CHAPTER 4: DISCUSSION

4.1 STATISTICS

An average of 120 paediatric and 95 adult acute leukaemia cases were admitted in UHKL each year (Table 7).

Table 21: Percentage of acute leukaemias in UHKL, phenotyped and reported.

<table>
<thead>
<tr>
<th></th>
<th>Paed</th>
<th></th>
<th>ALL</th>
<th></th>
<th>AML</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td></td>
<td>M</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>UHKL</td>
<td>27.7</td>
<td>19.3</td>
<td></td>
<td>4.2</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Phenotyped</td>
<td>24.2</td>
<td>21.9</td>
<td></td>
<td>3.4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Reported*</td>
<td>34.0</td>
<td>21.6</td>
<td></td>
<td>7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>M</td>
<td>F</td>
<td></td>
<td>M</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>UHKL</td>
<td>12.6</td>
<td>9.3</td>
<td></td>
<td>11.0</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Phenotyped</td>
<td>20.2</td>
<td>11.2</td>
<td></td>
<td>5.6</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>Reported*</td>
<td>7.4</td>
<td>4.8</td>
<td></td>
<td>24.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Callender and Bunch (1987)

Table 21 shows the incidence of acute leukaemia among adult and paediatric cases admitted in UHKL and reported in developed countries. The distribution of phenotyped cases were also included. The results showed a similar higher incidence of ALL among paediatric cases and a higher ratio for paediatric males. Even so, two times more adults with ALL were seen here. As reported, more adults than paediatric patients suffered from AML. The M:F ratio among AMLs in UHKL was almost equal, similar to that reported by Callender and Bunch (1987). Breakdown of cases collected for immunophenotyping was similar in percentage to the overall
number of patients admitted in UHKL, except among adult male
ALL and AML cases where twice and half the reported number
were collected, respectively (Table 21).

From Tables 4 (Chapter 1) and 9 (Chapter 3), the number of
ALLs phenotyped in UHKL and reported, according to phenotyp-
ic subgroups and four different age groups (Pui et al.,
1993) were compared. Values are shown in Table 22.

Table 22: Percentage distribution of ALLs among phenotypic
subgroups in UHKL and reported.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Infants (&lt;1.5yr)</th>
<th>Children (1.5-10yr)</th>
<th>Adolescents (&gt;10yr)</th>
<th>Adults (&gt;15yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Pre-B CD10+ UHKL</td>
<td>33.3</td>
<td>69.4</td>
<td>57.1</td>
<td>34.8</td>
</tr>
<tr>
<td>Reported*</td>
<td>57.0</td>
<td>82</td>
<td>68</td>
<td>56-580</td>
</tr>
<tr>
<td>CD10- UHKL</td>
<td>33.3</td>
<td>12.5</td>
<td>21.4</td>
<td>10.9</td>
</tr>
<tr>
<td>Reported*</td>
<td>33.0</td>
<td>4</td>
<td>8</td>
<td>200</td>
</tr>
<tr>
<td>T UHKL</td>
<td>0.0</td>
<td>12.5</td>
<td>21.4</td>
<td>47.8</td>
</tr>
<tr>
<td>Reported*</td>
<td>6.0</td>
<td>13</td>
<td>23</td>
<td>200</td>
</tr>
<tr>
<td>B UHKL</td>
<td>33.3</td>
<td>5.6</td>
<td>0.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Reported*</td>
<td>4.0</td>
<td>1</td>
<td>1</td>
<td>2-40</td>
</tr>
<tr>
<td>Total (UHKL) ALL</td>
<td>(3.3)</td>
<td>(40.9)</td>
<td>(5.7)</td>
<td>(28.4)</td>
</tr>
<tr>
<td>AML</td>
<td>(1.1)</td>
<td>(4.0)</td>
<td>(3.4)</td>
<td>(13.1)</td>
</tr>
</tbody>
</table>

* Pui et al. (1993) from table
@ Pui et al. (1993) from text
(values) = percent distribution of acute leukaemia (ALL + AML)
The infants:children:adolescents:adults ratio of cases phenotyped and reported were 1:12:2:8 and 1:14:3:16, respectively (Tables 4 and 9). Almost 60 (56/94) and 41% (82/202) of adult and paediatric cases of ALL and NHL, were collected for immunophenotyping, respectively. About the same number of adult (29%, 28/96) and paediatric (32%, 12/37) AML cases were also collected (Tables 7 and 8).

Because of the low number of infant cases collected for this study, no comparison was made. Among children, there was a larger number of CD10+ cases and a lower number of CD10- than reported. Among adolescents, distribution of cases in the various subgroups corresponded very well with reported values.

Adult patients suffered more (47.8%) from T-ALL and less from C-ALL than reported (Table 22). CD10- cases were reduced to half the expected number and appeared to be more prevalent among the younger age groups (Table 22).

Immunophenotypic subtypes differ according to geographical and ethnic settings. An improved socioeconomic situation and hygiene with altered patterns of infection in infancy results in an increased risk of CD10+ B-lineage ALL (C-ALL) in developed countries. Black African children have a significantly lower incidence of C-ALL and a higher incidence of T-ALL (Pui et al., 1993). These may not be the
reasons for the results observed here because the University Hospital, being one of only two referral centres, receives 'difficult' cases from all over the country. Increased T-cell cases among adults may be the result of a bias towards collection of suspected T cell samples for this study.

From Tables 10, 11, 12a, 13 and 15, the number of patients among the various ethnic groups were obtained and further tabled according to phenotypic subgroup, sex and age groups. Values for infants were not included because of the low number of infant cases collected for this study. Results were presented in Table 23. A chi-square test of the adult population showed random distribution of patients suffering from B, T and myeloid leukaemia among the three main races (0.25>p>0.10). Among paediatric patients, 71, 77 and 100% of Malay, Chinese and Indians, respectively, suffered from B cases, specifically C-ALL.

The distributions of sexes were random except among adult T-ALL cases where more males were observed (p<0.001).

In general, Null-ALL, C-ALL and B-ALL predominated among paediatric patients while T-ALL, AML and CLL predominated among adults. CLL is rare locally. From a total of 747 cases of leukaemia, CLL formed only 0.9% (Ng et al., 1990). This is similar to the total reported here, 0.9% (Table 7).
Table 23: Distribution of the number of immunophenotyped cases according to ethnic group, sex and age.

<table>
<thead>
<tr>
<th>Ethnic</th>
<th>Sex</th>
<th>1-11yrs.</th>
<th>≥12yrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Null C-ALL</td>
<td>T-ALL</td>
</tr>
<tr>
<td>Malay</td>
<td>M</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Chinese</td>
<td>M</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Indian</td>
<td>M</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td>M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

M - male; F - female
4.2 CLINICAL FEATURES

The occurrences of mediastinal mass (Med. Mass.), organomegaly [spleenomegaly (Spleno) and hepatomegaly (Hepato)] and adenopathy (LN) among different subgroups were listed below. Percentages were obtained from Tables 16, 17 and 18 except for the total white blood cell counts which were obtained from Tables 10, 11, 12 and 15.

Mediastinal masses were seen in only half the expected number (Borowitz and Falletta, 1988) but all were among T cell leukaemias. Lymphadenopathy occurred most frequently among T cell patients and least among AML patients as is indeed reported (Hoffbrand and Pettit, 1988). Hepato- and splenomegaly were observed most frequently among the T cell cases followed by null-ALL, AML and least in C-ALL.

Table 24: Percentage of clinical features among the phenotypic subgroups.

<table>
<thead>
<tr>
<th></th>
<th>Med. Mass</th>
<th>LN</th>
<th>Spleno</th>
<th>Hepato</th>
<th>WBC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>21.4</td>
<td>92.6</td>
<td>84.0</td>
<td>85.2</td>
<td>61.1</td>
</tr>
<tr>
<td>Null-ALL</td>
<td>0.0</td>
<td>87.5</td>
<td>62.5</td>
<td>87.5</td>
<td>21.0</td>
</tr>
<tr>
<td>C-ALL</td>
<td>0.0</td>
<td>55.5</td>
<td>22.2</td>
<td>44.4</td>
<td>17.3</td>
</tr>
<tr>
<td>AML</td>
<td>0.0</td>
<td>46.2</td>
<td>38.5</td>
<td>53.8</td>
<td>57.1</td>
</tr>
</tbody>
</table>

* >50X10^9 per L

Researchers find C-ALL has the most favourable prognosis followed by Null-ALL and T-ALL (Callender and Bunch, 1988). The values above correspond well with the prognosis of each subgroup. The occurrence of hepato- and splenomegaly in T cell patients were associated with inferior prognosis.

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(Shuster et al., 1990). The most important single prognostic factor at presentation is WBC count, followed by age. Patients with common ALL and high WBC count do almost as poorly as patients with T-ALL with similar counts. No studies have been done on T-ALL patients with low counts to see if they do as well (Borowitz and Falletta, 1988). Total white blood cell count of > 50x10^9 per L was seen most often among T-ALL patients followed by AML, null ALL and C-ALL (results obtained from individual tables: Tables 10, 11, 12a and 15).

Concordance between cytochemistry tests and surface marker studies, in regard to distinguishing ALL from AML, was very good. Of 181 immunophenotyped cases, only four (2.2%) results differed. N-14 (Table 10) was initially diagnosed as T cell leukaemia, since the blood sample was positive for asid phosphatase. This test is positive mainly in T ALL samples but are also observed in non-T cell cases (Borowitz and Falletta, 1988). The sample was, however, negative for four T cell markers (including T2) but was positive for HLA-DR and B4. The high positivity for B4 (95%) and negative value for Calla, identified it as an immature B cell. These cells tend to be rearranged for the TCR genes. The three other samples (T-3, 9 and 14; Table 12b) were diagnosed as AML cases because they were all positive for the peroxidase test. Surface marker analysis, however, showed them to be early T cells. Lineage infidelity or the expression of
markers of two different lineages on a single sample is known to exist and may be explanatory for samples such as the above. PAS block positivity is usually shown on cells carrying the C-ALL antigen (Hoffbrand and Pettit, 1988). PAS was positive in 59.6% (31/52) of C-ALL cases. They were block positive alone or with other forms of positivity.

4.3 IMMUNOPHENOTYPING AND GENE ANALYSIS

Mature T cells found in peripheral blood and lymphoid organs are derived from the thymus where their immature counterparts undergo selective processes. Thymocytes are themselves descended from primitive pluripotent stem cells in the bone marrow. To positively identify the haematopoietic stem cell, which should be very similar to its ascendent in fetal life, abortuses were recovered to extract for liver and bone marrow cells. These cells are extremely rare (<0.01% of total cell population) but have high proliferative potential (HPP) (DiGiusto et al., 1994). For now, cells are identified based on surface expression of putative markers supplemented by morphology. Flow cytometric analysis on florescent activated cell sorter (FACS) is then used to isolate or fractionate cells for further studies including cell culture.

CD34, a marker of pluripotent haematopoietic stem cells, is found on 0.1 to 1.0% of CD38-, lineage- [negative for mature markers such as GPA (glycophorin-A, erythroid cells), CD3 (T cells), CD14 (monocytes), CD19, CD20 (B cells) and CD56
CD34+, CD38- LDFL cells are mostly positive for CD33. HPP-CFC are found on cells which express CD33. CD33+ cells, however, are heterogenous for the expression of HLA-DR. The majority (92.4%) are HLA-DR positive. About 6% are dim while 1.5% are negative for the complex. Cells that are CD33- and HLA-DR- contain no CFC (Muench et al., 1994). CD34hi cells from fetal liver, seeded into isolated fetal thymic lobes of severe combined immunodeficient (SCID) mice and cultured in vitro, give rise to mature T cells and dendritic cells. No B cells or monocytes were found (Plum et al., 1994).

In the fetal bone marrow, CD34 positive cells form an average of 26% low density, lineage- cells. Approximately, 20% of these are CD34hi and CD38-. Similar to fetal liver cells, they are CD33lo, but unlike fetal livers, do not express HLA-DR. These fetal bone marrow cells were able to repopulate human bone fragments and human fetal thymus engrafted into SCID mice with B and myeloid and T cells, respectively (DiGiusto et al., 1994).

Terstappen et al. (1991) studying the adult bone marrow
divided CD34+ cells (1% of bone marrow aspirates) into four subsets, PI, PII, PIII and PIV. Only the group with the brightest expression of CD34 and negative for CD38 (PI) was highly enriched for progenitor cells not committed into any lineage. These cells were CD33 negative and formed 1% of CD34+ cells. Stem cells in the adult bone marrow are negative for HLA-DR, as discussed by Muench et al. (1994). Multidimensional flow cytometry showed lineage specific markers (CD71 for erythroid, CD33 for myeloid and CD10 for B cells) were found especially on cells brightly positive for CD38 but dim for CD34 (PIV) with CD33 already seen on PII cells (Terstappen et al., 1991). Thus, it appears that phenotype for the dominant population of stem cells varies during ontogeny as does the responsiveness of these cells to growth factors. In all stages, HPP cells are CD34hi and CD38-. In the fetal liver, these cells appear to be positive for both CD33 and HLA-DR. In the fetal bone marrow, they are still positive for CD33 but negative for HLA-DR. In the adult bone marrow, however, progenitor cells are negative for both CD33 and HLA-DR.

Myeloid and B cells undergo maturation in the bone marrow. The heterogeneity of stem cells (for example in the expression of HLA-DR and CD33) was also observed in both AML and B-cell samples here. About 26% (11/43) of AML cases were negative for HLA-DR (<20% positive cells). Kita et al. (1993) studying 210 AML patients also found about 30% to be negative for HLA-DR. Of seven samples tested for all four
myeloid antigens, six expressed My7, My9 or My7 and My9. These phenotypes are similar to normal HPP-CFC in the fetal and postnatal bone marrow. The seventh sample, M-28, further expressed Mol. This My7+, Mol+, HLA-DR- expression is exactly the same as normal promyelocytes observed on 8% of AML cells (FAB M3) (Foon and Todd, 1986) (Table 15).

Samples positive for HLA-DR included all six M7 cases. Fourteen other cases were tested for all four myeloid antigens. Fifty percent of these (7/14) were positive for My7, My9 or My7 and My9 only, corresponding to myeloid committed progenitor cells capable of limited proliferation (L-CFC) (Foon and Todd, 1986). Two cases did not express My7 or My9 but expressed My4 (M-8 and M-20). The rest further expressed Mol, My4 or Mol and My4 (Table 15).

A smaller number of B cells were negative for HLA-DR, about 6% in Null (1/17) and Galla (5/77) cases. Ten percent (1/10) of mature B cells (Smig+) were negative for HLA-DR (Table 13). The cell morphology of 71% (5/7) HLA-DR- cases had at least 10% of FAB type L2 compared to only 46% (29/63) cases with L2 among samples positive for HLA-DR (Tables 10, 11 and 13).

The CD7 antigen is expressed on 63% of GPA- LDFL cells of which 11% were bright and 52% dull. CD7 bright cells were CD34- and also coexpressed CD56, making them natural killer
cells. Subsets of this population were positive for CD2 and CD8. Both the CD7 dull and CD7- populations have high levels of CD34 and HLA-DR and also expressed CD33 but were negative for all other T lineage markers (Barcena et al., 1993). The CD7 dull and CD7- populations closely resemble a newly identified group among AMLs, classified as minimally differentiated AML (AML-M0). Cells in this group expressed only My7, My9 or My7 and My9. On most of these cells, CD7 is also present. CD34 and Tdt, both markers of immaturity, are also expressed. All are highly positive for HLA-DR (Kita et al., 1993; Stasi et al., 1994). The absence of RAG-1 (recombinase gene-1) or RAG-2 indicate these cells are not undergoing any gene rearrangement. A few cells in both the CD7- and CD7 dull populations are CD10 positive (Barcena et al., 1993).

The seven HLA-DR+ AML samples here which expressed My7, My9 or both (M-4, 10, 17, 22, 23, 34 and 43) had no indication of CD7 positivity. This may be because the presence of endogenous peroxidase made it difficult to detect antigen present in low density. None of the AML cases here possessed CD10 either (Table 15). Three of the AML cases above (M-17, M-23 and M-43) were further investigated for TCR gene rearrangement (Table 20). No sign of rearrangement was observed in TCR gamma, beta and alpha. Only one sample, M-23, had rearranged the delta gene. A fourth sample (M-18), which was negative for HLA-DR and positive for only My9 also showed very little sign of rearrangement. Reis et al.
(1989) noted that the TCR delta gene rearranges occasionally in AMLs. The lack of rearrangement corresponds well with the lack of RAG on early cells. Slight rearrangement in the other samples may be reflective of the different origins of myeloid cells, for example, from bone marrow or thymus, discussed below.

Only two Calla+ samples positive for HLA-DR+ coexpressed myeloid antigens (C-28 and C-58, Table 11). Again, CD7 was not detected. DNA analysis of the B cells showed them to be extensively rearranged not only for the delta gene but also for the gamma and beta genes. None, however, deleted the delta locus, though the 6.0 kb band was lost on all occasions. Germline, rearranged and deleted configurations were observed on the J delta gene. B cells tend to attempt incomplete assembly of the V-delta gene which possibly represents an early, though abortive attempt at lymphocyte differentiation. These rearrangements per se do not seem to have any significance on commitment to cell lineage (Biondi et al., 1990). The TCR alpha gene appeared unrearranged. The only immature B cell sample had an R1 type of rearrangement (Table 20).

CD7- fetal liver cells precede the CD7 dull stage. Differentiation of CD7- cells in fetal thymic organ culture (FTOC) resulted in 39% of CD7+ cells while all CD7 dull cells remained positive. These cells then gave rise to mature T
cells. Both CD7- and CD7 dull cells have myeloid proliferative potential (Barcena et al., 1993).

The most immature fetal thymocytes form only a fraction (1-3%) of triple negative (CD3-, CD4-, CD8-) cells. They are CD34hi, CD7hi and CD2- (Barcena et al., 1993).

The most immature cells in the postnatal thymus have been identified as CD7+, CD4lo, CD44+ (a cell adhesion/homing molecule), CD2- and CD8-. TCR genes are still in germline (Kisielow and von Boehmer, 1995). None of this group of cells were represented among the samples here. The closest leukaemic case reported was the cell line P30/OKUBO. It was found to be unrearranged for the alpha, beta and gamma genes. The only T cell marker the cell line expressed was T1 (CD5). (It was rearranged for the Ig heavy chain gene.) This cell line was postulated to be the earliest stage of T cell or B cell development (Sangster et al., 1986).

The earliest changes to these early cells involved downregulation of CD4 accompanied by the initiation of a period of slow exponential growth. It is here that the first TCR gene rearrangement event occurs (Kisielow and von Boehmer, 1995). Kurtzberg et al. (1989) recognized this group to have pluripotent potential and is not irreversibly committed to the T lineage. Under different conditions, granulocyte-macrophage, mixed, erythroid, T and B cells may be generated. That these were thymocytes was proven by the presence

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of mediastinal masses on the majority of patients. No myeloid markers were expressed on these cells. TCR gamma and beta were rearranged in 57% and 29% of samples, respectively.

Cells of this phenotype (category I) formed less than one third 10/34 (29.4%) of T cell cases collected here. CD5 (T1) is weakly expressed at this stage, and was detected on only one sample. HLA-DR was seen in only half the cases of this group and was present at relatively low percentages (34-43%) (Table 12a). Representatives of this group showed no sign of mediastinal mass which was apparent on more mature groups (Table 17). DNA analysis of the cells showed early forms of rearrangement. The J delta probe revealed rearranged configurations in 3/5 (60%) cases while the gamma gene was rearranged in 3/6 samples (50%). The beta gene was rearranged in 2/5 (40%) cases. Agreeably, beta gene rearrangement which occurs after CD7 expression takes place before other T cell associated surface molecules generally appear (Pittaluga et al., 1987). Changes on the alpha gene were observed only in EcoRI digested DNAs, showing R1, R2 and R3 types of rearrangement (Table 19; Figure 14). This may have resulted from rearrangement in the delta gene rather than true rearrangements in the alpha gene (elaborated under Results). The larger number of cases in this group reflects the proliferative stage which is a target for leukaemogenesis (Janossy and Campana, 1988).
At the end of this period of expansion, cells begin to express IL-2R alpha (a T cell response gene), CD2 (Rothenberg, 1992; Kieselow and von Boehmer, 1995) and CD5. Half of these thymocytes express HLA-DR. Most (57.4%) are dull for CD10 while CD33 is observed in only a low proportion of cells (Barcena et al., 1993). Marquez et al. (1995) observed CD34 high, CD7 high, CD2+, TN, CD1- early postnatal thymocytes expressed 20% of CD33 low and 82% of HLA-DR. Six patients (T-11 to 16, 17.6%) had this phenotype. The low expression of CD5 (T1) was not observed on all of the samples. HLA-DR, however, was highly expressed on these cells (ranging from 37-80%). On a single patient (T-12), My9 was detected in a low proportion (23%) of cells (Table 12a). TCR delta and gamma were rearranged more extensively on cells of this category with 3/4 cases showing new restriction fragments with the J delta probe and 5/5 rearranged for the gamma gene (BamHI digest). Half (2/4) the samples were rearranged for the TCR beta gene. TCR beta gene can be fully rearranged before the cell acquire CD8 and CD4 (Rothenberg, 1992). The TCR alpha gene had only the R1 type of rearrangement. The inactivation of the gene was obvious from the fact that three of the samples were at germline for two to three enzyme digests (Table 19).

A fraction of CD44+, CD2+, double negative (DN) cells express gamma-delta TCRs on their surface (Kieselow and von Boehmer, 1995). No monoclonal antibody against TCR gamma-
delta was used here. However, the high expression of CD3 (80%) but negative value for WT-31 indicated that the leukaemic cells of T-17 must express a TCR gamma-delta chain. The association of CD3 (T3) and TCR is an obligate requirement for surface expression of TCR. CD1, CD4 and CD8 were negative while the sample expressed a weak level of CD5 (Table 12a). CD4-CD8- gamma-delta cells in the peripheral blood lacked the expression of CD1 (McIntyre and Sigaux, 1989). Also, the TCR gamma-delta CD3+ populations in human peripheral blood are either negative or express low levels of CD5 (T1) (Brenner et al., 1988). Both alleles in the sample were rearranged for the TCR delta gene. Northern blot shows that only one recombination is transcribed (Hata et al., 1989). Rearrangement on the 3′ side of the 12 kb band (detected by C-delta probe) suggested involvement of V-delta3 and J-delta1 (J-delta probe). V-delta3 lies about 2-3 kb 3′ of the C-delta locus (Hata et al., 1989) and is probably situated on the 6.3 kb HindIII germline fragment. V-delta3 rearrangements are detected by a C-delta probe (Hata et al., 1989). Band sizes, however, did not correspond to those suggested in the report by Griesinger et al. (1989). The gamma gene was also rearranged in both alleles, one involving C1 and the other, C2. TCR beta and alpha gene studies were not carried out on this sample (Table 19). Even though only 5-10% of CD4-,8-,5dull thymocytes express surface CD3 associated gamma-delta receptor (Korman et al., 1989), gamma-delta cells make up approximately 20% of all T-
ALLs and between one third and one half of CD3 positive T-ALLs (MacIntyre and Sigaux, 1989).

CD7+, CD2+, CD44-, DN thymocytes represent the latest developmental stage which can give rise to gamma-delta T cells. Before developing into DP cells, T cells express the TCR beta chain in association with a newly recognized protein, gp33. This pre-TCR alpha gene does not undergo rearrangement. The heterodimer is expressed in conjunction with the CD3 complex. Upon activation, the T cells continue development and begin expression of CD4 and CD8 (Kisielow and von Boehmer, 1995)

Before acquisition of the CD4 antigen, CD1 molecule is expressed. CD1 (T6) is a thymic associated marker. Cells undergoing maturation in the thymus express the T6 marker for a period of time (Janossy and Campana, 1988). T-21 showed an elevation of only CD1 molecules.

The sequential expression of CD1 and CD4 was observed when CD34 high, CD7 high, CD2+, TN, CD1- cells from the postnatal thymus were cultured in the presence of IL-7. These thymocytes which were about 20% CD33 low and 82% HLA-DR+ expressed intermediate levels of CD44 (CD44 int.) segregated into two populations, one consisting of large cells expressing bright levels of CD44 while the small cells were negative for CD44. (The original population may have consisted of two different populations with an overlapping phenotype.
For example, CD33 was expressed on 20% of the original population. Upregulation of CD44, also occurred on only 20% of the total population.) While the expressions of CD1 and CD4 on the large cells were bright, they were intermediate on the smaller cells (Marquez et al., 1995). T-22 was positive for CD1 and CD4.

Transition from DN to DP thymocytes may begin with either one of the two coreceptors followed by the other, as occurs in mice (Kisielow and von Boehmer, 1995) and observed in human (Janossy and Campana, 1988). T-23 expressed 86% of CD8, however it was negative for CD1. On the other hand, the lack of CD1 suggested cells of this sample may have been derived extrathymically especially since 30-40% of peripheral TCR alpha-beta thus formed are CD8+ (Rothenberg, 1992). HLA-DR was no longer expressed on these cells (Table 12a). Not much results on gene rearrangement was obtained in these cases. Analysis of TCR gamma gene showed them all to have rearranged into the second constant region, C2 (Table 19). Among leukaemic samples, about an equal number of cases had been reported for the immature single positive phenotypes (CD4:CD8 = 8:7). Of a total of 124 cases, they formed about 6% each. Except for two instances, CD2 was positive on all of these cases. CD1 appeared on some but not all of the samples. The TCR delta, gamma and beta genes were all rearranged on these samples (Flug et al., 1985; Waldmann et al., 1985; Greenberg et al., 1986; Foroni et al., 1987; Hara
et al., 1988a; Griesinger et al., 1989).

Three patients (T-18 to 20) expressed low levels of CD4 and CD8 (15-39%) without CD1 (Table 12a). Results from gene analysis suggested that these samples should be placed between DN and DP subgroups since the delta gene had not been deleted (alpha gene was at germline) and C1 and C2 types of rearrangement were observe in the gamma gene. While more and more complex phenotypes observed in leukaemic cases (Borowitz and Falletta, 1988) can now be easily positioned into the map of differentiation, discrepancies still exist such as the above. They may be extrathymically derived. They may also be normal counterparts of processes such as when mature CD8 cells convert to CD3+,CD4-,CD8- under certain conditions (Seder and Le Gros, 1995).

The small cells in the study (above) then began expression of CD8 to become DP cells. They lacked B, natural killer, myeloid antigens and the HLA-DR molecule. In this study, cells with this phenotype were categorized as group VI. They formed 26.5% of T cell cases (T-24 to 32). While no longer proliferating, they are the most numerous cell type in the thymus (Rothenberg, 1992). Similar to its normal counterpart, all were HLA-DR negative, expressed CD1 (T6) (except for one case), CD5, and in most cases showed high values for CD4 and CD8. We saw also that CD3 begins to be expressed at low levels in correspondence with the appearance of TCR alpha-beta molecules (WT-31+). The only excep-
tion was on T-31 (CD3=22%, WT-31=94%). T-32 had downregulated CD1 while still high for CD4 and CD8 (Table 12a). The TCR delta loci were deleted in 5/8 samples and on at least one of two alleles in the other three samples. This was supported by the detection of only one rearranged fragment with the J delta probe. All alleles rearranged the TCR gamma gene. The TCR beta genes were also all rearranged except one (T-24) which was uncertain because of its large band size. TCR alpha rearrangement takes place on DP cells. Gp33 is downregulated to allow pairing of the TCR alpha protein with the TCR beta protein which are then heterogeneously expressed on DP T cells (Kisielow and von Boehmer, 1995). Thus it is in this group of cells that real changes to the TCR alpha gene were observed (T-24 and 27). Even so, the changes were very slight as there were only an addition (T-24) and a deletion (T-27) of a single band (Table 19).

Studies on rearrangement of the alpha chain gene have been limited due to the great distance over which the J segments are dispersed. In order to detect all rearrangements, it will be necessary to isolate the entire J region and to generate representative single copy probes. A second way to detect rearrangement in the alpha region is to use other forms of electrophoresis and restriction enzymes that cut infrequently.

T-33 and 34 were both positive for CD3 and WT-31 and single
positive for CD4. Thus, they belonged to the major group of mature T cells found in the peripheral blood. Davey et al. (1986) discovered mature T cell leukaemias expressed CD3, CD4 or CD8 and with rare exceptions, no longer expressed 3A1 (CD7), as were evident on the two samples here (T-33 and 34). The same was also observed by Denning and Haynes (1988). T-34 was a suspected case of Sezary syndrome. Immunophenotyping confirmed a mature phenotype. Gene analysis, however, found that all TCR genes were at germline (Table 19). The dissociation between genotype and phenotype, where an immature genotype is associated with late activation markers are frequently observed among Hodgkin's Disease. In B cells transformed with Epstein Barr Virus (EBV), the resulting cell lines which were at different stages of gene rearrangement were all in possession of a late phenotype. Similarly, virally induced transformation of T cells can also occur as many herpesviruses and retroviruses induce lymphoproliferation in T cells (Herbst et al., 1989).

CD4-,CD8-,TCR alpha-beta+ cells reported to form 2-12% of DN cells in the peripheral blood were not seen among any of the samples here, probably due to its low number. In vitro culture of this cell showed it may have the capacity to undergo nonlymphoid (myeloerythroid) differentiation (Kurtzberg et al., 1989).

A small fraction (<1%) of hematopoietic non-T cells are normally present in the human postnatal thymus. The study by
Marquez et al. (1995) showed that the larger cells then downregulated the expression of CD7 and expressed high levels of myeloid-related antigens such as CD11b, CD13 and CD33 as well as HLA-DR. Many were CD14+ (My4).

Three T cell samples (T-3, 9 and 14; Table 12b) were initially diagnosed as AML (two FAB, M4) by using the myeloperoxidase stain. Immunophenotyping identified them as T lineage, being positive for T2 or T2 and T11. Results from gene analysis showed T-3 to be in germline when probed with the J delta 1 while T-9 and 14 were rearranged. T-3 was again at germline for the gamma gene, whereas the other two had undergone rearrangement, though only minimally on T-9. Rearrangements were detected on the beta genes of T-3 and 14 with 3/4 alleles rearranging to an 8 kb band. While an R1 type of rearrangement was detected on T-3, no sign of rearrangement was found on the alpha gene of the other two samples (Table 19). Two indistinct groups of AML appear to exist, one which maintains TCR genes at germline (also observed on AML samples here, Table 20) and the other which have early forms of TCR rearrangements (observed in the T/AML samples mentioned above and also in M-23, Table 20). These may correspond to myeloid cells derived from the bone marrow and thymus respectively. CD7+, CD1-, TN thymocytes positive for myeloid markers also tend not to rearrange the TCR gamma and beta genes in a sequential manner as observed in T-3 (rearranged for TCR beta but not TCR gamma) and
reported by Cross et al. (1988) and Jensen et al. (1991) with 3/10 and 4/5 samples, respectively. [Cross et al. (1988) studied samples also expressing CD2.] Early thymocytes negative for myeloid markers tend to rearrange sequentially, as evident on the nine samples researched by Kurtzberg et al. (1989) where only one was asynchronous involving TCR beta and alpha. The lack of Ig and TCR gene rearrangement in cases of pure myeloid phenotypes and the rearranged Ig genes on mixed B-myeloid phenotypes were also observed by Sobol et al. (1985).

By virtue of the small number of functional V genes in both the TCR delta (three to six) and TCR gamma (seven to eight) locus, analysis with a J probe (which is known to be used more frequently than the rest) reveals on Southern blot restriction fragment sizes which can be used to identify the V segment involved. J-delta1 was used for the delta locus (Griesinger et al., 1989; Hata et al., 1989) while J-gamma1 was used for the gamma locus (Chen et al., 1988; Hata et al., 1989; Casorati et al., 1989). For comparison, other J segments were also often used. Otherwise, each V segment of the delta locus (Takahara et al., 1989) and gamma locus (Chen et al., 1988) were used directly as probes. Gene analysis with the J-delta1 probe here showed the segment to be rearranged in the majority (12/19) of cases. (Three cases were deleted for the gene.) However, no distinctive pattern was observed. Furthermore, most did not correspond to any reported band size. On the other hand, the bulk of
double negative cell DNA digested with BamHI revealed bands of 10.5 and 11 kb when probed with J-delta1 (Takihara et al., 1989). Of the ten alleles digested with BamHI here, nine rearranged to bands of about 10 or 11 kb (Table 19).

V-gamma segment usage among the samples here could not be determined, as JP is used infrequently (Brenner et al., 1988). The JP fragment was not detected most of the time due to its small size and the high stringency wash carried out here. However, the position of V9 allowed us to determine whether the V segment used was 3' or 5' to V9 (Figure 3). In five samples (T-11, 12, 14, 18 and 30) four of which were immature DN thymocytes, the V9 segment in at least one of the alleles remained at germline. V-J rearrangement here must have involved V segments 3' to V9 that is V10, V11 or V12. Two other samples (T-16 and 27) involved rearrangement to V9. The rearranged fragment in T-16 corresponded in size (2.4 kb) to a V9 J1.3 recombination (Leber et al., 1989a). In eight cases, the fragment containing V9 was deleted (detected on EcoRI or HindIII) with no rearranged band seen. There were two DN samples and six DP samples. These rearrangements must have involved V segments 5' to V9 including V8. Results obtained here were in contrast to those reported by Chen et al. (1988) where immature single positive and double positive cells rearranged more often to V segments 3' to V9 than did more immature (CD7+ only) or more mature (CD3+) cells.
The ratio of C-beta1 to C-beta2 rearrangement was observed to vary in a highly significant way between phenotypic subgroups (Foroni et al., 1987). The same was observed here for both TCR beta and gamma. A comparison of category I, II, V and VI showed the ratio of C1:C2 for alleles carrying the beta and gamma genes to be 1:1, 3:1, -, and 1:7 and 1:3, 8:2, 0:4 and 1:11, respectively.

Overall results showed there was concordance in the rearrangement of the four TCR genes in that all samples that were rearranged for TCR alpha and beta were also rearranged for TCR gamma and delta.

The peripheral blood samples in this study in many instances did not reveal bands of germline configuration. This was also observed by Takihara et al. (1988) and Cossman and Uppenkamp (1988) because cells of the peripheral blood have undergone rearrangement or deletion of the genes. Most references use germline DNA from non T cell lines (Takahara et al., 1988; Tkachuk et al., 1988) or normal bone marrow cells (Yanagi et al., 1985; Yoshikai et al., 1987). Unfortunately, these sources were unavailable here.

It is important to continue to refine definitions of leukaemic subtypes because different cells respond differently to treatment. The immortality of stem cells render them resistant to conventional chemotherapy and the presence of
risk factors (eg. immature markers) would quickly identify patients in need of alternative therapy.

The study of hematopoietic stem cells is necessary as they can be used as a source for the reconstitution of immune cells in extensively purged bone marrow transplanted into severely immunocompromised patients (DiGiusto et al., 1994). Furthermore, they are targets for gene therapy (Plum et al., 1994).

The Southern blot technique is a relatively insensitive technique in that in order to detect a band on an autoradiograph, there must be at least 10 copies of the same rearranged gene present in the sample. In cases in which each cell bears a different rearrangement, no band is detected as the representation of any one particular pattern is below the level of detection. Proliferation of any one cell to represent approximately 1% of the population allows the gene of this family to be detected (Slingerland et al., 1988). Normal peripheral blood is made up of matured T cells with a diverse repertoire for antigen recognition. With no one distinctive population, no bands are detected on a Southern blot (Cossman and Uppenkamp, 1988). In the normal peripheral blood other white blood cells such as mature B cells and myeloid lineages make up 20-30% of the population. Being germline configuration (in most cases), germline fragments may be detected on the blot.
The polymerase chain reaction (PCR) technique is able to detect 0.01% to 0.001% of a clonal marker out of 10 cells (Cave et al., 1994). It is not only much more sensitive than the Southern blot technique but also simpler, more rapid and non-radioactive. Thus, it is very helpful for skin biopsy samples which have low numbers of malignant cells (Bottaro et al., 1994) and the monitoring of minimal residual disease (MRD) to evaluate the efficacy of the therapy and predict impending relapse (Cave et al., 1994).

Lineage specific markers were found to be not so specific after all. As we already know, immunophenotyping markers such as CD7 is expressed on 5-10% of AMLs. CD5 is distributed on the vast majority of B cells in fetal lymphoid tissues and represent a normal subset of B cells in peripheral blood and in secondary lymphoid organs (Banchereau and Rousset, 1992). CD13 antigens are expressed on B cells while the common-ALL antigen is expressed on granulocytes. Even cytoplasmic CD3 was found on natural killer cells (Barcena et al., 1993; Muench et al., 1994). Gene rearrangement to show lineage specificity was also upsetted since both Ig and the TCR genes were found rearranged on all types of acute leukemia (Griesinger et al., 1989; Biondi et al., 1990). One method in use now to identify clonality is by demonstrating the existence of a clonally restricted VJ junction. This method employs the PCR technique (Bottaro et al., 1994).
With better techniques available, phenotypes once explained as aberrant are now recognized as actual stages in T cell maturation. Similarly, better techniques in gene analysis will show inconsistent rearrangements to be normal. A large number of rearrangements may be unproductive attempts which cannot be selected if not by a neoplastic event. We should not forget the 90% of cells deleted in the thymus during thymic selection. The negativity or autoreactivity of some of these cells may be the cause of malignancy in leukaemic cells.