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Contribution of the expression of ICAM-1, HLA-DR and IL-2R to the diagnosis of acute rejection in renal allograft aspirative cytology

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Abstract Acute rejection is associated with a poor long-term prognosis for renal allografts. Sequential fine-needle aspiration cytology (FNAC) has been used to monitor rejection. However, FNAC diagnoses rejection only when the infiltrating cells are already damaging the graft and, in some borderline cases with a low increment of inflammatory cells in the graft, FNAC lacks the specificity to diagnose rejection. In these cases, the number of inflammatory cells within the graft can decline, stabilize or increase with time. In this study, we sought to determine whether the analysis of the expression of ICAM-I, HLA-DR and IL-2R along with borderline FNAC results increases the specificity to diagnose rejection. Of 117 FNAC samples taken from 24 patients after renal transplantation, 85 (72%) were considered suitable for cytological analysis. Of these patients, 9 (37%) did not suffer an acute cellular rejection (ACR) episode and 15 (63%) had at least one ACR episode. ICAM-1 and IL-2R were studied using an immune-peroxidase technique. The ICAM-1 results are expressed as the percentage of tubular cells in the aspirate stained with this marker and the IL-2R results are expressed as the absolute number of positively stained lymphocytes in the whole cytopreparation. With a total corrected increment (TCI) of > 3 there was a sharp increase in the specificity index for rejection that reached almost 100% at a TCI of ≥ 4. Sensitivity for rejection at this level was only 20%. Between a TCI of 2.5 and 2.9 the sensitivity increased to 75%, with specificity for rejection around 75%. There was an upregulation of ICAM-1 and IL-2R when FNAC diagnosed rejection but with a large overlap of the results when compared either to normal graft or acute tubular neurosis. The mean TCI during the week preceding the rejection episode was 2.5 and the TCI reached a mean value of ≥ 3 only during rejection. The peak ICAM-1 and IL-2R expression occurred during the week preceding the clinically evident rejection episode. The expression of ICAM-1 by ≥ 70% of the tubular cells increased the specificity for rejection of a TCI of ≥ 2.5 to 100%. In the same way, the specificity for rejection increased up to 90% when eight to ten IL-2R-positive lymphocytes were seen along with a TCI of ≥ 2.5. There was no further increase in specificity after that. A specificity index of 100% for rejection could be obtained for moderate levels of both ICAM-1 (70% or more tubular cells) and IL-2R (eight or more lymphocytes). ICAM-1 expression in 70% or more tubular cells and/or IL-2R expression in eight or more lymphocytes was found in 58% of the FNAC aspirates with a TCI between 2.5 and 2.9. In conclusion, the

expression of IL-2R in lymphoid cells and ICAM-1 in tubular cells was upregulated during rejection episodes and the upregulation preceded both the clinical and the routine FNAC diagnosis of rejection by 1 week. The ddition of these markers to the FNAC increased substantially the specificity of the FNAC to diagnose rejection.

Key words Graft rejection · Aspirative cytology · Renal transplantation · ICAM-1 · Interleukine-2 receptor

Introduction

Acute rejection is associated with a poor long-term prognosis for renal allografts [1]. Sequential fine-needle aspiration cytology (FNAC), as proposed by Häyry and Willebrand [2], has been used to monitor allografts for acute cellular rejection (ACR). However, FNAC diagnoses rejection only when an unequivocal amount of infiltrating cells are already damaging the graft and, therefore, in some borderline cases (with a low increment of inflammatory cells in the graft), FNAC lacks the specificity to diagnose rejection. In these cases, the number of inflammatory cells within the graft can decline, stabilize or increase over time. Consequently, there is the need for a parameter that identifies ongoing rejection before it develops fully.

Some molecules directly involved in the rejection mechanism, for example HLA class-II antigens, intercellular adhesion molecule-1 (ICAM-1) and interleukin-2 receptor (IL-2R), are upregulated within the graft during rejection [3–10] and usually before that the final rejecting cells start destroying the graft [5, 8, 9].

However, in almost all published studies that have reviewed the expression of these molecules in FNAC during an acute rejection episode, the results obtained with these markers have been analysed separately from those of the routine FNAC morphology. This approach requires that the sole expression of these molecules in the aspirates, independent of the number and the type of infiltrating cells, is able to distinguish acute rejection from other events occurring immediately after renal transplantation. Also, upregulation of these molecules has been seen during infection and even in stable grafts [10].

In this study, we sought to determine whether the addition of the expression of ICAM-I, HLA-DR and IL-2R along with the usual FNAC morphology can contribute to the detection of acute rejection earlier than the FNAC cytological results alone.

Patients and methods

During a period of 4 months, 24 patients received consecutive renal transplants at our unit. Recipients were 8 females and 16 males with ages ranging from 12 to 67 years. Of these 24 patients, 16 received cadaveric and 8 living-related kidneys, and 21 patients were on cyclosporin A/azathioprine/prednisone while 3 were on azathioprine/prednisone only; 23 were first transplants. None of these pairs were HLA-identical.

In these patients, 117 FNAC samples were obtained according to the method of Häyry and Willebrand [2]. The aspirates started to be collected 4 days after transplantation on an every-other-day basis. Aspirates were mixed with 10 ml cell culture medium (RPMI-1640 (Gibco, EUA) enriched with 5 ml/l 20% human albumin, 25000 U/L heparin and 1 ml 1 *M* Hepes.

The mixture was centrifuged at 800 rpm for 8 min and resuspended in phosphate-buffered saline.

Twelve samples (six peripheral blood and six renal aspirate) of 100 µl each were centrifuged on slides with absorbant pads in a Cytospin no. 2 at 500 rpm for 10 min. The material obtained was air-dried for 5 min and then was stained for microscopic analysis (May-Grünwald-Giemsa). If at least one cytopreparation (cytoprep) contained seven or more renal tubular cells it was considered adequate and then analyzed and compared with the blood sample, to obtain a total corrected increment (TCI) of inflammatory cells.

The FNAC samples were classified pathologically as:

1. *Normal Graft (NG)*: TCI < 2.5 and no changes in renal tubular cells
2. *Acute tubular necrosis (ATN)*: TCI < 2.5 and ischaemic changes in renal tubular cells
3. *Incipient immune activation (IIA)*: 2 · 5– < 3.0 regardless of the changes in tubular cells
4. *Acute cellular rejection (ACR)*: TCI ≥ 3 or the occurrence of more than three blast cells in the whole cytoprep.

In the adequate FNAC samples, the above routine was repeated for indirect immunoperoxidase staining with monoclonal antibodies ICAM-1 (British Biotecnology), IL-2R (Dakopatts, M731) and HLA-DR (Incor USP, Brazil).

Six renal slides were numbered and then frozen (–70°C) for a subsequent blind study without knowledge of the clinical evolution and/or the previous cytological results of the FNAC. The frozen slides were stained using the avidin-biotin peroxidase method as described elsewhere [11]. Positive and negative controls were obtained for each preparation. ICAM-1 results are expressed as the percentage of the total tubular cells in the whole cytoprep positively stained and the IL-2R results are expressed as the absolute number of positively stained lymphoid cells in the whole cytoprep.

At our institution, acute rejection is diagnosed on the basis of a protocol that requires clinical diagnosis in addition to confrimation with either FNAC or renal biopsy. Methylprednisolone (i.v. 1 g/day for 3 consecutive days) is used to treat rejection episodes without changing the baseline immunosuppression. For the purpose of this study, two nephrologists with wide experience in renal transplantation seperately reviewed each patient's record. This analysis took into consideration the whole clinical course, response to rejection treatment, cyclosporin blood levels, the course of the renal allograft function after each medical action, and the FNAC/renal biopsy results. By reviewing the patient's clinical course, the physicians defined the day on which the process of rejection had possibly started. The first day of any acute rejection episode was defined as day "zero", the previous days as –1, –2 etc. and the subsequent days as day + 1, d + 2 etc. This retrospective clinical classification was used as the "gold standard" to analyse the profiles of ICAM-

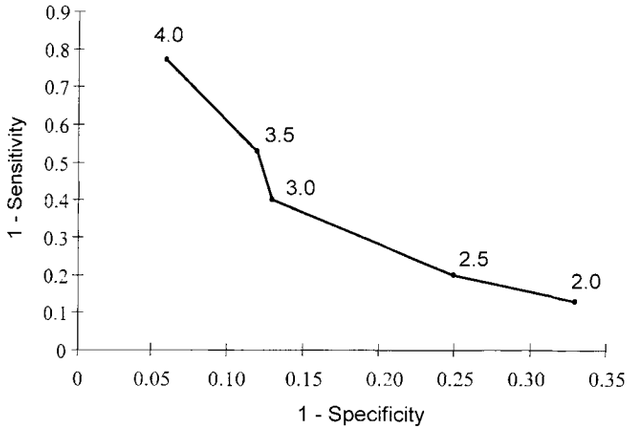

Fig. 1 ROC curve for five different levels of TCI and the diagnosis of rejection

1, HLA-DR and IL-2R during the course of a rejection episode. Consequently, the FNAC samples were grouped as follows:

1. *Never rejecting (NR)*: Cytopreps from patients who never showed a rejection episode
2. *-7 to -1 days*: Cytopreps within the week preceding a rejection episode
3. *0 to + 7 days*: Cytopreps within the week following a rejection episode
4. *> + 7 days*: Cytopreps more than 7 days after a rejection episode treatment

Specificity was calculated as TN/(FP + TN) and sensitivity as TP/(TP + FN), where TN is the number of true negative cases, FP the number of false positive cases, TP the number of true positive cases and FN the number of false negative cases. Data are presented as means ± SD. ANOVA was used to compare the variance between groups with the help of the software STATS-2. A *P*-value < 0.05 was accepted as significant.

Results

Nine patients (37%) did not suffer an ACR episode and 15 (63%) had at least one ACR episode. No steroid-resistant rejections were seen in the whole group. Of the 117 FNAC samples, 85 (72%) were considered adequate (seven or more renal cells/sample) for cytological analysis. In 70 renal aspirates, corresponding to 82% of the adequate FNAC samples, adequate slides for ICAM-1 and IL-2R analysis were also obtained.

Figure 1 shows the specificity and sensitivity indexes for rejection of the many TCI in our study population. With a TCI of > 3 there was a sharp increase in the specificity index for ACR that reached almost 100% at a TCI of ≥ 4. However, sensitivity at this level was only 20%. Between a TCI of 2.5 and 2.9 the sensitivity increased to 75%. However, at these moderate TCI values, specificity for ACR was around 75% only.

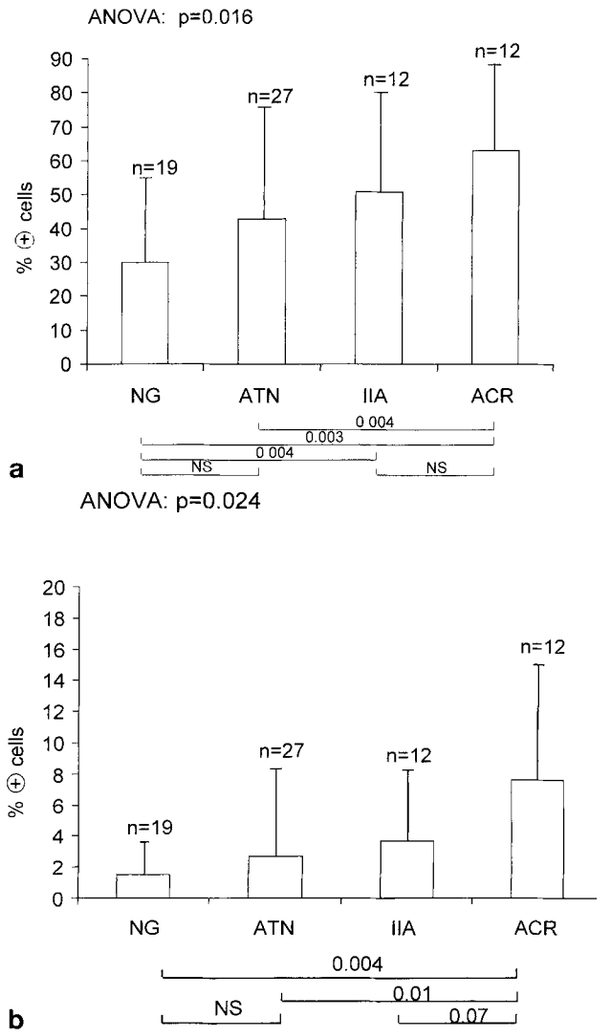

Fig. 2 **a** Relationship between ICAM-1-positive tubular cells and FNAC diagnosis. **b** Relationship between IL-2R-positive cells and FNAC diagnosis

Figure 2 a, b shows how ICAM-1 and IL-2R were expressed in the FNAC diagnosis. There was a highly significant increase in cells stained positively for either ICAM-1 or IL-2R in IIA and ACR when compared with NG and ATN. No differences were seen between IIA and ACR or between NG and ATN. Although HLA-DR slightly increased, during IIA and ACR diagnosis, this increase did not reach statistical difference (NG 42 ± 32%, ATN 48 ± 35%, IIA 51 ± 33% and ACR 56 ± 27%; *P* = NS).

Figure 3 shows the profiles of the TCI values and the expression of IL-2R and ICAM-1 according to the day of the rejection episode. The TCI increased steadily up to the day of rejection and diminished after treatment. The mean TCI during the week preceding the re-

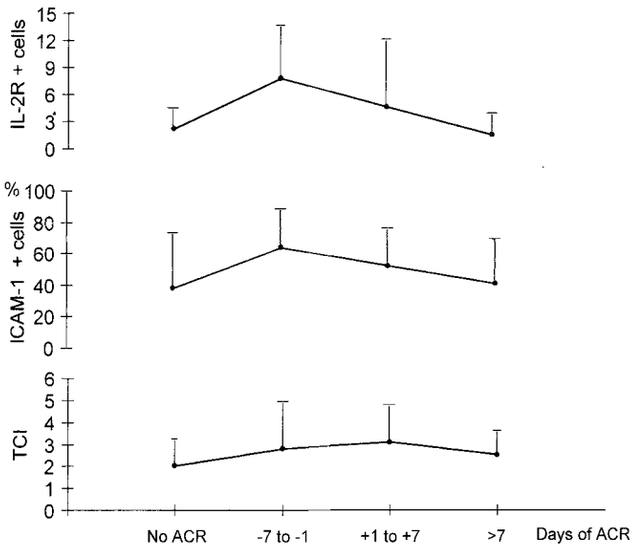

Fig. 3 Temporal relationship between ACR and IL-2R and ICAM-1 staining

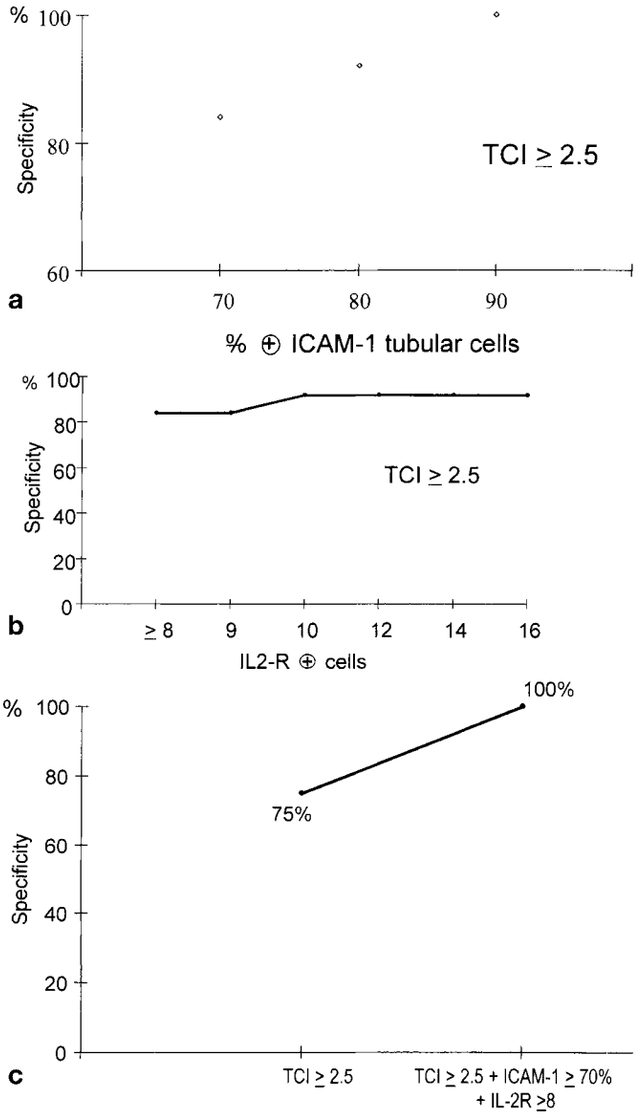

Fig. 4 **a** Specificity for rejection and ICAM-1 upregulation. **b** Specificity for rejection and IL-2R upregulation. **c** Specificity index with ICAM-1 expression in 70% or more cells and IL-2R in eight or more for TCI ≥ 2.5

jection episode was 2.5 and reached a mean value of ≥ 3 only during rejection. On the other hand, the peak of ICAM-1 and IL2-R expression occurred during the week preceding the clinically evident rejection episode. There was a slight decrease during the rejection episode and then a return to baseline values after treatment.

Figure 4a shows the increase in the specificity for ACR when ICAM-1 expression in 70% was added to a TCI of ≥ 2.5. The specificity increased to 100% when progressively higher values of ICAM-1 expression were added. Figure 4b shows the increase in the specificity for ACR, for a TCI ≥ 2.5, when eight or more cells positively stained for IL-2R are found in the cytoprep. The specificity for rejection increased up to 90% when eight to ten IL2-R-positive cells per cytoprep were seen. There was no further increase in specificity after that. Taken together, a specificity index of 100% for rejection could be obtained for ICAM-1 expression by 70% or more tubular cells and IL-2R expression by eight or more lymphocytes (Fig. 4c). We did not use HLA-DR results to recalculate specificity because they did not reach statistical differences in the many FNAC diagnosis.

Figure 5 shows the frequency of finding ICAM-1 expression by 70% or more tubular cells or IL2-R expression by eight or more lymphocytes together with a TCI in the range 2.5 to 2.9 and also with a TCI of ≤ 2.4. The expression of ICAM-1 by 70% or more tubular cells per cytoprep was found in only 45% of the FNAC samples, and the expression of IL2-R by eight or more lymphocytes per cytoprep was found in only 37% of the samples. However, taken together, ICAM-1 expression in 70% or more tubular cells and/or IL-2R in eight or more lymphocytes was found in 58% of the FNAC samples with a TCI between 2.5 and 2.9.

Discussion

This study shows that the analysis of the quantitative expression of ICAM-1 and IL-2R in renal aspirates along with FNAC cytological indexes can substantially increase the specificity of renal aspirates in diagnosing

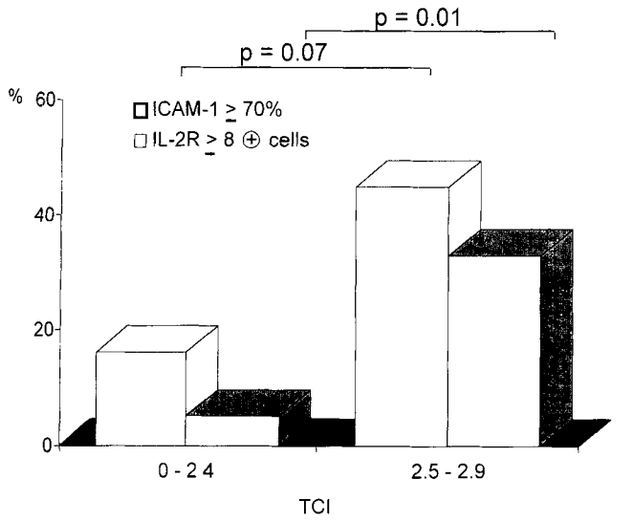

Fig. 5 Frequency of ICAM-1 and IL-2R upregulation in FNAC aspirates

ACR in borderline or ongoing rejection in renal allografts.

Only light to moderate rejection episodes were seen in this group of nonsensitized patients as shown by the absence of steroid-resistant rejections. Accordingly, the TCI values were smaller than usually seen in other periods and other patients [12, 13]. This probably happened because of the continuous monitoring of the renal allografts, with FNAC samples being collected almost every other day, allowing us to diagnose earlier subclinical rejections and to treat them during their onset.

This approach enabled us to detect borderline TCI (2.5–2.9) in FNAC samples. Borderline FNAC samples can either progress to a full rejection or can completely remit with time. Although a borderline TCI has a large sensitivity for rejection, it lacks specificity and therefore increases the number of unnecessary treatments and their unfavourable consequences. Because of this, a more specific marker for rejection is required.

IL-2R expression in lymphoid cells is a marker for activated lymphocytes [14]. During routine analysis of FNAC aspirates, blast cells largely contribute to a higher TCI. The expression of IL-2R in lymphocytes represents an earlier stage of blast changes before they can be seen cytologically in the FNAC aspirate [14]. In the same way, the upregulation of ICAM-1 in tubular cells precedes the cellular phase of rejection since LFA-1/ICAM-1 interaction appears crucial to this process. T-cell clones from LFA-1-deficient individuals have a reduced rate of migration through cultured endothelial cells [15] and antibodies against LFA-1 intensively reduce lymphocyte migration [16]. ICAM-1 is constitutively expressed in endothelial cells of the normal kidney. Nevertheless, acute rejection is associated with a diffuse staining of proximal tubules and focal staining of distal tubules and collecting ducts [10]. Also, the use of anti-ICAM-1 antibodies inhibits T-cell-mediated injury in vivo [17].

In this study, we showed that the quantitative analysis of both IL-2R and ICAM-1 expression can be used as specific markers of rejection. However, the expression of these markers was interpreted only in the presence of some renal cellular infiltrate to increase the sensitivity of the method. We used the cellular infiltrates as a screen for rejection with a high sensitivity rate and the upregulation of ICAM-1 and IL-2R to increase the specificity for rejection in borderline cases. Both ICAM-1 and IL-2R were shown to be upregulated during acute rejection and during IIA compared with normal grafts or ATN. The same results have also been found by others [4, 6, 8, 9]. Nevertheless, there was a marked overlap of results that prevents these markers being used as sole markers of rejection.

The approach of analyzing the expression of these two immunomarkers only for borderline TCI is a novel. Most previous studies have tried to obtain high sensitivity and specificity indexes for rejection using the expression of these antigens as sole markers [18–20]. In our opinion this policy is not appropriate because the upregulation of these markers can be seen in other situations, for example in cytomegalovirus and bacterial infections [18, 19]. Also, the isolated expression of these markers does not necessarily mean that the cellular infiltrative phase of acute rejection will develop. On the other hand, by associating these markers with a few invasive cells one can speculate that very early rejection is already ongoing, justifying treatment.

For these reasons, we believe that the expression of ICAM-1 and IL-2R, is only valid as an indicator of rejection in the presence of cell infiltration that suggests rejection but has not yet fulfilled the established criteria for rejection. This rationale can be extended not only to a borderline TCI in FNAC samples, but also to borderline changes in renal allograft biopsies [21].

The continuous monitoring of these markers during a rejection episode demonstrated that beyond being upregulated during rejection, the expression of both ICAM-1 and IL-2R in renal aspirates preceded the clinical and FNAC diagnosis of rejection by a couple of days (Fig. 2). Fuggle et al. have also demonstrated that increased expression of ICAM-1 precedes a rejection episodes by 1 week [3]. Furthermore, Willebrand et al. have detected increased ICAM-1 expression in tubular cells at the very beginning of blastogenic rejection [6].

More impressive than the finding of the expression of these two molecules was the enhanced specificity for the diagnosis of rejection they gave to a borderline TCI of ≥ 2.5 (Fig. 3). Either ICAM-1 in more than 90% of cells or IL-2R in more than ten lymphoid cells resulted in a specificity index for rejection of 100%. Even small-

er numbers of the two markers taken together could achieve the same indexes (Fig. 3).

Finally, this kind of interpretation could not be valid if the findings of the expression of ICAM-1 in 70% or more tubular cells and IL-2R in eight or more lymphocytes were rare. The analysis of the frequency of these markers showed that at least one of them can be encountered in as many as 58% of the renal aspirates with a TCI of > 2.5. This frequency, in our opinion, largely validates the use of these markers, mainly because the immunoperoxidase method has to be performed only when one finds a borderline TCI. At our institution, it takes approximately 4 h to obtain the immunoperoxidase-stained slides.

In contrast to previous reports [6, 10, 22], we could not demonstrate any significant difference in the expression of HLA-DR during rejection episodes compared with the expression in ATN or stable grafts, although it increased in the former. Moolenar et al. have also found nonsignificant upregulation of HLA class-II antigens during rejection [23]. Likewise, Fuggle et al. found that 26% of rejection episodes do not have marked expression of HLA class-II molecules [3]. This could explain why increased expression during rejection did not reach statistical significance.

In conclusion, the expression of IL-2R and ICAM-1 in cytopreps was upregulated during rejection episodes and preceded both the clinical and the routine FNAC diagnosis of rejection by approximately 1 week. When borderline TCIs were found in FNAC aspirates, the evaluation of these two markers increased markedly the specificity of FNAC to diagnose rejection.

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