated after cardiac transplantation and frequently exhibit peak intensity near the time of clinically evident rejection. Whether HRA appearance and activation of cellular mechanisms of rejection may in some way be causally related, however, remains to be defined.

Acknowledgments

Margaret E. Billingham, M.D., Stanford University, performed the pathologic interpretation of all the endomyocardial biopsies. In addition, we thank her for her encouragement and helpful suggestions in initiating this project. We also thank Howard Brown for technical assistance, and Eve Finestein for assistance in preparing the manuscript.

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_Circulation_. 1979;60:629-637
doi: 10.1161/01.CIR.60.3.629

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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