Flow cytometry quantitation of peripheral blood T-cell subsets as a monitor of renal allograft rejection[1]

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In an attempt to predict acute renal allograft rejection, the authors prospectively studied peripheral blood T-cell subsets in 38 patients (an average of 4.2 determinations per patient) during the early posttransplantation period using the fluorescence activated cell sorter (FACS II) and monoclonal antibodies reactive with the total T-cell population (OKT 3), helper T cells (OKT 4), and suppressor T cells (OKT 8). All patients were treated with prednisone and azathioprine according to standard protocols, and all patients were initially treated with horse anti-human lymphocyte globulin (ALG) for an average period of 12.6 days. The rejection group consisted of 22 patients who rejected the allograft within one month of the last T-cell subset measurement. The rejection group, but not the nonrejection group, showed a significant increase ($P < 0.0005$) between the average T-helper to T-suppressor ratio (T4/T8 ratio) during the ALG (T4/T8 = 1.36) and post-ALG (T4/T8 = 2.30) therapy periods. Predictive value analysis showed that no patient with a peak T4/T8 ratio of less than 1.3 after cessation of ALG had a clinical rejection episode. However, each patient whose peak T4/T8 ratio after cessation of ALG was greater than 2.3 times his own average T4/T8 ratio while receiving ALG therapy (incremental T4/T8 index) had a subsequent episode of rejection. In the rejection group there was a rising trend in the mean T4/T8 ratio as the day of rejection approached (1.92, 2.11, 2.15, 2.47, and 3.18 during days 15–21, 8–14, 5–7, 3–4, and 1–2 before rejection, respectively).

Index terms: Antibodies, monoclonal · Flow cytometry · Kidney, transplantation · Surveillance, immunologic · T lymphocytes

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Many different immunologic assays have been applied to the problem of monitoring renal allograft recipients in an

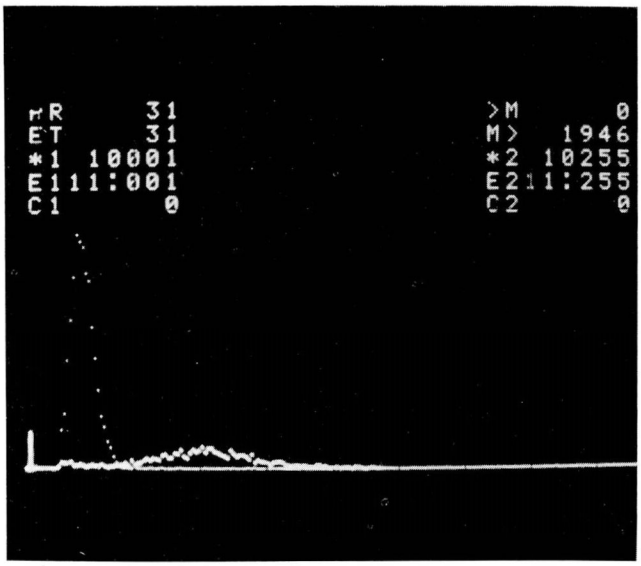

Fig. 1. Computer overlapped histograms of the T-helper (left) and T-suppressor (right) populations showing a marked increase in T4/T8 ratio. The *y*-axis corresponds to the number of lymphocytes, and the *x*-axis to arbitrary units of fluorescence intensity on a linear scale. The areas under the curves, which can be integrated electronically, are proportional to the number of lymphocytes in each population. In this particular case, T4 = 60.1%, T8 = 18.6%, and T4/T8 = 3.23.

attempt to detect a prerejection or early rejection state.[1–3] The reliable detection of such a high-risk state would allow modification of the immunosuppressive therapy early enough to possibly abort clinical rejection episodes and diminish subsequent damage to the graft. Immunologic tests that require the use of donor antigens or cells[4,5] are theoretically attractive, but tend to be technically cumbersome.[3] Non-donor-specific assays have included enumeration of total T- and B-lymphocyte concentrations, lymphocyte transformation tests with nonspecific mitogens, dinitrochlorobenzene skin tests, and recently, measurements of T-cell immunoregulatory subsets.[6–11] It has been proposed that alteration in the numeric balance of circulating helper and suppressor T-lymphocytes occurring after renal transplantation may reflect the immune response of the recipient to foreign donor antigens. Estimation of this balance between helper and suppressor T cells by enumeration of OKT 4^+ (inducer-helper) and OKT 8^+ (suppressor-cytotoxic) T-lymphocytes[12] expressed as the T4/T8 ratio, may allow the recognition of patients who are at either especially high or low risk of rejection of the allograft.

The current study evaluated the relationship between the T4/T8 ratio and subsequent acute renal allograft rejection episodes. The results suggest that both high- and low-risk groups can be identified.

Patients and methods

Patient selection

The group analyzed consisted of 38 renal allograft recipients, representing 67% of the kidney transplants performed at the Cleveland Clinic between February and October 1981. These patients were selected for analysis based on the performance of at least one T4/T8 ratio determination during the hospital course. Eight of the 38 patients were living related donor allograft recipients, and 30 received cadaveric kidneys. The latter more homogenous subgroup was also separately analyzed. The underlying renal disease included some form of glomerulonephritis (17), obstructive or reflux-associated nephropathy (7), diabetes mellitus (5), parenchymal malformation (4), hypertension with nephrosclerosis (3), and end-stage renal disease (2). For 32 patients, this was the first renal allograft, and for 6 it was the second.

Patient treatment

Preoperative and postoperative management was standardized by protocol. All patients were given azathioprine 3 to 5 mg/kg in the 12 hours before transplantation and were then maintained on 1.5 to 2 mg/kg/day thereafter. One gram of intravenous methylprednisolone (IVMP) was administered in divided doses on the day of surgery. Postoperatively, all patients also received intravenously equine anti-human lymphocyte globulin (ALG) produced at the University of Minnesota for an average time of 12.6 ± 2.7 days at a dose of 15 to 30 mg/kg/day. Cadaver allograft recipients were randomized to a high (2 mg/kg/day) or a low (30 mg/day for two months) initial prednisone dose with a tapering schedule thereafter. Rejection episodes were treated either with intravenous methylprednisolone, one gram in divided doses daily, or with a second 10-day course of ALG at a dose of 20 mg/kg/day while maintenance immunosuppression with azathioprine and prednisone was maintained.

Diagnosis of rejection

Renal allograft rejection episodes were clinically defined on the basis of a rise in serum

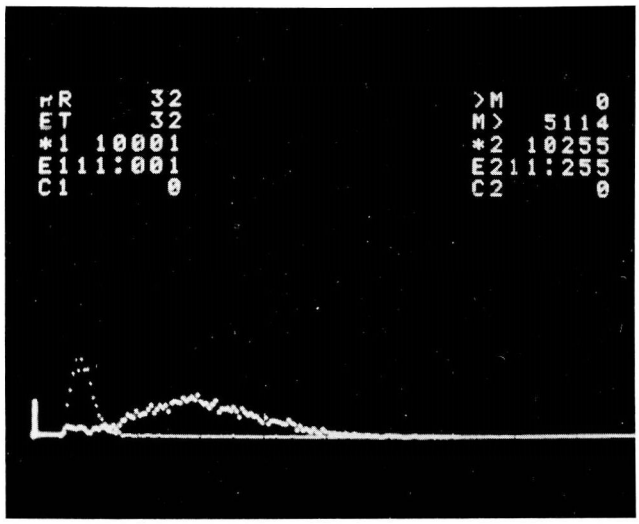

Fig. 2. Similar histograms showing a marked decrease in the T4/T8 ratio. T4 = 20.9%, T8 = 51.5%, and T4/T8 ratio = 0.41.

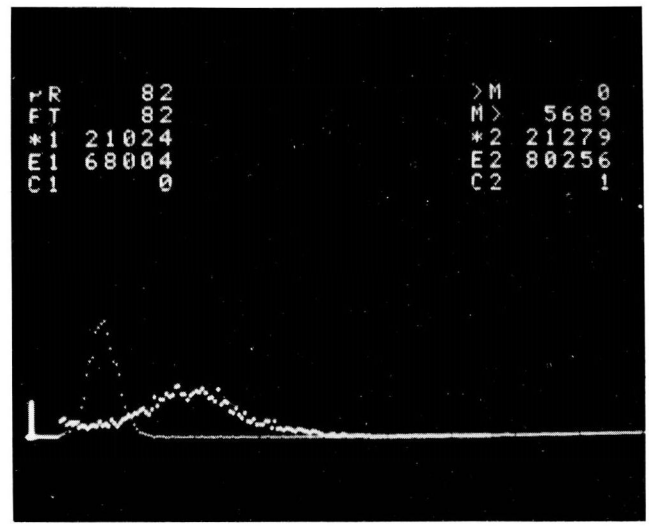

Fig. 3. These histograms show a normal T4/T8 ratio = 1.82.

creatinine levels, decrease in urine output, transplant pain or tenderness, fever, and radionuclide flow changes. For analytic purposes, rejection was considered to have occurred if and when antirejection treatment was instituted. Recipients experiencing a rejection episode within one month of the final T4/T8 ratio determination were included in the "rejection" group, and other patients were included in the "no rejection" group, regardless of later clinical events.

T4/T8 analysis

A total of 158 blood samples from transplant recipients were analyzed for T4/T8 ratios, an average of 4.2 determinations per patient. Of these, 55 were during a time when the patient was receiving ALG therapy (a mean of 1.4 tests per patient during ALG), and 103 ratios were measured after the patients had completed the ALG course, but before any rejection episode commenced (a mean of 2.7 tests per patient after ALG).

Peripheral blood T cells (T3), T-helper lymphocytes (T4), and T-suppressor lymphocytes (T8) were quantitated using the fluorescence activated cell sorter (FACS II) (Becton Dickinson, Mountainview, Calif.) and mouse monoclonal antibodies: OKT 4, T helper-inducer; OKT 8, T suppressor-cytotoxic (Ortho Diagnostics, Raritan, N.J.). After initial purification with Ficoll-Hypaque, the lymphocyte preparations were divided into aliquots and incubated with the monoclonal antibodies and stained with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Cappel Labs, Westchester, Pa.). As controls, lymphocytes were processed as previously described but omitting the incubation step with monoclonal antibodies. The FACS was adjusted with glutaraldehyde-fixed chicken red blood cells, and appropriate gating to exclude undesired contaminating cells was carried out on the basis of forward light scatter. At least 10,000 cells from each preparation were counted, and the number of T cells (T3), T-helper lymphocytes (T4), and T-suppressor lymphocytes (T8) was calculated by electronically dividing the 100× fluorescence profile by the total scatter profile of the test sample and subtracting similar profile of the control sample. The helper to suppressor T-cell ratio (T4/T8) was calculated by dividing the percentage of T4 positive mononuclear blood cells by the percentage of T8 positive mononuclear blood cells. Examples of computer superimposed T helper (T4) and T suppressor (T8) fluorescence histograms are depicited in *Figures 1–3*.

Absolute T3, T4, and T8 lymphocyte counts were calculated by multiplying the fraction of fluorescent mononuclear cells, detected with the appropriate monoclonal antisera, by the absolute lymphocyte count derived from an automated total white blood cell count (Coulter S^+) and an automated differential lymphocyte determination (Hematrak).

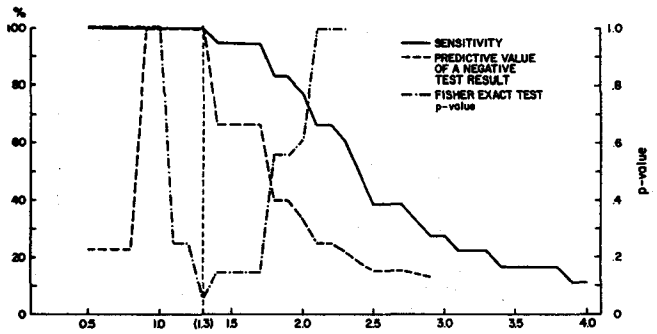

Fig. 4. Predictive value analysis of peak T4/T8 ratio in the first week after cessation of anti-human lymphocyte globulin treatment, in 30 cadaveric allograft recipients. This diagram shows that a T4/T8 ratio of 1.3 is associated with optimal sensitivity, optimal predictive value of a negative test result, and a minimum *P* value.

Statistics

The data were analyzed using both the time-averaged mean and peak T4/T8 ratios. For comparisons within and between groups, the mean T4/T8 ratios during various defined time periods throughout the hospital course were calculated, with each patient contributing one mean T4/T8 value for a given time interval to the overall group mean ratio. Comparison within groups used the paired t test, and between-groups comparisons were based on the (nonpaired) Student's t test. An overall significance level of 0.05 was divided by 100 to compensate for total number of individual statistical comparisons (131) to generate an individual α-level of 0.0005 for each hypothesis test, meaning that the calculated *P* value for each comparison had to be < 0.0005 to be considered significant.

Predictive value analysis was performed using a PDP-15 computer and standard definitions.[13] This calculation used the peak T4/T8 ratio recorded during the first week after ALG therapy was discontinued, or the peak T4/T8 ratio from any time during the remainder of the hospitalization following cessation of ALG until a rejection episode developed or the patient was discharged. An additional calculation used the peak T4/T8 ratio divided by that same patient's mean T4/T8 ratio from the time period during which ALG treatment was given (incremental T4/T8 index), which represented an index of how much the patient's T4/T8 ratio increased following the discontinuation of ALG infusion. Predictive value tables were constructed and the sensitivity, specificity, predictive value of a positive test result, predictive value of a negative test result, and false-positive and false-negative percentages were calculated. A Fisher's exact test was performed on the 2×2 contingency table. This technique resulted in choosing the optimal T4/T8 ratio or incremental T4/T8 index, which generated the most medically significant classification of patients into high and low risk of groups for rejection. Such data were generated at multiple hypothetical T4/T8 ratio cutpoints between 0.5 and 4.0, at intervals of 0.1. A sample graph showing the variation in sensitivity, predictive value of a negative test result, and the *P* value from the Fisher's exact test is shown in *Figure 4.* The most discriminative T4/T8 level was selected from this tabulated or graphed data, and represented the T4/T8 ratio that maximized the efficiency of classification of patients into rejection and no rejection groups, and that produced a local minimum *P*-value from the Fisher's exact test.

Table 1. Mean T4/T8 ratios at various time periods before rejection

Days before rejection	Cadaveric transplant patients			Total group of recipients		
	Mean T4/T8	SD	n	Mean T4/T8	SD	n
1–2	3.18	2.04	4	3.18	2.04	4
3–4	2.37	0.97	10	2.47	0.98	11
5–7	2.12	0.53	9	2.15	0.51	10
8–14	2.11	0.76	11	2.11	0.76	11
15–21	1.96	0.84	9	1.92	0.80	10

Mean = mean T4/T8 ratio in patients who rejected, at various time intervals before rejection, *SD* = standard deviation, and *n* = number of patients contributing T4/T8 data to that time period.

Results

A total of 38 patients were studied. Of these, 22 experienced a clinical allograft rejection episode within one month of the date of the final T4/T8 ratio determination, and 16 did not. The rejection group included 12 males and 10 females, had a mean age of 32.1 ± 12.2 years, and had an average of 2.50 ± 1.01 HLA-AB and 0.68 ± 0.75 HLA-DR loci mismatched with respect to the donor. These patients received ALG for a mean time of 13.09 ± 2.37 days following the day of transplantation. The group of patients who did not reject the allograft consisted of 10 males and 6 females, had an average age of 33.6 ± 13.0 years, and was mismatched for 0.81 ± 1.17 HLA-AB and 0.60 ± 0.63 HLA-DR loci. This group was treated with ALG for an average

of 11.88 ± 2.99 days following transplantation. Of the above parameters, only the number of mismatched HLA-AB loci were significantly different between the two groups, with a *P* value of 0.00003 by Student's t test. There was no demonstrable statistical difference in the mean T4/T8 ratios after ALG treatment between the group of recipients with 0–1 HLA-AB mismatches (T4/T8 = 2.22 ± 1.23) and the group with 2–4 HLA-AB mismatches (T4/T8 = 2.27 ± 0.78).

The subgroup of 30 patients who received cadaveric allografts included 21 patients who experienced a clinical rejection episode within one month of the final T4/T8 ratio measurement, and 9 patients whose posttransplant course was free of rejection. There were no significant differences between the cadaveric allograft patients who did or did not experience rejection with respect to age, sex, number of HLA-DR loci mismatched, and the length of time during which ALG was administered postoperatively. The mean number of mismatched HLA-AB loci was 2.52 ± 1.03 in the group with rejections, and 1.11 ± 1.36 in the group without rejections, with $P = 0.004$ by Student's t test. Thirteen of the cadaveric allograft patients were managed postoperatively under the high-dose steroid protocol. Seven of these patients experienced rejection of the allograft, and 6 did not. Fourteen of the 17 patients managed under the low-dose steroid protocol had rejection episodes. This apparent difference in the frequency of rejection between the high- and low-dose steroid groups was not significant by either the chi-square or Fisher's exact test.

The mean T4/T8 ratios increased in a monotone fashion as the day clinical rejection was recognized approached (*Table 1*). The relatively large standard deviations of the data precluded attainment of statistical significance by this trend. At no time during the posttransplant hospital course did the mean T4/T8 ratio significantly differ between the group of patients who rejected and those who did not (*Table 2*).

The mean T4/T8 ratios were relatively lower in all groups analyzed during the time when ALG was infused (*Table 3*). The increase in the T4/T8 ratio was numerically greater and significant by the paired t test in the group of patients who eventually demonstrated rejection episodes.

Predictive value analysis identified a T4/T8 ratio of 1.3 as the most discriminant level. Six of 38 patients demonstrated T4/T8 ratios that were less than 1.3 at all times after cessation of ALG therapy, and none of these 6 patients experienced a rejection episode (*Fig. 5*). In this overall group of 38 patients, both the sensitivity and the predictive value of a negative result were 100% (*Table 4*). The predictive value of a positive test result (having a peak T4/T8 ratio greater than

Table 2. Comparison of mean T4/T8 ratios between recipients who did not reject allograft

	Patients who rejected			Patients who did not reject			t test
	Mean	SD	n	Mean	SD	n	*P* value
Cadaveric transplant patients (n = 30)							
During ALG	1.37	0.68	15	1.70	0.62	5	0.350
1st wk after ALG	2.46	0.76	18	2.49	1.32	6	0.935
2nd wk after ALG	2.67	1.46	9	2.88	1.15	4	0.806
Any time after ALG	2.38	0.79	18	2.24	1.23	9	0.714
Total group of recipients (n = 38)							
During ALG	1.38	0.66	16	1.53	0.65	8	0.597
1st wk after ALG	2.49	0.75	19	2.12	1.21	10	0.314
2nd wk after ALG	2.67	1.46	9	2.44	1.48	11	0.733
Any time after ALG	2.42	0.79	19	2.05	1.14	16	0.268

Mean = T4/T8 ratio, *SD* = standard deviation, *n* = number of patients contributing data during this time interval, and *ALG* = anti-human lymphocyte globulin.

Table 3. Increases in the mean T4/T8 ratios after cessation of ALG therapy

	Rejection group			No rejection group		
	Mean	SD	n	Mean	SD	n
Cadaveric transplant patients						
During ALG	1.35	.61	13	1.70	.62	5
1st wk after ALG	2.42*	.61	13	2.40†	1.45	5
Any time after ALG	2.24*	.48	13	2.45†	1.48	5
All ALG-treated patients						
During ALG	1.36	.59	14	1.53	.65	8
1st wk after ALG	2.47*	.61	14	2.00†	1.32	8
Any time after ALG	2.30*	.51	14	2.09†	1.38	8

* These T4/T8 ratios are significantly different from the "during ALG" values, by the paired t test, with $P < 0.0005$.

† These T4/T8 ratios are not significantly different from the respective "during ALG" values.

ALG = anti-human lymphocyte globulin, *Mean* = mean T4/T8 ratios, *SD* = standard deviation, and *n* = number of patients contributing data to this time period.

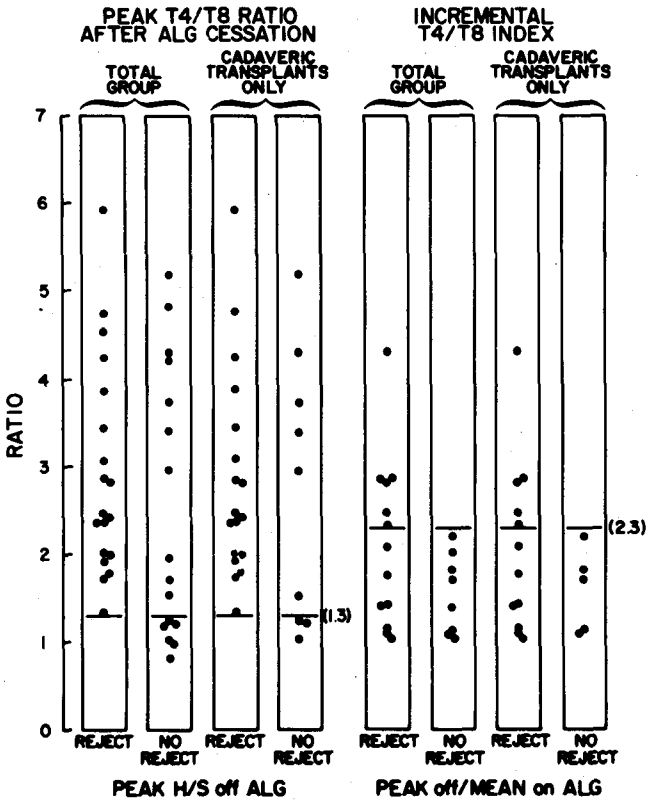

Fig. 5. This scattergram depicts the T4/T8 ratios and the incremental T4/T8 index in the various populations studied.

1.3 at any time after ALG therapy was halted) was only 66%, in that only 19 out of the 29 such patients rejected the allograft.

Twenty-one of the 38 patients had sufficient data to allow calculation of an incremental T4/T8 index (*Fig. 5*). Six patients had an index greater than 2.3, and each of these patients subsequently experienced a clinical rejection episode, making both the specificity and the predictive value of a positive test result 100% (*Table 5*).

Discussion

Prior investigations have suggested that the balance of helper and suppressor T lymphocytes in the peripheral blood may be related to an underlying process of renal allograft rejection.[6–11] Studies using either monoclonal antibodies[6–11] or IgM and IgG Fc receptors[11] to identify helper and suppressor T cells have concluded that a normal to high ratio of helper to suppressor cells is associated with an increased risk of rejection, whereas recipients who maintain a depressed helper to suppressor ratio have a lower risk of rejection. The data from the current study tends to confirm these suggestions. The T4/T8 ratio has a large variance in both rejecting and nonrejecting groups of graft recipients, and the degree of overlap between these two populations is great. A T4/T8 ratio of 1.3 was found to have the greatest discriminative value, and allowed recognition of a group of recipients with a low risk of rejection. No patients with a peak T4/T8 ratio less than 1.3 after cessation of ALG therapy experienced a rejection episode in the time frame of this study. The converse, however, was not true. Recipients with a post-ALG peak T4/T8 ratio of over 1.3 had a 66% chance of experiencing a rejection episode. Given that 58% of the total group of 38 patients had rejection episodes anyway, a high T4/T8 ratio did not materially increase the risk of rejection. The posterior probability was not markedly different from the prior probability. No T4/T8 level could be identified that was associated with a high positive predictive value for rejection.

Examination of the mean T4/T8 ratios showed that, as a group, the recipients who experienced rejection episodes tended to have a larger numeric increase in the ratio following cessation of ALG infusion. This suggested that it might be possible to identify patients who had a high risk of rejection by establishing a baseline T4/T8 ratio during the time of ALG infusion and comparing later T4/T8 ratios to that individual reference level. The incremental T4/T8 index reflects the degrees of increase in the T4/T8 ratio after the period of posttransplant ALG immunosuppression is over, compared to that same individual's baseline ratio while receiving ALG. The most discriminative incremental T4/T8 index was 2.3, in that each of the 6 recipients whose peak index was greater than this level experienced a subsequent rejection episode, making the predictive value of a positive test 100%. A peak incremental T4/T8 index of less than 2.3 was less helpful in establishing the prognosis for rejection, in that only 50% of recipients with an index less than this value were free of subsequent rejection episodes. Since predictive values are dependent on prevalence, we cannot project our study data onto other kidney transplant patient populations unless the frequency of rejection in those populations is similar to that of our group.

The only major pretransplant difference between the rejection and nonrejection group of recipients was in the relative extent of HLA-AB

mismatching. Whether this difference alone, independent of a developing allograft rejection episode, could contribute to the trend towards higher T4/T8 ratios noted in the rejection group is unknown, but no significant differences in the T4/T8 ratios were found between recipients with relatively good and relatively poor HLA-AB matching.

Multiple factors could account for the large variance in the T4/T8 ratios. A superimposed infection, especially with cytomegalovirus,[14] can affect the T4/T8 ratio. The underlying etiology of the original renal disease may have an influence on the ratio.[15] The T4 and T8 markers are not specific for helper and suppressor lymphocytes. It is possible that relatively immature T lymphocytes, containing both T4 and T8 surface markers, may be released into the peripheral circulation during profound ongoing immunologic stimuli, leading to an inaccurate estimate of the numeric helper to suppressor T-cell ratio.[7] Inaccurate estimations may also be related to the technical difficulties involved in gating out contaminating cells in blood samples obtained from this particular patient population. The biologic variability of the T4/T8 ratio from hour to hour and day to day has not been well characterized. Also, pharmacologic immunosuppressive agents can affect circulating T-cell subsets.[16]

It may prove useful to investigate the relationship of other markers of immune activation to the risk of rejection, such as activated T-helper and activated T-suppressor lymphocytes using more accurate gating techniques, more specific antibodies, and multicolor fluorescent measurements. Flow cytokinetic analysis, using DNA and RNA fluorescent probes, might also provide useful information of immune activation. Active developmental work in the field of immunologic monitoring is underway in our laboratory. Sequential monitoring of specific immune activation by flow cytometry may allow the identification of both high- and low-risk groups of transplant recipients and may allow modified immunosuppressive regimens to be specifically tailored for individual patients.

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Table 4. Predictive value analysis of peak T4/T8 ratios, using T4/T8 = 1.3 as discriminant level

	Sens (%)	Spec (%)	PVP (%)	PVN (%)	FP (%)	FN (%)
Cadaveric transplant patients						
1st wk after ALG	100	33	82	100	67	0
Any time after ALG	100	33	75	100	67	0
All ALG-treated patients						
1st wk after ALG	100	40	76	100	60	0
Any time after ALG	100	38	66	100	62	0

Sens = Sensitivity, *Spec* = specificity, *PVP* = predictive value of a positive test result, *PVN* = predictive value of a negative test result, *FP* = false-positive ratio, *FN* = false-negative ratio, and *ALG* = anti-human lymphocyte globulin.

Table 5. Predictive value analysis of the incremental T4/T8 index (peak T4/T8 after ALG)/(mean T4/T8 during ALG) using 2.3 as the discriminant level

	Sens (%)	Spec (%)	PVP (%)	PVN (%)	FP (%)	FN (%)
Cadaveric transplant patients (n = 30)	46	100	100	42	0	54
All ALG-treated patients (n = 38)	50	100	100	53	0	50

ALG = anti-human lymphocyte globulin, *Sens* = Sensitivity, *Spec* = specificity, *PVP* = predictive value of a positive test result, *PVN* = predictive value of a negative test result, *FP* = false-positive ratio, and *FN* = false-negative ratio.

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