MONOCLONAL ANTIBODIES

Diagnostic and Therapeutic Use in Tumor and Transplantation

Edited by

Satya N. Chatterjee

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Contents

1 *T Lymphocyte Subsets in Renal Allograft Recipients*

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It has been shown repeatedly that subsets of immunoregulatory T cells in peripheral blood undergo considerable shifts after organ allotransplantation. However, the biological and clinical importance of these shifts still remains a matter of controversy. For example, it has been suggested that T_H:$T_{C/S}$ ratios in kidney allograft recipients may reflect nothing more than the efficacy of immunosuppressive treatment and are not directly related to immunologic effector function. This assumption has been contradicted by the observation of Ellis and co-workers[1] that specific anti-donor CML unresponsiveness is associated with periods of decreased T_H:$T_{C/S}$ cell ratios.

The clinical importance of T_H:$T_{C/S}$ cell ratio in immunological monitoring of kidney allograft recipients also remains controversial. After the original observation by Cosimi and co-workers[2] that patients with a stable ratio of above 1.3 are at greater risk of developing acute rejection compared to patients with low ratio, numerous deviations from that rule were reported, and the feasibility of T-subset ratio in prediction or confirmation of rejection was questioned.[3,4]

In part, this controversy can be attributed to technical reasons, especially difficulties in preparing pure lymphocyte suspension from a patient's blood sample, and evaluation of fluorescence in a sample containing lymphocytes at various stages of blastic transformation. In addition, the results are affected by different immunosuppressive regimens and the times when blood samples are drawn in the posttransplant period.

MATERIALS AND METHODS

Cells: Mononuclear cells were prepared from 14 ml heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation. Adherent cells were removed by plastic adherence technique. Lymphocytes were separated into T and B cells by AET-treated sheep red blood cell (SRBC) rosetting.[5]

Monoclonal antibodies: Aliquots of purified T lymphocytes were incubated for 30 minutes at 4C with the following monoclonal antibodies:

Leu-4 (marking all mature T cells), Leu-3a + b (helper/inducer subset), and Leu-2a (cytotoxic-suppressor subset). In several experiments, HLA-DR antibody was used as well to identify activated T cells. After thorough washing, antibody-treated cells were incubated with fluorescein-labeled goat anti-mouse IgG (TAGO, Burlingame, CA) following the procedure of Becton-Dickinson. Labeled cells were enumerated using fluorescent microscopy. At least 200 cells per sample were counted. Reproducibility of results obtained with such preparations was 0.93.

RESULTS

In our first set of experiments, we compared T-subset ratios in *long-term recipients* of allografts. Fifty-one transplant patients who had survived for two years or longer were studied in this group. All patients in this group were on immunosuppressive regimens of prednisone and azathioprine. The overall mean T-subset ratio was 1.97 ± 1.57, not different from normal controls: 1.93 ± 0.72. Patients were divided into groups according to kidney function.

Patients with good kidney function (below 200 μmol/L) had a mean T_H:$T_{C/S}$ ratio of 1.89 × 0.94. In patients with impaired but stabilized kidney function, the ratio was increased to 2.77 ± 3.49. However, a very wide range of variation in ratios was observed in this group, so that the differences between the two groups are not statistically significant. The ratio in the patients who have subsequently lost the transplant actually showed the lowest ratio (1.54 ± 0.55). Whether this signifies the decrease of immunological activity and cessation of chronic rejection, or is just a chance variation, it is difficult to say at the moment. Our observations in long-term recipients seem to be in agreement with the conclusions of Ellis and co-workers[1] and Colvin and co-workers[6] that in long-term grafts the correlation between T_H:$T_{C/S}$ ratio and risk of graft dysfunction does not apply.

In the second series of experiments, we studied the T-subset ratios in 38 *recently* transplanted patients less than one year after transplantation. The mean ratio per patient was calculated on the basis of 3–4 samples obtained at various intervals after surgery. The overall mean of T-cell subset ratio in this group was 2.17 ± 1.50. Data were analyzed separately for three subgroups: patients who had smooth posttransplant courses without rejection episodes; patients with one or more episodes of reversible rejection; and patients who subsequently underwent irreversible rejection. Patients with no rejection episodes had the lowest ratio (1.46 ± 1.43). In patients with reversible rejection, it was elevated to 2.52 ± 1.43 and with irreversible rejections, to 2.97 ± 1.15. The difference between the non-rejecting group and both groups with rejections was statistically significant (see Figure 1).

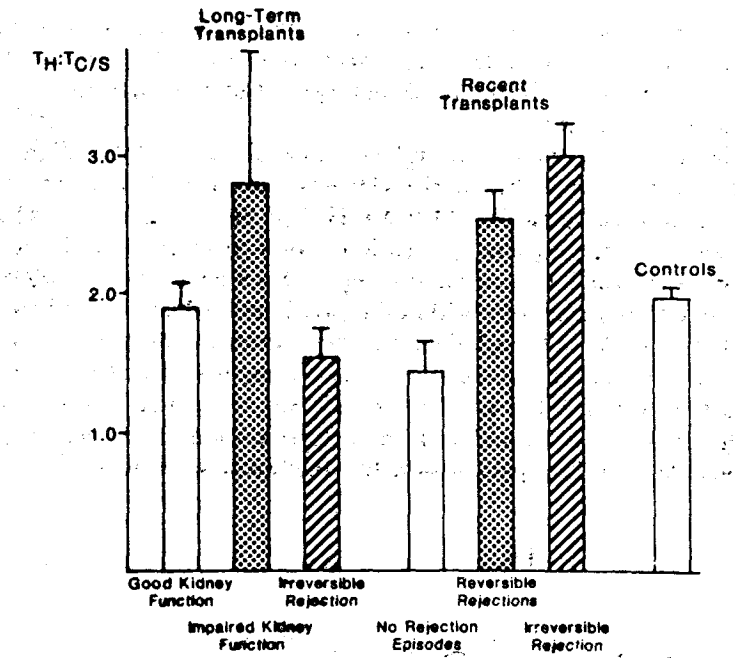

Figure 1 T_H:$T_{C/S}$ cell ratios in kidney allograft recipients and normal controls. Each bar represents mean T_H:$T_{C/S}$ cell ratio ± SE in a group of patients or controls. Differences between groups of long-term recipients are nonsignificant. Differences between nonrejecting group of recently transplanted patients and both groups with rejection was statistically significant ($p < 0.001$); difference between groups with reversible versus nonreversible rejections was not significant.

Thus, in recently transplanted patients, the correlation between the ratio and kidney function is obvious. However, the more important concern is the feasibility of enumeration of T-cell subsets in individual patients as a means of predicting the risk of graft rejection or detecting an ongoing rejection. This latter problem becomes increasingly important in patients on cyclosporine immunosuppression, since this drug is known to have nephrotoxic side effects. This means that when the increase in creatinine levels occurs, it may reflect either a rejection or cyclosporine toxicity. Obviously, the treatment in the two cases would differ considerably. To a certain extent, cyclosporine toxicity could be monitored by measuring serum cyclosporine levels, but individual variation in cyclosporine tolerance makes it very difficult to establish effective, yet safe doses for each patient. We have reasoned that if, indeed, an increase in T-subset ratio reflects activation of the patient's immune system, immunologic rejection should be accompanied by elevated ratios. In the case of cyclosporine toxicity, such elevation would not be expected. We have performed sequential T-subset determinations in 10 recently transplanted

patients. Blood samples were obtained before transplantation and then every week or, when possible, twice a week after surgery for two months. All patients were on cyclosporine (20 mg/kg) and alternate-day prednisone (1.5 mg/kg) treatment.

Figure 2 shows the posttransplant course of patient T.H., aged 15, whose end-stage renal disease (ESRD) was caused by membrano proliferative glomerulonephritis (MPGN). He had been on dialysis for half a year and had received nine transfusions. An increase in creatinine level from 90 to 150 μmol/L occurred starting on day 9 posttransplant. Levels of serum cyclosporine were within the safe limits, which we take to be 0.1 to 0.2 μg/ml (even though there may be considerable patient-to-patient variation). The increase in creatinine was accompanied by an increase in T-subset ratio from 2.4 to 4.8. This episode was interpreted as a rejection and the patient was treated with methylprednisolone, after which the creatinine leveled. The patient now has stable kidney function.

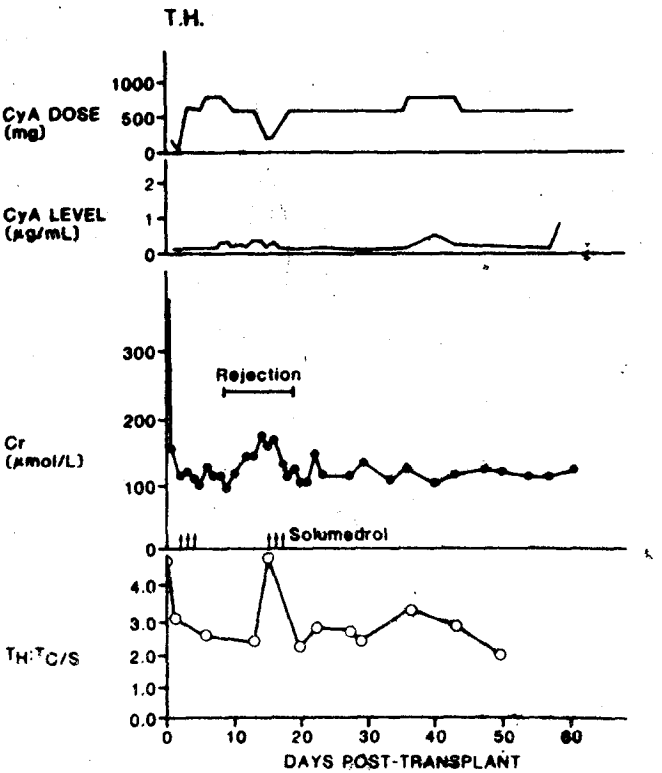

Figure 2 Posttransplant course of patient T.H., showing rejection episode accompanied by increase in T_H:$T_{C/S}$ ratio. CyA = cyclosporine, Cr = serum creatinine.

Figure 3 shows the posttransplant course of patient D.P., whose ESRD was due to Wegener's granulomatosis. He had been on dialysis for one year and was multitransfused. His serum creatinine started to rise on day 13 and reached the peak of 360 μmol/L on day 29. His serum cyclosporine levels were at that time close to 1 μg/ml. This was retrospectively interpreted as cyclosporine nephrotoxicity, not rejection. In this patient, the elevation in creatinine was not accompanied by a rise in T-subset ratio; it remained low during the whole observation time, never exceeding 0.7 μg/ml. No evidence of viral infection was found in this patient.

Figure 4 represents an example of an uneventful posttransplant course. Patient T.T., whose ESRD was due to MPGN, had been on dialysis for one week and was multitransfused (5 units) prior to transplantation. There were only slight fluctuations in his serum creatinine levels and T-subset ratio. There is no evidence of cyclosporine nephrotoxicity, although on several occasions his serum cyclosporine levels were as high as 0.6 μg/ml, nor was there evidence of rejection.

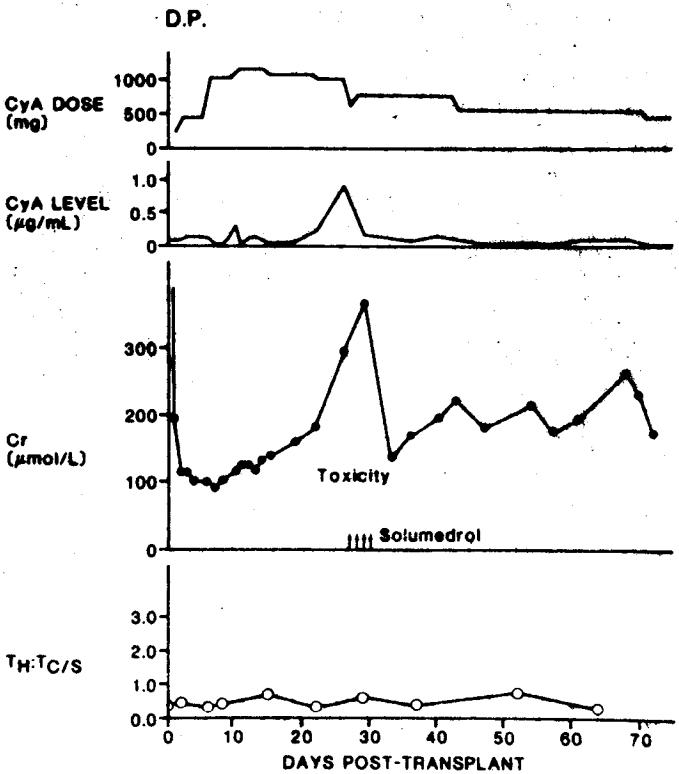

Figure 3 Posttransplant course of patient D.P., illustrating nephrotoxic effect of cyclosporine. Abbreviations: same as Figure 2.

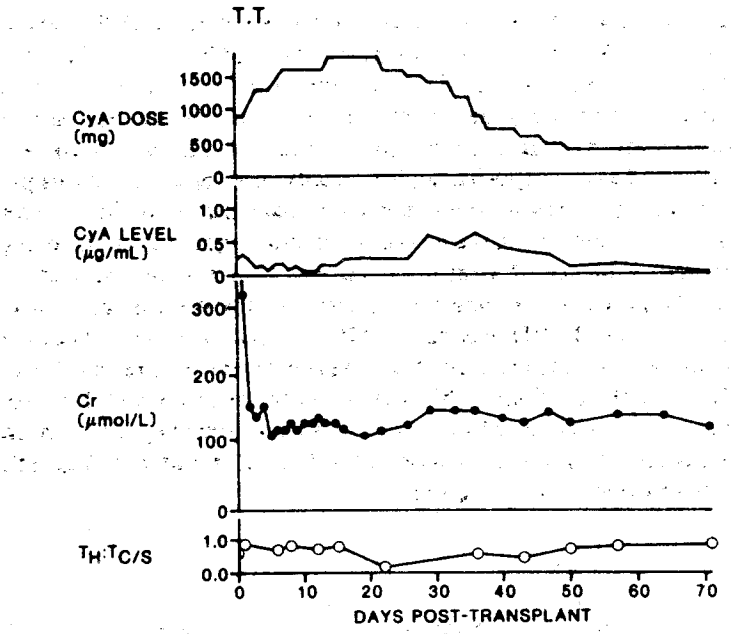

Figure 4 Patient T.T., uncomplicated posttransplant course. Abbreviations: same as Figure 2.

Figure 5 shows the posttransplant course of the second transplant of D.O., whose ESRD was caused by type 1 diabetes mellitus. She received a kidney from a cadaveric donor mismatched for one HLA-B and one DR antigen. At first, she was treated with cyclosporine. After the initial drop of creatinine immediately after transplantation, her creatinine rose progressively to plateau at the level of 800–900 μmol/L, with some fluctuations despite several courses of methylprednisolone. The initial levels of serum cyclosporine were not indicative of nephrotoxicity. Later they reached the level of 0.7 μg/ml and stayed high for several days, after which it was assumed to be cyclosporine nephrotoxicity and she was switched to azathioprine. Kidney function, however, was not improved and on day 57 the patient started dialysis.

Throughout her posttransplant course, the ratio was high, never dropping below 2.2. Peak ratio was 5.9. It is interesting to note that her first graft was also rejected within two months. That course had also been accompanied by consistently high T-subset ratios.

Several investigators have pointed out that, when monitoring kidney allograft recipients on the basis of T-subset changes, it is important to relate the changes in the ratio to pretransplant levels. Therefore, we have determined pretransplant ratios in all recently transplanted patients. We find no correlation between posttransplant T-subset ratios, expressed as

percentages of pretransplant levels, and subsequent kidney function. However, in retrospective analysis, we can generalize that patients with low pretransplant ratios had a more quiescent posttransplant course, with fewer rejections and lower creatinine values. It is possible that the pretransplant T-subset ratio may reflect immunological reactivity, and that a high pretransplant ratio may indicate a "high responder" to stimulation, either by previous transplant or blood transfusions.

We have examined the correlation of pretransplant ratios to the number of rejection episodes and the frequency of positive donor-specific lymphocyte mediated cytotoxicity (LMC) during the first four months after transplantation in 13 patients, using a T-subset ratio of 2.0 as a dividing line. As Table 1 shows, patients with a pretransplant ratio below 2.0 had a mean number of rejections of 0.2, whereas patients with a ratio over 2.0 had, on average, 1.4 rejections. The latter group also showed positive LMC at the time of clinical dysfunction in 10.3% of assays, whereas LMC was consistently negative in those low pretransplant ratios.

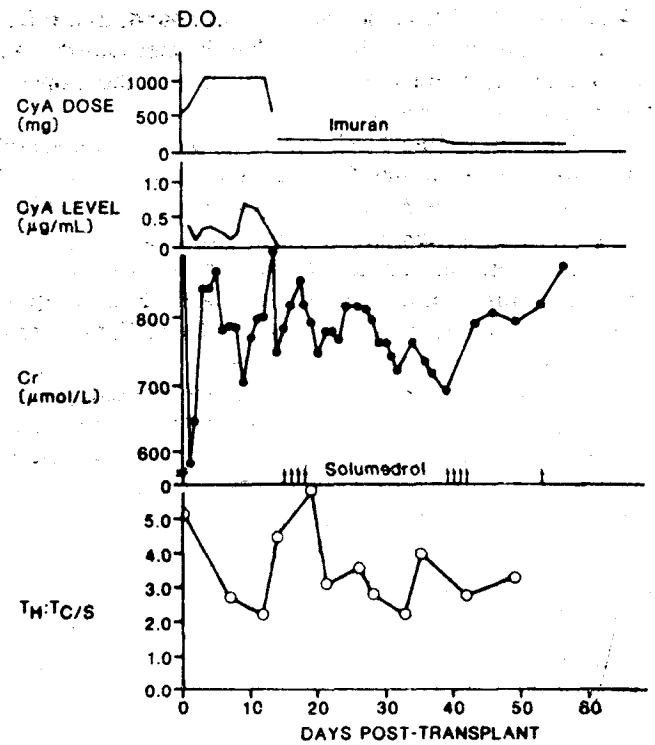

Figure 5 Posttransplant course of patient D.O., irreversible rejection. Abbreviations: same as Figure 2. Dx = dialysis.

Table 1
Effect of Pretransplant T_H:$T_{C/S}$ Ratio on Immunological Reactivity Post Transplant

T_H:$T_{C/S}$ Pretransplant	Number of Patients	Average Number of Rejection Episodes per Patient	% Positive LMC Assays in Relation to Phases of Clinical Dysfunction
< 2.0	5	0.2	0 (26)*
> 2.0	8	1.4	10.3 (39)*

*Number of assays for the group.

DISCUSSION

The value of T-subset determination as a means of immunological monitoring of kidney allograft recipients has been investigated in many transplant centers. Although a great degree of controversy is still present, the general consensus seems to be that a normal or high T_H:$T_{C/S}$ ratio is associated with an increased risk of rejection, whereas a low ratio signifies lower risk rejection.[1,2,7] This association is not found, as a rule, in long-term allograft recipients, which suggests that the mechanisms of graft acceptance in long-term recipients may be distinct from those operative during the immediate posttransplant period.[1]

Our findings concerning T-cell subsets in patients transplanted over two years ago did not reveal significant differences in T-cell ratio between long-term recipients grouped according to kidney function (Figure 1). In recently transplanted patients, on the other hand, the T_H:$T_{C/S}$ ratio is correlated to kidney function, the ratio being significantly lower in patients with stable kidney function than in recipients with rejection episodes and with irreversible rejection. However, the results of T_H:$T_{C/S}$ ratio determination should be interpreted with caution.

It has been pointed out that the ratio can be decreased by a superimposed infection, especially cytomegalovirus (CMV),[8] although it is not yet clear whether altered immunologic status precedes or follows viral infection. Colvin et al[6] have described CMV-associated glomerulopathy resulting in irreversible graft injury. In this group of patients, low ratios (below 1.0) were observed consistently. Thus, it appears that, in recently transplanted patients, T_H:$T_{C/S}$ ratio can serve as an indicator of the immunological status of a recipient, provided that the case is not complicated by viral infection.

Another factor that should be taken into account when evaluating the T_H:$T_{C/S}$ ratio is the possibility of selective redistribution of specific subpopulations: for example, sequestration of cytotoxic lymphocytes, at the onset of a rejection, from the circulation into the renal allograft. Analysis of lymphocytes obtained from kidney biopsy specimens, both

in terms of T-subset ratios and their functional activity, would provide more direct information on the involvement of various T-lymphocyte subsets in the rejection process. This information would make the conclusions based on numerical balance of T-cell subsets in peripheral blood more meaningful. Studies of this nature are now being initiated in our laboratory.

In patients on cyclosporine, as mentioned above, it is very important to be able to distinguish between immunological rejection and cyclosporine nephrotoxicity. Cyclosporine blood levels are not completely adequate for this purpose.

Our data show that sequential measurement of T-subset ratio may, on occasion, allow differentiation between rejection and nephrotoxicity: an increase in serum creatinine levels due to toxicity is not accompanied by changes in T-subset ratios, whereas a marked increase in the ratio is noted in association with rejection episodes (Figures 2, 3 and 5). It must be emphasized that, for reliable results, blood samples must be obtained frequently and on a regular basis from the very beginning of the posttransplant period and continued for two to three months posttransplant.

Our studies have also shown a correlation between T_H:$T_{C/S}$ value pretransplant, and the number of rejection episodes and incidence of donor-specific cell-mediated reactivity (Table 1). No such correlation was found by Carter et al[3] in their comparison of numbers of patients with mean lymphocyte subpopulation values above and below pregraft means to incidence of rejection episodes. However, it is not only the shift in the ratio after transplantation compared to pretransplant value, but the absolute value of pretransplant ratio that affects the graft outcome. When, in our studies, we expressed posttransplant values as percentages of pretransplant level, no correlation with kidney function was found; when patients were grouped according to pretransplant ratio, the effect of that ratio became apparent.

We are aware, of course, that the number of patients followed is still too low to reach any firm conclusions. However, we intend to pursue this line of investigation by studying ratios in prospective recipients of kidneys from living related donors who are under the program of donor-specific blood transfusions. Sequential study of donor-specific and nonspecific reactivity in such patients should provide more information as to the value of pretransplant ratio in the prediction of graft outcome.

A large part of the controversy existing so far in this area can be explained by technical factors, as mentioned above. In addition, it becomes increasingly clear that T-cell subsets, as defined by presently available monoclonal antibodies, are functionally quite heterogeneous. For example, the so-called helper-inducer subset is now known to be composed of inducers of help, inducers of suppression, functional suppressors, and even cytotoxic effectors against DR antigens.[9,10] It seems reasonable to expect

that, with the development of new monoclonal antibodies that allow dissection of T cells into subsets with more precisely defined functions, more effective immunological monitoring of organ allograft recipients will become possible.

ACKNOWLEDGMENTS

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2 *Phenotyping of Leukemia with Monoclonal Antibodies Using a Microcytotoxicity Test*

R. Billing

Leukemia and lymphomas are heterogeneous hematopoietic malignancies corresponding to their various cellular origins. The subclassifications of these diseases have been shown to be important in the clinical diagnosis and choice of therapy. Previously this subclassification required several costly, sophisticated, individual, lengthy procedures such as histological straining; enzyme assays such as terminal deoxynucleotidyl transferase; detection of sheep erythrocyte receptors by E-rosette formation; surface membrane immunoglobulin determination by fluorescent binding assays and chromosomal markers. The availability of monoclonal antibodies against cell surface differentiation antigens has made possible a rapid and precise approach to leukemia and lymphoma phenotyping. Each subclass of leukemia has a unique set of cell membrane antigens that can be detected by monoclonal antibodies in a cytotoxicity test to phenotype leukemia. The antibodies plus positive and negative controls are predotted at appropriate dilutions on a 60- or 72-well tissue typing tray. The general specificities of the antibodies are as follows: T ALL, Pan T (T1, T11), Ia, Smlg positive cells, monocytes, myeloid cells, AML, blast cells, common ALL (gp26 and gp100). The microcytotoxic tray methodology allows for the addition of new antibodies as they become available. The pattern of reactivity of the lymphoproliferative cells against the panel of antibodies determines the subclass. Over 90% of random leukemias could be identified into the following subclasses: T ALL, B ALL, common ALL, T and B CLL, AML, promylocyte leukemia, CML blast crisis (lymphoid and myeloid types).

Lymphoproliferative diseases (leukemia and lymphoma) have been recognized since the early 19th century and are presently the seventh leading cause of death from cancer. Research in this area has been steadily accelerating since the early 1960s, stimulated by the availability of human leukemia cell lines in culture. At that time researchers began to standardize characterization, nomenclature and classification of the various forms of this disease in four main morphological subclasses: acute lymphocytic leukemia (ALL), acute myelocyte leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelocytic leukemia (CML). In the 1970s, subclassification of these diseases has become more sophisticated and has

been shown to be important in the clinical diagnosis and choice of therapy.[1,2] Previously, this subclassification required several costly, sophisticated, individually, lengthy procedures such as histological staining; enzyme assays such as terminal deoxynucleotidyl transferase (TDT); detection of sheep erythrocyte receptors by E-rosette formation; and surface membrane immunoglobulin determination by fluorescent binding assays and chromosomal markers. The availability of monoclonal antibodies against cell surface differentiation antigens has presented a new approach to leukemia and lymphoma phenotyping[3-6] and also the identification of normal leukocyte subpopulations.[7,8]

The phenotyping of human leukemia and lymphoma cells has since become a fundamental, albeit expensive, research procedure. Labeled monoclonal antibodies in concert with fluorescent cell sorters are routine fixtures in large, well-funded research laboratories. However, the continual development of complement-fixing cytotoxic monoclonal antibodies now places this technological capability within reach of all. Using the proven microcytotoxic technology so well discussed in the literature and routinely used by tissue-typing laboratories all over the world, the researcher can phenotype human leukemic and lymphoma cells with a relatively inexpensive inverted phase microscope.

METHODS

Cell Preparation

1. Collect 10 ml of whole blood (or in the case of lymphoma, remove and process an appropriate node) into a 10 ml heparinized vacutainer tube containing 2 ml of RPMI tissue culture media with Hepes or equivalent and mix.

2. Centrifuge the tube for 10 minutes at 2000 rpm (733 g) in a Sorval GLC-2B or equivalent motor.

3. Using a Pasteur pipette, remove the buffycoat and mix with an equal volume of HBSS and layer a maximum 2 ml of the buffycoat-media mixture over 1.5 ml of Ficol Hypaque, the refractive index (RI) of which is 1.3545, contained in a 5 ml tube.

4. Centrifuge the tube for 10 minutes at 2000 rpm (733 g), again using a Sorval GLC-2B or equivalent rotor.

5. Using a Pasteur pipette, remove 1 ml of interface and place in a Fisher tube and spin at 4000 rpm in a Fisher or equivalent centrifuge. This will pellet out the leukemia and mononuclear cells.

6. Decant supernatant and lyse residual red blood cells with ammonium chloride; buffer if necessary.

7. Resuspend and rinse pellet twice in 1.0 ml HBSS or McCoy's media.