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Morphometric image analysis and eosinophil counts in human liver allografts

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Abstract Histology of liver allografts is the gold standard for diagnosis of acute cellular rejection. However, scoring the severity of rejection and distinguishing it from other infiltrations is not easy. Only one group has evaluated biopsies morphometrically and also suggested that eosinophils are a specific diagnostic feature. We quantitated eosinophil count in 92 biopsies in a group of 25 patients and, in another group of 30 patients, used morphometric image analysis to measure the cross-sectional area and cell density in each portal tract in day 5 protocol liver biopsies. Rejection was diagnosed by pathological evaluation confirmed with clinical and biochemical graft dysfunction graded histologically into mild or moderate-to-severe. The control groups were five patients with no rejection, nine patients with CMV infection, and eight biopsies in eight patients for whom the cause of the liver dysfunction was obscure. The cross-sectional area, the inflammatory cell count of each portal tract and the mean portal tract inflammatory cell density (cells/mm^2) increased with the severity of rejection. In each case the regression coefficient was statistically signifinant. Correlating the mean of the total inflammatory cell count with the mean of the portal inflammatory cell density (cell/mm^2) gave far better separation of the mild rejection and moderate-to-severe rejection groups. Eosinophils were specific for the presence of acute cellular rejection and increased with the severity of rejection. They were absent in the no rejection group, in the CMV group and in those with obscure liver dysfunction. The eosinophil count fell markedly following treatment of rejection. We conclude that morphometric image analysis can be used to quantify acute cellular rejection and that eosinophils are a specific feature of acute cellular rejection.

Key words Liver transplantation, morphometry, eosinophils

Introduction

Serial pathologic evaluation of liver allografts combined with monitoring of allograft function is the accepted standard method for diagnosing acute cellular rejection after liver transplantation. The most characteristic histological features typically form a diagnostic „triad": a mixed portal inflammatory infiltrate, inflammatory damage to small and medium-sized bile ducts and venous endothelial inflammation or „endothelitis" [6]. The clinical features and biochemical changes are nonspecific [2, 13], such that when rejection is clinically suspected to be the cause of dysfunction of the transplanted liver, histological study may instead suggest a different cause for the symptoms and biochemical disorder [24]. However, both intraobserver and interobserver varia-

tion in the histopathological assessment of liver allograft rejection has been documented [7]. Histological features thought to be important in the evaluation of liver allograft biopsies for acute rejection are reliably reproduced only among a group of experienced pathologists [7]. Only one group has reported a morphometric evaluation of liver biopsies in 58 liver transplant recipients, and it was also suggested that eosinophils were a specific diagnostic feature of acute liver allograft rejection [10]. We have attempted to quantify accurately acute cellular rejection in liver allografts by using morphometric image analysis and to assess the value of eosinophil counts in portal tracts for the diagnosis and assessment of severity of acute cellular rejection. We have compared these results with the published system of Demetris et al. [6] and with a Royal Free scoring system [12].

Patients and methods

We retrospectively reviewed 30, day 5 protocol, percutaneous allograft biopsies in 5 patients without cellular rejection (control group) and in 25 patients in whom acute cellular rejection was diagnosed by qualitative pathologic evaluation [6], combined with allograft dysfunction, defined as 2 consecutive days of rising bilirubin and transaminase values. Each biopsy was graded qualitatively as: grade 1, consistent with acute cellular rejection; grade 2, mild acute cellular rejection; grade 3, moderate acute cellular rejection or grade 4, severe acute cellular rejection [6]. Other causes of graft dysfunction were excluded. We also scored the severity of acute cellular rejection using an in-house system based on portal inflammatory infiltrate, venulitis, eosinophils and bile duct damage with a range of 0–12 points [12]. As further control groups, we reviewed nine percutaneous allograft biopsies in nine patients who were confirmed as having cytomegalovirus (CMV) hepatitis (clinically with histochemical stains and/or virological culture) and eight biopsies in eight patients in whom the diagnosis of liver dysfunction was obscure. None of the control group patients had received methylprednisolone for the treatment of acute cellular rejection within the previous 2–4 weeks.

We used morphometric imaging for analysis of the portal areas. All of the portal tracts seen in each biopsy were evaluated. Each patient had one 5-day protocol biopsy analyzed. The video image analysis used was a microcomputer fitted with a video processing card using chromatic image analysis software (Chromatic colour image analysis system, Leading Edge, Australia). The images were captured using a RGB colour video camera attached to a microscope mount. The system was separately calibrated for all objectives of the microscope, allowing dimensional features to be measured in real rather than arbitrary units. The image analysis system used is capable of differentiating on the basis of colour and density of staining, and can count cells in portal tracts [20]. The programme uses the following steps:

1. Correct image to account for the camera's white balance and microscope bulbs' heat colour.
2. Convert image to complementary colour to eliminate white background.
3. Roll image colours to avoid operator needing to look at blue on black.
4. Detect cells by their nuclei on the basis of colour and a size limit.
5. Measure field, recording cell numbers, nuclear area and total area used for measurement.

The mean of each morphometric value was determined in all biopsies, and the unpaired *t*-test was used to determine the statistical significance of the differences. The regression coefficient was applied, as appropriate.

Eosinophil counts were counted microscopically per portal tract in 92 consecutive biopsies in a different group of 25 liver transplant patients. Each of the haematoxylin and eosin-stained allograft biopsy specimens that was retrospectively reviewed for acute rejection was re-evaluated in a blind fashion for the number of eosinophils in each portal tract. For comparison we used nine biopsy specimens from nine patients with CMV hepatitis and eight biopsy specimens from eight patients with dysfunction of obscure cause.

All patients assessed for rejection had been treated initially with ATG, 2.5 mg/kg, as a single agent for the first 10 days after transplant, with cyclosporin being introduced only at day 5. Maintenance immunosuppression included cyclosporin (4 mg/kg per day intravenously, 10 mg/kg day orally), prednisolone (beginning at 1 mg/kg per day) and azathioprine (1.5 mg/kg per day). Acute rejection episodes were treated with three daily doses of 1 g methylprednisolone intravenously. Resistant episodes (more than two cycles of methylprednisolone) were treated with OKT_3 antibody for 15 days.

Results

There were obvious differences between the day 5 no rejection and rejection groups as well as the other control groups in all of the morphometric variables and eosinophil counts/portal tract (Table 1).

The cross-sectional area of each portal tract increased with the cell area (severity of rejection: Fig. 1). The values for the control group fell within the range of patients with rejection. The regression coefficient was 0.81 ($P < 0.01$) for the moderate-to-severe rejection group and 0.67 ($P < 0.01$) for the mild rejection group. The mean measured portal tract cross-sectional area in the moderate-to-severe group was 146263.70 μ^2 as compared to 61679.14 μ^2 in the mild rejection group (a 237 % increase; $P < 0.001$) and it was 49688.35 μ^2 in the no rejection group ($P < 0.001$; Table 1).

The inflammatory (mononuclear cells, eosinophils and polymorphs) cell count of each portal tract also increased with the severity of rejection (Fig. 2). The regression coefficient was 0.89 ($P < 0.001$) for the moderate-to-severe rejection group and 0.8 ($P = 0.001$) for the mild rejection group. The mean cell count was 585.48 per portal tract in the moderate-to-severe rejection group as compared with 116.62 per portal tract in the mild rejection group (a 502 % increase; $P < 0.001$), and it was 57.61 per portal tract in the no rejection group ($P < 0.001$; Table 1).

The mean nuclear area (μ^2) occupied by inflammatory cells in each portal tract increased with the severity of rejection: 2280.43 μ^2 in the mild rejection group as compared with 18487.53 μ^2 in the moderate-to-severe rejection group ($P < 0.001$) and it was 1151.17 μ^2 in the no rejection group ($P < 0.001$; Table 1).

Table 1 Morphometric measurements

Group	Mean portal area (μ^2)	Mean cell count	Mean nuclear area (μ^2)	Mean density (cells/mm^2)	Mean eosinophils No/portal tract
Mild rejection (n = 13)	61679.14	116.62	2280.43	1890.70	0.41
Moderate-to-severe rejecton (n = 14)	146263.70	585.48	18487.53	4002.90	27.38
CMV (n = 8)	82958.61	219.79	5183.64	2649.36	0
Non-specific (n = 8)	67857.31	226.55	9377.23	3338.56	0
No rejection (n = 5)	49688.35	57.61	1151.17	1159.40	0

$P < 0.001$ between mild and moderate-to-severe rejection groups for mean portal area and mean cell count

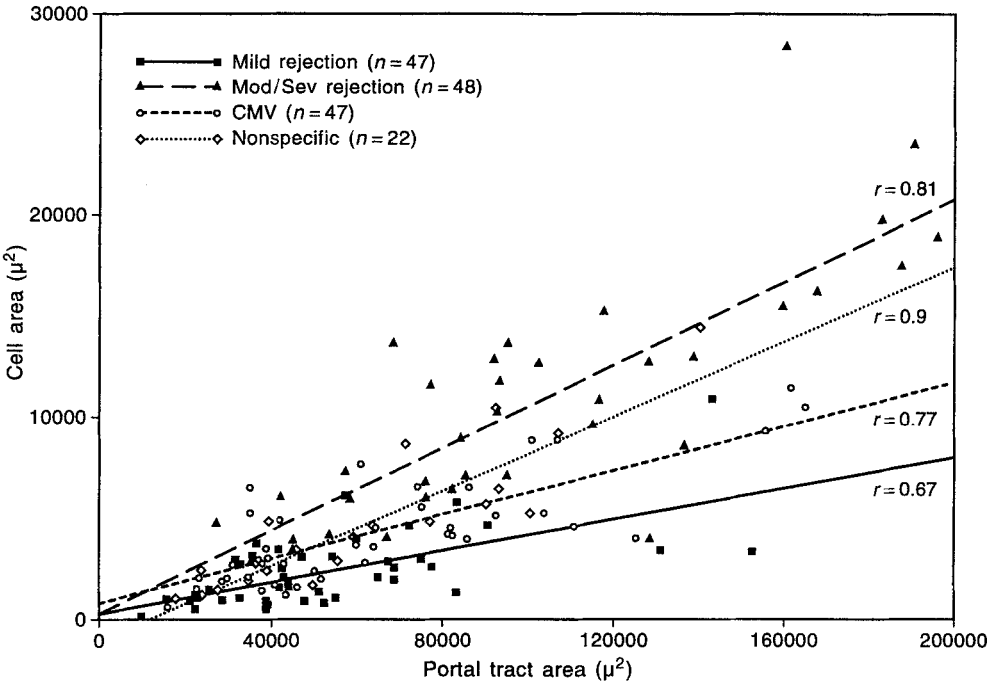

Fig. 1 Total inflammatory cell area versus portal tract area (μ^2). The cross-sectional area of each portal tract increased with the severity of rejection (the values for the control groups lie within these values). The regression coefficient is 0.81 ($P < 0.01$) for the moderate to severe rejection group and 0.67 ($P < 0.01$) for the mild rejection group

The mean portal tract inflammatory cell density (cells/mm^2) also increased with the severity of rejection: 1890.70 (cells/mm^2) in the mild rejection group as compared to 4002.90 cells/mm^2 in the moderate-to- severe rejection group ($P < 0.001$), and it was 1159.40 (cells/mm^2) in the no rejection group ($P < 0.001$; Table 1). The values for mean nuclear area occupied by inflammatory cells and mean portal tract inflammatory cell density of these morphometric measurements in the control groups (CMV group and the non-specific group) fell in-between the mild and moderate-to- severe rejection group ($P = 0.033$; Table 1).

Correlating the mean of the total inflammatory cell count with the mean of the portal inflammatory cell density (cells/mm^2; Fig. 3) separated the mild rejection group from the moderate-to-severe rejection group in more cases with little overlap ($P = 0.033$).

We found good agreement between the published scoring system of Demetris et al. [6], the Royal Free in-house scoring system [12] and the morphometric parameters in assessing the severity of acute cellular rejection, with an 86 % agreement with Demetris et al. and a 92 % agreement with the Royal Free system.

Eosinophil counts were quantified in 92 consecutive biopsies in another group of 25 liver transplant patients. Eosinophils were specific for the presence of acute cellular rejection in the context of this study (Table 1) as they were absent in the CMV group, in those with obscure liver dysfunction and in the no rejection group of patients. The eosinophil count increased with the degree of rejection. The mean eosinophil count per portal tract in the mild rejection group was 0.41 (range 0–2), and it was 27.38 (range 5–95) per portal tract in the moderate-to-severe rejection group (Table 1; $P < 0.001$). The eosinophil count in the second biopsy fell markedly following treat-

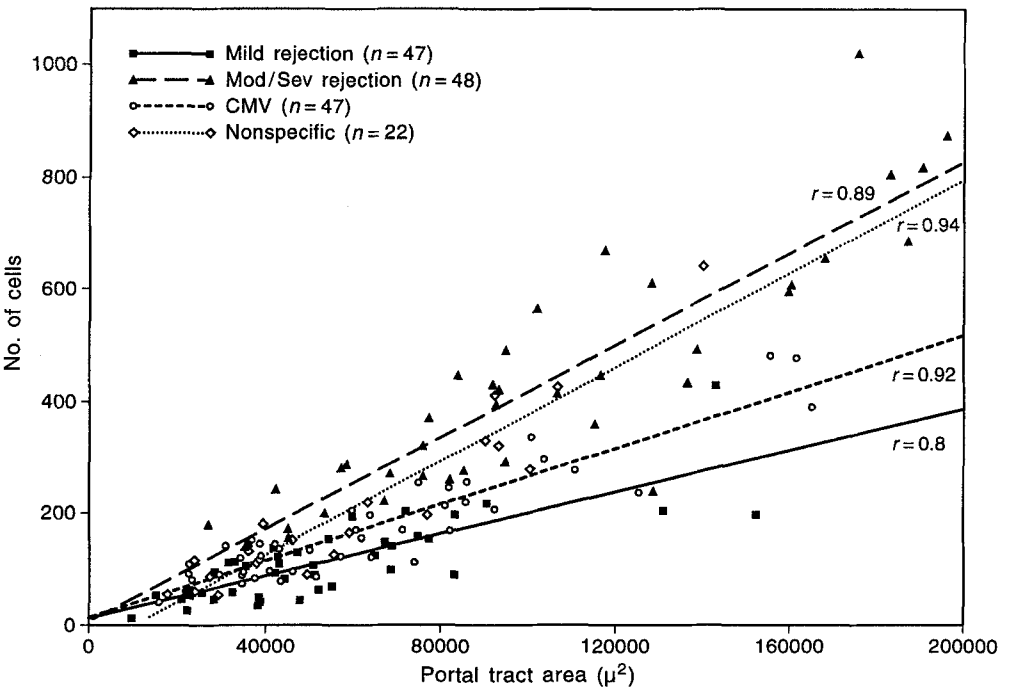

Fig. 2 Total inflammatory cell count versus portal tract area (μ^2). The cell density of each portal tract increased with severity of rejection (the values for the control group lie within these values). The regression coefficient is 0.89 ($P < 0.001$) for the moderate-to-severe rejection group and 0.8 ($P = 0.001$) for the mild rejection group

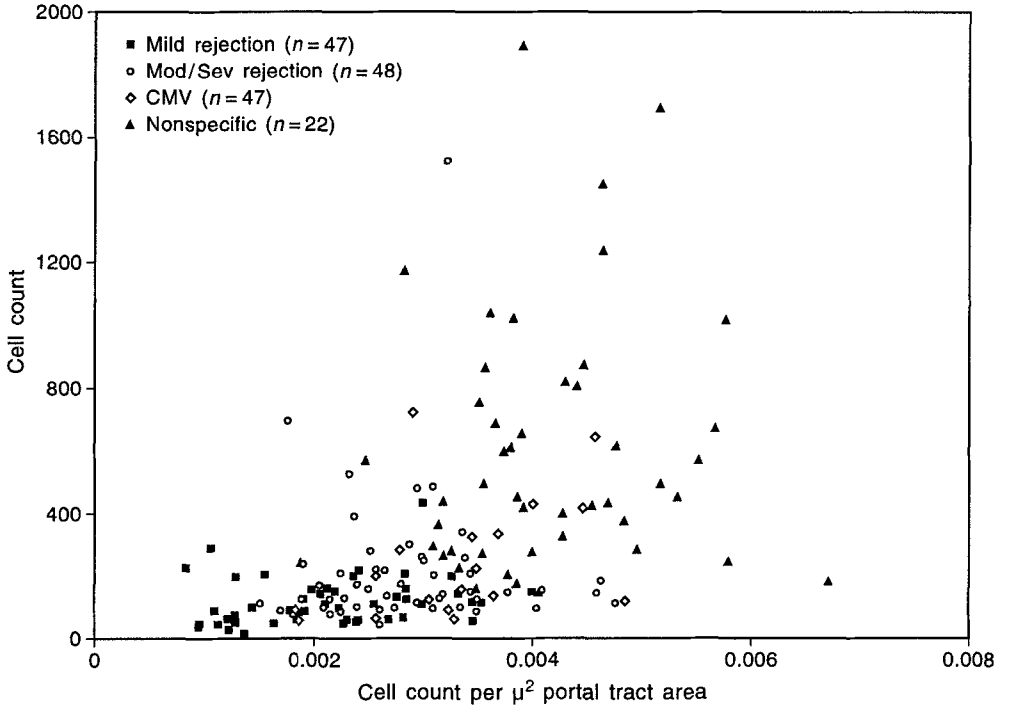

Fig. 3 Total inflammatory cell count versus portal inflammatory cell density. The correlation of the total inflammatory cell count versus portal inflammatory cell density, separated out the mild rejection from the moderate-to-severe rejection group ($P = 0.033$)

ment of rejection (Fig. 4) in the moderate-to-severe rejection group ($n = 14$). The second biopsy was performed to assess the histological response to treatment in all patients. A third biopsy was only performed in those patients ($n = 6$) whose response in the second biopsy was inadequate or who had no response.

Discussion

Morphometric inflammatory cell analysis has been evaluated in renal allografts [4, 19] but has not been shown to be reliable in predicting acute allograft rejection. There is evidence in liver allografts that inflammatory

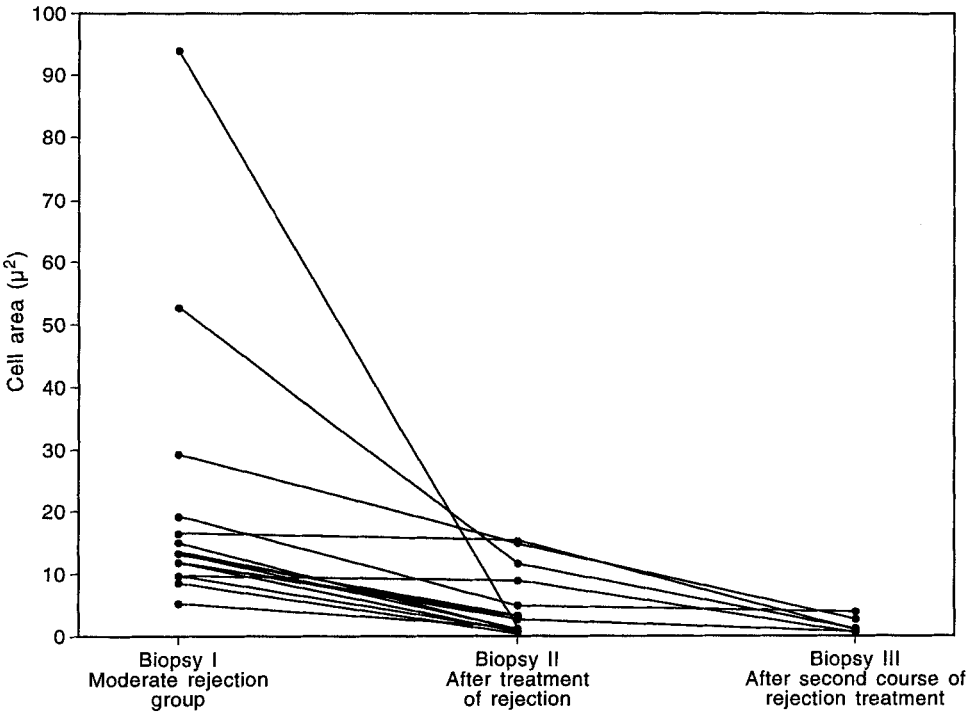

Fig. 4 Eosinophil count and response to treatment for acute cellular rejection in the moderate-to-severe rejection group. The eosinophil count fell markedly following treatment of moderate-to-severe rejection in 14 patients. The second liver biopsy was performed in all to assess histological response. The third biopsy was performed to confirm response to further treatment after no response or inadquate response in the second biopsy ($n = 6$)

cell analysis is reliable in quantifying acute rejection in both clinical and experimental models [15, 18, 22]. In a single study of quantitative and morphometric study of portal tract infiltrates [10, 25], the average portal tract number and density of all inflammatory cells, as well as the percentage of inflammatory cells in relation to the total number of cells, were significantly increased in biopsies associated with acute rejection [10].

To date, all morphometric analyses published have used the optical micrometer to calculate the width and length of each portal tract [9, 10]; the cross sectional area is calculated as the width times the length [10]. However, as the portal tract is irregular in shape, the portal tract area cannot be measured accurately and the consequent estimates of cell density will also be inaccurate. By using the chromatic image analysis software, we were able to overcome this and to measure accurately the cross-sectional area, the cell count of each portal tract and the cell density. Since the previous report suggested that eosinophils, rather than neutrophils and lymphocytes, are the best variable predictive of rejection [10], we measured all inflammatory cells in each portal tract as well as the number of eosinophils.

Our study shows that the mean cross-sectional area of portal tracts increased with the severity of rejection (Fig. 1): the moderate-to-severe rejection group increased by 237 % as compared with the mild rejection group (Table 1). The mean cell count per portal tract also increased significantly with the severity of rejection (Fig. 2): the moderate-to-severe rejection group increased by 502 % as compared with the mild rejection group (Table 1). These findings are similar to those reported by the only other group that has evaluated liver biopsies morphometrically in 58 liver transplant recipients [10]: the average number of neutrophils per portal tract increased by approximately 100 %, and the average number of lymphocytes per portal tract increased by approximately 70 % in acute cellular rejection compared to no rejection [10]. The area of portal tracts was also significantly increased by greater than 50 % in liver allograft biopsies associated with acute rejection [25].

We found that both total inflammatory cell count and portal area increased with increasing severity of rejection. Therefore, we assessed whether the severity of rejection correlated with inflammatory cell density and, indeed, found that the inflammatory cell count per portal tract correlated with the severity of rejection (Fig. 3). We chose patients with no rejection, patients with proven CMV infection and patients with obscure abnormalities of liver function tests as groups for comparison, and these groups were statistically significantly different in all morphometric prameters ($P < 0.01$ for cell area and cell count in the no rejection group, $P < 0.01$ for cell area and cell count in the CMV group and $P < 0.001$ for cell count in the obscure dysfunction group).

Eosinophils were specific for the presence of acute cellular rejection. Only one group has previously suggested that eosinophils are a specific diagnostic feature

of acute liver allograft rejection [10] that were consistently predictive of acute rejection following receiver operating characteristic curve analysis (sensitivity 82%–86%, specificity 91%–92%) [10].The eosinophil count increased with the degree of rejection and was zero in the control groups (Table 1). The counts fell markedly following treatment of rejection (Fig. 4).

The presence of eosinophils during acute rejection in our study and previous studies [10] suggests their possible role as effector cells in immunologically mediated liver allograft injury. The eosinophils' role in the inflammatory response of rejection is poorly understood, but there is considerable evidence that it is important. Firstly, there is a role for eosinophil granule proteins in contact dependent antibody-mediated cytotoxicity [1]. Cytolysis may involve a secretory phenomenon whereby granule proteins are released at the site of contact between eosinophil and target cells [26]. Several basic proteins have been isolated from eosinophil granules, including the major basic protein [11] eosinophil cationic protein (ECP) [21], eosinophil protein-X [23] and eosinophil peroxidase [5]. ECP can form a functional channel in target cell damage mediated by eosinophils. Channel formation by granule proteins of immune effector cells may represent a general and effective mechanism of target cell killing [27]. A positive extra-cellular ECP staining was significantly higher in patients with hepatic allograft rejection than in patients with dysfunction from other causes [9]. Furthermore, corticosteroids reduce the number of blood eosinophils in an animal model [3] and in clinical studies [14], including studies of liver allografts [8, 10]. The mechanism by which corticosteroids induce eosinopenia is uncertain. It is suggested that they may produce eosinopenia by decreasing lymphocyte mediators of eosinophil proliferation such as interleukin-5 [16]. Interleukin-5 seems to be involved in the local cellular and molecular mechanisms that contribute to liver allograft rejection [17].

We conclude that morphometric image analysis can be used to quantify acute cellular rejection. Eosinophils within portal tracts indicate a diagnosis of acute cellular rejection specifically in this clinical context, and the eosinophil count parallels the severity and response to treatment. These findings could provide a basis for a reproducible diagnostic and quantitative method to assess allograft rejection, particularly when comparing different immunosuppressive regimens.

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