An in vitro analysis of murine thymocyte-stromal cell interactions

by

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ABSTRACT

This thesis is directed towards two separate but closely related goals; one involves the biology of precursor cells in T-cell development and the second involves the use of retroviral constructs for the establishment of thymic stromal cell lines and its application for studying differentiation of T-cells in vitro.

Using the mouse foetal thymic organ culture system, a quantitative assay was devised in which the relative abundance of T-cell progenitors among different populations was estimated by seeding decreasing numbers of precursors into alymphoid thymic rudiments. This limiting dilution approach combined with serial transfer of successfully recolonised precursor cells in organ culture, provided evidence that certain populations seeding the thymus have the potential for extensive cell divisions (up to 10-12 weeks).

Stromal cells from adult and foetal sources were transformed by a temperature sensitive (ts) mutant of SV40 and E1a 12S in order to study interactions between the developing thymocytes and specific elements of the microenvironment. Eighty four cell lines were generated and have been shown to contain properties related to their counterparts in vivo.

The thermolabile transforming agent (large T) allowed inactivation of the immortalising gene when the cells were switched to the nonpermissive temperature. Properties like the expression of MHC antigens and the ability to bind thymocytes could be induced by growing ts-derived clones at the nonpermissive temperature, which suggests that although cell proliferation had ceased, the cells were still metabolically active and had reverted to a more 'normal' non-transformed phenotype allowing certain molecules to be expressed at the cell surface.
Close interaction of the developing thymocytes with the stroma is essential for the development of functionally mature T-cells. Using a rosette assay unfractionated adult thymocytes were found to bind unstimulated E1a-derived clones resembling epithelial cells (15.5 and 15.18) and also to several other established clones after temperature switching and/or IFNγ treatment.

In co-culture with a multipotent bone-marrow derived stem cell clone (A4) it was shown that both proliferation and differentiation into certain myeloid lineages were supported. Furthermore, in co-culture with CD4⁺CD8⁻ thymocytes, with and without interleukin-7, the growth of this subset could be sustained for ~20 days. In addition, phenotypic changes of thymocytes in these cultures suggest that some of these lines may have the potential to induce differentiation of early precursors.

These findings indicate that established cell lines could be useful tools for studying maturation of T-cells in vitro and furthermore, to investigate distinct events in T-cell ontogeny governed by different stromal cell types.
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ABBREVIATIONS

BM Bone marrow
CD Cluster of differentiation
CFU-S Colony forming unit of spleen
CM Conditioned medium
Con A Concanavalin A
DC Dendritic cell
dGuo 2'deoxyguanosine
DN Double negative
DP Double positive
ECM Extra cellular matrix
EM Electron microscopy
FACS Fluorescence activated cell sorter
FCS Foetal calf serum
FITC Fluorescein isothiocyanate
FL Foetal liver
G-CSF Granulocyte colony stimulating factor
GM-CSF Granulocyte-Macrophage colony stimulating factor
HS Horse serum
HSA Heat stable antigen
ICAM Intracellular adhesion molecule
IF Intermediate filament
IFN Interferon
Ig Immunoglobulin
IL Interleukin
IL-2R Interleukin-2 receptor
i.t Intrathymically
i.v Intravenously
LFA Lymphocyte function-related antigen
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MΦ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>PBM</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Pgp-1</td>
<td>Phagocytic glycoprotein-1</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TNC</td>
<td>Thymic nurse cell</td>
</tr>
<tr>
<td>T-ROS</td>
<td>Thymocyte rosette</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
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CHAPTER 1

INTRODUCTION
1.1 General introduction
The immune system consists of multiple components that may become integrated in the process of generating any one of a number of distinct immune responses. Cells of the immune system have the ability to recognise and respond specifically to foreign antigens. Antigen-specific immune responses are mediated by two types of lymphocytes: B-lymphocytes which mediate humoral immunity via the production of immunoglobulins and T-lymphocytes which coordinate the humoral response and effect cellular immunity. The thymus gland plays a central role in the development of functional mature T-cells and in the specificity of the immune system. Stem cells migrate into the thymus where they undergo both differentiation and selection. Through mechanisms not fully delineated, T-cells are educated to be able to recognise antigens in the context of self encoded major histocompatibility (MHC) antigens and in addition to become tolerant to self-MHC.

This chapter will review aspects of T-cell ontogeny, T-cell differentiation and the influence of the thymic microenvironment on T-cell development.

1.2 T-cell ontogeny
The purpose of this section is to review issues concerning haemopoietic stem cell development. Haemopoiesis, the production of blood cells, begins early during embryogenesis and continues throughout life. A permanent pool of haemopoietic cells is necessary in order to replace cells as they die. Stem cells, the most primitive precursor cells, are responsible for generating progeny of all the myeloid and lymphoid lineages. Many studies have been undertaken in the search for such a 'true stem cell' with the dual capacity for self-renewal and differentiation.

During thymic development new stem cells have to be drawn from a stem cell pool to replace those that are dying and those that are differentiating into mature T-cells. It is well established that the embryonic thymic rudiment is seeded by cells of extrinsic
origin. However, many issues remain unresolved. Firstly, it is unclear whether in adult life there is a stem cell pool present in the thymus or whether stem cells have to be recruited, when necessary, from the bone marrow (BM) (the site for stem cell production during adult life) in order to maintain the production of T-cells. Secondly, are stem cells long-lived or do they have a limited life-span? Thirdly, are there internal cellular signals or external microenvironmental factors which determine the self renewal or differentiation of stem cells? In other words, are certain cells already committed to the T-cell lineage in the bone marrow or does the thymic microenvironment induce cells to differentiate along the T-cell pathway? Fourthly, how is the migration of bone marrow cells to the thymus regulated in terms of (i) factors which control migration, (ii) the number of cells which transit through the thymus? Lastly, how can T cell progenitors be identified and what are the phenotypic changes which take place during intrathymic development?

1.2.1 Origin of stem cells and their cellular diversity

In the late nineteenth and early twentieth centuries there were two theories about the origin of lymphocytes. Beard (1894) described that lymphoid cells originate from the thymic epithelium. A few years later another view was proposed by Hammar (1905) who postulated that the connective tissue surrounding the thymic epithelium was the source of lymphocytes. Both these theories were modified by several subsequent studies. But experiments carried out in the adult mouse by Harris et al., (1964) and Ford et al., (1966) and in the embryonic mouse and chick by Moore and Owen (1967), Owen and Ritter (1969) and Le Douarin and Jotereau (1975) demonstrated that the thymic rudiment was colonised with cells of extrinsic origin. Chick embryos of different sex can be distinguished by chromosome markers (Moore and Owen 1965) and this labelling technique was utilised in order to identify cells entering the thymus (Moore and Owen 1967). In parabiosis and transplantation experiments it was observed that blood borne cells colonise the thymic rudiment and that the first haemopoietic precursors originate from blood islets of the yolk sac before
intraembryonic angiogenesis. Later during embryonic development, after vascularisation has occurred, the foetal liver is the primary site for T-cell precursors (after day 11 of gestation in the mouse). Haemopoiesis begins in the spleen and bone marrow after day 15. In adult life the bone marrow is responsible for providing haemopoietic stem cells (Metcalf and Moore 1971).

During embryogenesis cells have to divide extensively in order to provide differentiated progeny or increase the total number of cells in the embryo. However, in adult life there are static cell populations that do not divide, 'transit' cells that show limited proliferation and cells with high proliferative capacity (Lathja 1979). The haemopoietic system is maintained throughout adult life by a population of immature precursors known as stem cells. A stem cell is capable of extensive proliferation and can generate more stem cells (self renew) as well as more differentiated progeny. Although these characteristics of stem cells have been recognised for some time, it is not known to what extent stem cells can self renew. In mammals and birds there appear to be a common multipotent or totipotent haemopoietic stem cell, which can give rise to erythrocytes, granulocytes, macrophages, megakaryocytes and platelets and lymphocytes. As multipotential stem cells differentiate they can give rise to progeny which are more restricted in their developmental potential and self-renewal capacity. Certain stem cells can differentiate along all myeloid lineages (pluripotential) and others are restricted to one or two to three myeloid lineages and can be referred to as unipotential and oligopotential, respectively (figure 1.1). The existence of such stem cells was shown by Ford et al., (1956) and Till and Mc Culloch (1961), who injected bone marrow cells into lethally irradiated mice. Here, these intermediate or late stage precursors were identified by producing nodules in the spleen composed of erythrocytes, granulocytes and platelet precursors. Further studies were conducted when low numbers of cells were injected. In these studies colonies from single marrow cells were found to contain both myeloid and erythroid cells (Wu et al., 1968). The precursor cell responsible for this colony formation is called CFU-S, the colony
forming unit of spleen. This limited range of developmental potential, where no lymphoid cells were observed, suggested that the CFU-S may not be the most primitive stem cell.

Multipotential stem cells, giving rise to myeloid as well as lymphoid lineages, have been demonstrated using a variety of approaches. The capacity to reconstitute the haemopoietic system was studied in irradiated mice (Kadish and Basch 1976; Ceredig and MacDonald 1982; Sharrow et al., 1983; Hirokawa et al., 1985). Multipotential stem cells have also been demonstrated by reconstituting the genetically haemopoietic stem cell-deficient mouse strain W/Wv (Russell 1949; Russell 1979; Capel et al., 1989). Studies using radiation-induced chromosomal translocations by Abrahamson et al., (1977) and Wu et al., (1968) have confirmed that myeloid and lymphoid cells can be derived from the same stem cell. Another recent method has employed the use of retroviruses to infect bone marrow cells. Provided that only one virus has infected each cell, a unique retroviral integration site for each clone is used as a genetic marker (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986; Snodgrass and Keller 1987 and Jordan and Lemischka 1990).

Using radiation-induced chromosomal translocations, it was difficult to identify precursor-progeny relationships. In other words, it was not possible to distinguish between reconstitution by true multipotential stem cells from those by more restricted progeny that have the capacity to repopulate a limited number of lineages. However, in addition to confirming the existence of multipotential stem cells, retroviral marking experiments have made it possible to address issues concerning lineage relationships and clonal stability. The data presented by Lemischka et al., (1986) and Snodgrass and Keller (1987) indicate that a variety of stem cell clones exist. By studying the unique retroviral integration sites the observation was made that certain clones are stable while others at first are predominant in the myeloid lineage and with time switch to become predominant in the lymphoid lineage. These clonal fluctuations could be a result of an
inability to differentiate between restricted progeny and progeny of multipotential stem cells since the life-spans of most progenitors are unknown. Alternatively, the time at which a clone can be detected in a particular lineage could be influenced to a certain extent by the need for differentiated cells within that lineage. Thus, it is possible that a clone derived from a multipotential stem cell could contribute progeny to a limited number of lineages at a certain time and then to other lineages at a later time.

In attempts to investigate the life-span of stem cell clones, recent studies have indicated that different types of behaviour of individual clones can be explained partially by the length of time after transplantation at which the analysis was carried out (Jordan and Lemischka 1990; Keller and Snodgrass 1990). Dramatic clonal fluctuations in lineage contribution was observed up to 4-6 months after reconstitution. However, it appeared to be a gradual expansion of a few stem cell clones until the sytem reached a state where variation in clonal contribution was limited (6-12 months post-transplantation) and a small number of totipotent clones dominated the haemopoietic system (Jordan and Lemischka 1990). These findings support the hypothesis that at least part of the haemopoietic system of irradiated reconstituted mice is maintained by long-lived stem cells. Long-lived stem cells have also been found in W/Wv animals by reconstitution of the haemopoietic system with retrovirally marked foetal liver (FL) cells or BM without imposed depletion by irradiation (Capel et al., 1989). Therefore, the large variability of stem cell clones reported by Lemischka et al., (1986) and Snodgrass and Keller (1987) could be partially explained by the length of time after reconstitution at which the animals were analysed (5 months and 20 weeks, respectively).

It is interesting to note that both foetal liver (day 14) and adult bone marrow derived stem cell clones of donor origin, showed identical behaviour (Capel et al. 1989; Jordan and Lemischka 1990). This implies that the adult and embryonic stem cells are induced similarly in the same environment independently of their origins. This finding raises the question of the role of the microenvironment in inducing differentiation.
Figure 1.1 A model for haemopoietic stem cell differentiation
Other approaches for defining the properties of totipotent stem cells have employed several separations on the basis of buoyant density, sensitivity to antimitotic drugs or the expression of cell surface antigens. A subpopulation of adult mouse bone marrow cells with low expression of the Thy-1 antigen (Thy-1lo) and lacking expression of granulocyte, macrophage, B or T cell lineages markers (Lin-) have been shown to contain increased numbers of CFU-S (Muller et al., 1986). Further division of this population into Sca-1+ and Sca-1- (stem cell antigen-1) subsets demonstrated a population of Thy-1loLin-Sca-1+ cells that has multilineage capacities (Spangrude et al., 1988). Approximately 1 in 5 Thy-1loLin-Sca+ cells injected intrathymically into lethally irradiated hosts will give rise to a significant thymocyte clone, while colony formation was apparent in the majority of cases when 10 or more of these cells were injected (Spangrude et al., 1988). Despite the fact that stem cell-containing bone marrow subpopulations have been defined, no unique markers of stem cell populations have yet been identified. However, by combining retroviral markers with immunological reagents it was possible to follow uniquely marked foetal liver cells (day 14 of gestation) when injected into irradiated hosts (Jordan et al., 1990). Foetal liver cells expressing the surface antigen recognised by the mAb AA4.1 (McKearn et al., 1985) (0.5-1% of the total population) were highly enriched in stem cell activity. With further characterisation of the cellular properties of this population it was demonstrated that cells AA4.1 selected, Linlo and unable to bind fibronectin (FNA-) and with a density of 1.065-1.070g/ml (Den1.065-1.070) had a totipotency that was ~500-1000fold enriched in vivo (Jordan et al., 1990).

A human membrane-associated glycoprotein, CD34, is present on bone marrow progenitors with the capacity to differentiate into all lymphohaemopoietic lineages (Andrews et al., 1986; Katz et al., 1985; Civin et al., 1984). Furthermore, CD34 is also expressed by endothelial cells (Watt et al., 1987). Although the function of CD34 is unknown, recent studies have shown that it may be involved in cell adhesion and/or migration (Fina et al., 1990). In addition, the CD34 antigen is phosphorylated on
serine residues by activated protein kinase C, which suggests it may play a part in signal transduction of early progenitors (Fackler et al., 1990).

1.2.2 Stem cell migration
A variety of strategies have been used to study colonisation of the thymus. Most experimental models aimed at determining the flow of bone marrow cells to the thymus during normal conditions or in special situations when the thymus is genetically lymphoid deficient or depleted by irradiation.

One model for studying migration to the thymus utilises two mouse strains, CBA/H and CBA/H-T6T6, which can be karyotypically distinguished by the presence of the chromosome marker T6. Parabiotic mice of these mouse strains were used by Harris et al., (1964) to study the flow of cells between different compartments in animals where the haemopoietic system had not been disturbed by irradiation. Similarly, Ford et al., (1966), Micklem et al., (1975a) and Micklem et al., (1975b) investigated to what degree thymus chimerism could be achieved by injecting bone marrow cells into a part-body irradiated recipient where the thymus was shielded. The results obtained from the parabiotic mice showed that the percentage of exchange between different haemopoietic compartments was greatest in the spleen and lymph node and much lower in the bone marrow and thymus. The low proportion of immigrant cells in the thymus (~12%) indicated that very few cells entered the thymus from the periphery during normal conditions. This finding is in agreement with results from intravenous injections (i.v) of bone marrow cells into unirradiated recipients where no or a very small number of donor cells were found in the thymus of the host (Micklem et al., 1968 and Takada and Takada 1971). A much higher degree of chimerism could be demonstrated in the thymus of animals subjected to irradiation followed by BM-transplantation. This indicates that the adult thymus recruits cells from the outside and that bone marrow cells contain cells with recolonising capacity (Ford et al., 1966; Micklem et al., 1975b).
These contradictory results suggest that the thymus lobes in part-body irradiated mice, despite being protected, are affected by radiation in such a way that the rate of immigration is increased compared to what can be observed in a normal adult thymus. One explanation might be that the thymus is depleted as a result of stress, induced by irradiation thus creating space to allow colonisation by stem cells. Alternatively, depletion of bone marrow in a certain part of the body might give a signal to activate a larger number of stem cell clones than during normal conditions, which would affect colonisation of the thymus.

Another extensively used model for studying thymus repopulation is the whole body radiated bone marrow chimera where the kinetics of progeny can be followed utilising allelic differences between host and donor cells at the Thy-1 or Ly-loci (Kadish and Basch 1976; Ceredig and MacDonald 1982; Sharrow et al., 1983; Hirokawa et al., 1985). All these studies showed very consistent results, the first wave of cells appear early and reach a maximum around day 10-16. These cells are of host origin and have clearly escaped radiation. The second wave of cells which are donor derived bone marrow cells can be found around day 10-12 after injection and predominate (>98%) after 3 weeks. The data presented here raise a number of questions about precursor migration in irradiated adult mice. Firstly, does an irradiated thymus function normally, i.e is the microenvironment altered as a result of radiation? Secondly, what is the cause of the difference in repopulation kinetics of radioresistant intrinsic thymocyte precursors and bone marrow derived extrinsic progenitors? Perhaps the difference is because intrathymic and bone marrow derived precursors represent different stages in the development (e.g bone marrow derived cells may need to mature before entering the thymus while intrathymic cells are already committed to the T-cell lineage and therefore can respond quicker to an activation signal induced by radiation to proliferate and differentiate). Alternatively, the number of spaces available for stem cells might be very few and thus already occupied by radioresistant thymocytes.
To circumvent the complex question of entry into the thymus another method of reconstitution has been developed where different precursor populations are injected directly into the thymus in an adoptive transfer system (Goldschneider et al., 1986). This allows the colonisation and proliferation potential of a given number of cells from a source of choice to be studied in a system independent of migration. In addition, this way of introducing cells into a recipient thymus offers a quantitative assay in which cells introduced by limiting dilution might provide a better estimate of the number of cells required for thymus repopulation. In a comparative study where rat progenitors were injected intrathymically (i.t) and i.v into recipients a 40-fold higher recolonisation activity could be observed when the cells were transferred i.t (Goldschneider et al., 1986). This finding demonstrated a higher sensitivity of receptiveness of i.t reconstituted mice, probably because there was no need for migration (Goldschneider et al., 1986). Moreover, when comparing the colonisation capacity of cells from different tissues it was shown that bone marrow cells contained the highest activity for reconstitution of the thymus followed by the spleen, the thymus and the lymph node. The observed lag period before donor derived thymocytes appeared in the thymus of an i.v bone marrow transplanted mouse was also apparent when cells were injected i.t. This finding suggests that the long lag period for thymic repopulation after i.v injection of BM cells is not only due to a delay while the BM is colonised, because even with a direct injection into the thymus BM-derived precursors take a longer time before proliferating. In addition, when the cells were introduced i.v, a permanent chimera was established while the number of donor cells in an i.t reconstituted mouse decreased with time. This could have been due to the absence of a pool of bone marrow stem cells in the mice where the donor BM cells are given i.t while i.v reconstituted mice would have a continuous supply of T-cell precursors in their BM with a potential to home into the thymus. This suggests that in order to establish long-term thymic repopulation, a continuous supply of precursors from colonised bone marrow is required.
Penit and Ezine (1989) also used the i.t transfer system to ask whether intrinsic radioresistant bone marrow and intrathymic radioresistant cells have different reconstitution and proliferation kinetics in irradiated mice compared to introduced (exogenous) BM cells and intrathymic radioresistant cells. By irradiating mice and not supplying them with any donor cells they were able to examine the regeneration in the thymus by endogenous radioresistant precursors. In these mice the regeneration of the thymus occurred in two waves. An increase in cell number was first observed at day 3-10 and these cells were most likely the surviving intrathymic precursor cells. After a period of relapse, a second wave of proliferating cells was found at day 25-32 leading to the conclusion that these were of bone marrow origin and had migrated to the thymus. In irradiated recipients injected i.t with exogenous BM, the kinetics showed that radioresistant host cells regenerated the thymus similarly as in non-reconstituted mice. However, exogenously derived BM cells were apparent in the thymus already after 2 weeks and increased up to 3 weeks. In contrast, endogenous BM in non-reconstituted mice were not found in the thymus until 4 weeks after irradiation. Clearly, the lag period observed in non-reconstituted mice was shorter in reconstituted mice. The delayed repopulation of BM-derived cells in non-reconstituted mice is perhaps a consequence of a time period required for the BM cells to be activated before homing to the thymus. I.t transferred BM cells would therefore be more readily available for colonisation.

Collectively, these results suggest differences in the properties of precursors related to their origins which may lie in their ability to respond by proliferation to the thymic microenvironment. Perhaps the thymic microenvironment is damaged by irradiation and therefore needs some time to recover before being able to provide the right environment for BM derived stem cells. However, this hypothesis does not seem likely with respect to intrathymic precursor cells which proliferate almost immediately (Penit and Ezine 1989). Another explanation for the delay in BM colonisation could be that a signal from intrathymic precursor cells is required for BM cells to proliferate.
A number of attempts have been made to estimate the number of precursors required to repopulate a thymus. However, because of the problems in determining the arrival of different precursor cells this has proved to be very difficult. Although several investigators have found BM-derived cells in the thymus early post-reconstitution it has not been possible to establish whether those cells were responsible for proliferation after the lag-period.

In reconstitution experiments using T6 chromosomally marked BM-derived donor cells the number of cells responsible for repopulation was calculated to be ~7 for bone marrow and ~1-2 for thymus (Wallis et al., 1975) when 5x10^4 to 5-10^7 cells were injected into heavily irradiated mice. Ezine et al., (1984) created Thy-1 congenic bone marrow chimeras by injecting 2x10^6 total BM consisting of different ratios (1:10 to 1:100) of donor to host Thy-1 type. Histological analysis demonstrated thymuses varying in clonal make up. A complete reconstitution by donor type cells was observed in only 2.5% of the total number of lobes examined while the remaining lobes showed no or focal reconstitution in different areas. These results indicate that there are very few stem cells present which are capable of populating a whole lobe and that the majority of cells have a limited capacity for proliferation (Ezine et al., 1984). The results obtained in these studies imply that within a given precursor population there is a small fraction of cells with the potential to enter and colonise the thymus. A similar conclusion can be drawn from in vitro recolonisation experiments. Foetal thymic lobes that have been depleted of lymphoid elements by treatment with 2'deoxyguanosine (dGuo) can be recolonised with T-cell precursors in vitro using a hanging drop method developed by Kingston et al., (1985). In this system a single cell (day 14 foetal thymocyte) was able to recolonise an alymphoid lobe at a frequency that was estimated to 2-3% (Kingston et al., 1985). Considering the small number of cells with this capacity they must be able to proliferate extensively since the thymus was restored and
the major T-cell populations found in normal ontogeny were present (Kingston et al., 1985).

These results indicate that the number of cells colonising the thymus at a given time is low. One hypothesis is that there are specific number of sites available in the thymus and that host and donor cells have to compete for a "vacancy" as discussed earlier. Alternatively, the rate of stem cell entry maybe very low or the rudiment may only be receptive at certain defined intervals as described in the chick/quail system.

Some of these hypotheses could perhaps be tested by performing in vitro recolonisation assays. The advantage would be that an almost unlimited number of alymphoid lobes could be used for recolonisation thus increasing the possibility of obtaining a statistically significant estimate of the seeding efficiency among different precursor cells. Furthermore, this system could provide a model system where a limited number of cells could be seeded and allow for progenitor frequencies to be calculated.

### 1.2.3 Chemotactic factors related to migration

Chemotaxis can be defined as a directed movement along an increasing gradient of an attractant (chemoattractant). Using the chick/quail chromosome marker system it was shown that stem cells of extrinsic origin migrated through the mesenchymal cells into the thymic rudiment before vascularisation had occurred (Le Douarin and Jotereau 1975). This observation indicated that the lymphoid cells had been attracted by a substance, probably secreted by thymic epithelium. In a subsequent study by Jotereau et al., (1980), a variety of in vivo and in vitro recolonisation methods were used, including a diffusion chamber technique, to investigate the potential of different precursor cells to colonise the thymus using the chick/quail system. The results suggested that a chemoattractant is released from the thymus at certain times since quail cells only enter the chick thymus during these periods. These periods of cell influx into
the embryonic chick thymus were shown to occur in cyclic waves by sequential grafting of quail thymus (Jotereau and Le Douarin 1982).

An alternative method of studying migration in vitro employs the measurement of chemotaxis under agarose. Pyke and Bach (1979) used a lymphoid-depleted thymus as an attractant and reported that foetal liver cells migrate in the assay. Another technique used for studying migration is the Zigmond chamber where the movements of single cells can be measured (Zigmond 1977). Slimane et al., (1983) showed by using this chamber that chick thymic stromal cells secrete substances that are involved in the homing of precursor cells. In order to dissect out which stromal component is responsible for this phenomena, Champion et al., (1986) fractionated different stromal cell types and tested their conditioned medium (CM) for chemoattractive substances in a Zigmond chamber as well as in a Boyden chamber (an alternative method for examining chemotaxis in vitro) (Boyden 1962). Pure epithelial cell cultures of quail thymus were shown to secrete chemoattractants in the 1-4kD range which resulted in a directed movement of bone marrow precursors along a gradient of CM. Furthermore, several heterogenous stromal cell cultures and purified epithelial cells of neonatal mouse thymus have been shown to produce chemoattractive factors for T-cell precursors in vitro (Potworowski and Pyke 1985; Taubenberger and Haar 1987b; Taubenberger and Haar 1987a; Klussmann and Haar 1988; Imaizumi et al., 1987).

A rat epithelial cell line (IT-45RI) (Itoh et al., 1982) has also been described to secrete a polypeptide that will attract rat juvenile bone marrow derived cells to migrate in a Boyden chamber (Imhof et al., 1988). This factor was named Thymotaxin and rat bone marrow cells responding in vitro were also found to colonise the thymus in irradiated recipients when injected i.v (Bauvois et al., 1989). In an attempt to characterise the responder cells to Thymotaxin, Deugnier et al., (1989) isolated a small fraction of bone marrow cells moving towards the factor in vitro. When this population of cells is co-cultured with thymic stromal cells it acquires T-cell differentiation antigens
which might indicate that they are already committed to the T-cell lineage. Recently Thymotaxin was found to be identical to rat β2-microglobulin (Dargemont et al., 1989) and furthermore, β2-microglobulin isolated and purified from plasma of adult chicken has also been shown to mediate chemotaxis during colonisation of chicken embryos (Dunon et al., 1990).

1.2.4 Prothymocytes

The degree of differentiation of thymocyte precursors or prothymocytes at the time they become distinct from totipotent stem cells or at the time they enter the thymus is not clear. However, prothymocytes are usually referred to as 'thymus migrating cells' with a capacity to repopulate the thymus of an animal whose endogenous precursor population has been depleted by irradiation (Lesley et al., 1985). Thymocytes can be divided into four major subpopulations on the basis of expression of the differentiation antigens CD4 and CD8. Within the foetal and adult thymus, the subset that is lacking CD4 and CD8 (CD4<sup>-</sup>CD8<sup>-</sup>) (3-5% of total mouse adult thymocytes) (Scollay et al., 1984), contains precursor activity and can give rise to all the other thymocyte populations both in vivo (Fowlkes et al., 1985) and in vitro (Ceredig et al., 1983a; Kingston et al., 1985). In an attempt to identify precursors within the heterogenous CD4<sup>-</sup>CD8<sup>-</sup> population several investigators have studied the expression of cell surface markers in order to define an early T cell progenitor. The phagocytic glycoprotein-1 (Pgp-1) (CD44) (Hughes et al., 1981) is expressed on both myeloid and lymphoid cells (Trowbridge 1986). In reconstitution experiments in vivo it appeared that Pgp-1<sup>+</sup> bone marrow cells contained prothymocytes homing to the thymus (O’Neill 1989). This capacity was also found among Pgp-1 expressing adult thymocytes (Trowbridge et al., 1982; Hyman et al., 1986). Another surface antigen of interest is the heat stable antigen (HSA), a marker that is identified by the antibodies J11d (Bruce et al., 1981), B2A2 (Scollay et al., 1984) and M1/69 (Springer et al., 1978). When CD4<sup>-</sup>CD8<sup>-</sup> thymocytes were divided into HSA<sup>+</sup> and HSA<sup>-</sup> subpopulations and tested for their recolonisation potential in vitro and in vivo, only the HSA<sup>+</sup> subset was found to
contain precursor activity (Crispe et al., 1987; Pearse et al., 1989). However, even the most immature cells within this population had partially rearranged T-cell receptor genes (Crispe et al., 1987; Pearse et al., 1989) which indicates that there is probably an earlier T-cell precursor which is not yet identified. Taking this into consideration, Wu et al., (1991) reported a thymocyte population with TCR genes in germline configuration and with repopulation kinetics suggesting that it was an earlier precursor cell than the CD4^-CD8^- cells already engaged in TCR gene rearrangement. Phenotypically this population was negative when stained for CD8, CD3 or IL-2 receptors (IL-2R) but expressed Thy-1, HSA and CD4 (at a low level). By examining the differentiation that occurred after reconstitution it appeared that this population was at a developmental stage in between the bone marrow derived pluripotent cell and an already committed intrathymic precursor.

A limited number of culture systems have been reported where cells resembling prothymocytes could be propagated in vitro. Pro-T lymphocyte clones were established from bone marrow of adult mice and could be maintained when cultured in the presence of interleukin 3 (IL-3) and interleukin-4 (IL-4) and IL-2 or IL-4, respectively (Palacios et al., 1987; Pelkonen et al., 1988; Palacios and Pelkonen 1988). Following i.v transfer into irradiated recipients or co-culture with thymic stroma these cells were able to differentiate and acquire T-cell specific markers indicative of a prothymocyte. Furthermore, Chervenak and Altazan (1990) reported on adult bone marrow cells maintained in IL-3 with a capacity to reconstitute irradiated recipients when injected i.t but not i.v. This population appeared to contain pre-T-cells which had lost their thymus homing capacity. From this data it is not clear whether some cells still had multiple choices or if all cells were committed to the T-cell lineage. Moreover, 50% of these cells stained positive with a Thy-1 marker but the exact phenotype of these cells is not yet identified.
From these data and from the evidence presented by Spangrude et al., (1988) it appears that multipotent cells and T-cell precursors share certain characteristics like the expression of cell surface Thy-1. However, cells with the phenotype Thy-1lowLin'Sca-1+ cannot be classified as prothymocytes since they are capable of differentiating into multiple lineages (Spangrude et al., 1988) and do not only give rise to T-cells. In order to distinguish multipotent cells from cells already committed to a T-cell lineage it will be necessary to determine whether the prothymocyte phenotype is acquired in the bone marrow, after the cell has left this site, or if specific microenvironmental influences determine the destiny of a cell. For this purpose further purification of different cell fractions will be required in order to establish lineage relationships of the haematolymphoid system. Furthermore, environmental factors, such as diffusible signals, growth and inhibitory factors as well as extracellular matrices produced by stromal cells of various origin, probably play an important role in the regulation of stem cell behaviour and will need to be investigated in more detail. The influences of the microenvironment will be discussed in section 1.3, 'The thymic microenvironment'.

1.2.5 Intrathymic differentiation

T-cell development proceeds from early immature precursors to mature T lymphocytes. In order to understand at what stages the developing thymocytes shape their repertoire in terms of how they see antigen/peptides in association with MHC molecules and how commitment to certain phenotypes and functions are regulated, it is important to define subsets of maturing cells within the developing thymus. Thymic subsets can be defined by their expression of a number of cell surface antigens. Their expression is reviewed in the following section.

**CD4 and CD8:** The T-cell differentiation and function associated markers CD4 and CD8 (L3T4 and Lyt-2 in mouse) play a key role when identifying the pathways of developing thymocytes. Early in embryonic development (day 13-14 of gestation), the predominant cell type lacks both these antigens and can be referred to as double
negative (DN), CD4<sup>-</sup>CD8<sup>-</sup>. During ontogeny the DN population progresses to expressing both CD4 and CD8 (from day 16 of gestation and onwards in foetal life) and is therefore referred to as double positive (DP). Before the cells mature into the DP stage it has been shown that an intermediate phase occurs at which the cells express CD8, (CD4<sup>-</sup>CD8<sup>+</sup>) (Guidos et al., 1989b; Penit and Vasseur 1989; Shortman et al., 1988; Smith 1987). This proposal is in agreement with studies on irradiated rat thymus where CD4<sup>-</sup>CD8<sup>+</sup> cells in the cortex rapidly differentiated into CD4<sup>+</sup>CD8<sup>+</sup> thymocytes during the recovery period (Paterson and Williams 1987). A transient intermediate expressing CD4 (CD4<sup>+</sup>CD8<sup>-</sup>) prior to the DP stage has also been reported after BM transplantation into irradiated Thy-1 congenic mice and in cortisone treated mice (Matsumoto et al., 1989; Hugo et al., 1990). Recent studies have shown that the proportion of immature single positive (SP) cells CD4<sup>+</sup> or CD8<sup>+</sup> (CD3<sup>-</sup>) is mouse strain dependent and that the difference is probably related to the BM-derived cells rather than the microenvironment (Matsumoto et al., 1991). Furthermore, both SP subsets were able to give rise to DP cells in short term cultures. In contrast to these results which favour an intermediate step, no transient phase of either CD4 or CD8 expressing cells was observed between the DN and DP stage by (Wilson et al., 1989). The DP population contains precursors for the mature CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> single positive (SP) (Guidos et al., 1989b; Fowlkes et al., 1988; Smith 1987) subsets and during embryonic development SP appear at day 17-18 of gestation and increase in numbers until the time of birth (day 19-20) (Ceredig et al., 1983a; Kisielow et al., 1984; van Ewijk et al., 1982). The SP population is found mainly in the medulla. In adult mice the cortical DP is the predominant cell type while the DNAs represent only 3-5% of the total thymocyte population.

A number of minor and major subsets with coordinated expression of the CD4 and CD8 differentiation antigens in conjunction with the CD3/TCR complex or other surface molecules have been described by several investigators and the sequence and importance of these different populations will be discussed in sections to follow.
Heat stable antigen (HSA)

The heat stable antigen is a marker that has been used for identifying different stages during differentiation of both B and T-lymphocytes (Bruce et al., 1981). Expression of HSA separates DNs into two subsets differing in a number of properties. All precursor activity is contained within the DN HSA+ population of which most are actively cycling cells (Crispe et al., 1987; Ewing et al., 1988; Pearse et al., 1989; Scollay et al., 1988). The DN HSA- subset on the other hand shows no or little colonisation capacity in vivo and in vitro reconstitution experiments (Crispe et al., 1987; Shimonkevitz et al., 1987; Pearse et al., 1989). The majority of cells within the DN HSA- are believed to have a very slow turnover (Egerton et al., 1990; Scollay et al., 1988). Single positive cells (CD4-CD8+ and CD4+CD8-) whether HSA expressing or not, are mostly mature CD3 expressing cells but the CD4+CD8+HSA+ subset contains a minor immature population lacking CD3 (Shortman et al., 1988) that might belong to the transient intermediate CD8+ stage.

Interleukin-2 receptor (IL-2R): The IL-2R is composed of two receptors, one high affinity (75kD) and one low affinity receptor (55kD) (Smith 1988). Due to lack of mAbs recognising the high affinity receptor, most studies have looked only at the low affinity receptor. Unfractionated adult thymocytes contain 2% IL-2R+ cells while 50% of adult DN cells and most immature (day 14-15 of gestation) foetal thymocytes express this receptor (Ceredig et al., 1985). Treatment of mouse foetal thymic organ cultures with exogenous monoclonal antibodies (mAb) recognising IL-2 receptors (p55) results in growth of fewer thymocytes while foetal liver fragments preincubated with this mAb will still recolonise a dGuo-treated alymphoid lobe in a transfilter system (Jenkinson et al., 1982; Jenkinson et al., 1987) and give normal cell recovery. These results indicated that the IL-2R is probably not expressed before cells enter the thymus but plays an important role for the developing thymocytes. However, Plum and De Smedt (1988) repeated the same experiment and did not observe a growth inhibition
which would suggest that IL-2 - IL-2R interaction is not crucial for immature thymocytes to develop in an organ culture. This argument is in agreement with the finding that IL-2R+ cells are not found in most rat strains (Paterson and Williams 1987). Adult DNIL-2R- thymocytes can reconstitute the thymus of an irradiated mouse when the cells are transferred i.v while DNIL-2R+ fail to do so (Hyman et al., 1986). However, both these populations are capable of seeding the thymus if introduced intrathymically into the recipient (Shimonkevitz et al., 1987). Kinetic studies of the progeny however show that the IL-2R+ cells differentiate more rapidly into DP and SP cells than the IL-2R- subset (Shimonkevitz et al., 1987) and therefore resemble a more mature prothymocyte that is unable to home to the thymus. The DN IL-2R+ cells lose their IL-2 receptors prior to expressing CD4 and CD8 while ~30% of the IL-2R- population has to acquire IL-2R transiently before differentiating (Shimonkevitz et al., 1987).

**CD3/TCR:** An early event in commitment to the T-cell lineage is the rearrangement of genes coding for the T-cell antigen specific receptor. The TCR genes, encoding four distinct polypeptides (α, β, γ and δ), are rearranged and expressed sequentially during thymic ontogeny and appear on the cell surface of developing thymocytes as αβ or γδ heterodimers (Davis and Bjorkman 1988). The TCR is associated non-covalently with CD3 which is composed of 3 chains (γ, δ, ε) and with a homodimer or heterodimer of subunits of the ζ family (ζ-ζ, ζ-η, ζ-Fce γ subunit) (figure 1.3) (Klausner et al., 1990). During murine foetal ontogeny, the γδ heterodimer is the first TCR to be expressed in the murine foetal thymus (day 14 of gestation) (Havran and Allison 1988; Bluestone et al., 1987). This is followed by cells bearing αβ receptors at day 17 (Roehm et al., 1984; Snodgrass et al., 1985a; Snodgrass et al., 1985b). After day 17 of gestation, γδ positive thymocytes decline rapidly in numbers and αβ+ cells become the predominant cell type during late ontogeny and in adult life when >65% of total thymocytes are TCRαβ+ (~1-10% γδ+). Studies concerning the relationship of αβ and γδ receptors have indicated that they are of distinct lineages since intrathymically
injected γδ+ thymocytes lacking the markers CD4 and CD8 did not develop into αβ expressing cells. Furthermore, γδ cells have not been shown to give rise to αβ+ cells in vitro or when transferred intrathymically (Chien et al., 1987; Nakano et al., 1987; Scollay et al., 1988).

1.2.6 Differentiation pathways of phenotypically distinct subsets within the thymus

There are a number of model systems which have been used to investigate intrathymic T-cell differentiation. (i) irradiated mice reconstituted with precursor cells introduced i.v or i.t; (ii) regeneration of the thymus from radioresistant intrathymic precursors; (iii) DNA labelling in adult steady state thymus and (iv) mouse foetal thymic organ culture, unmanipulated or depleted of lymphoid elements and recolonised by precursors. A schematic view of T-cell differentiation pathways described in this section is shown in figure 1.2.

The precise sequence of the acquisition of T-cell specific cell surface markers is unclear but recent developments utilising a wide variety of approaches have provided excellent assays for studying the events occurring in the thymus. As already described, the DN (CD4-CD8-) population is the most immature cell type in the developing thymus. By studying this subset and characterising phenotypic changes by cross-correlating a range of other surface markers, the following differentiation pathways have been suggested. In kinetics studies of BM reconstituted Thy-1 congenic mice donor cells were found between day 0-6 after injection to have a phenotype which resembled a BM-derived prothymocyte. However, Thy-1 expression was brighter than had been described previously for prothymocytes (Lesley et al., 1990). At day 6-7 the majority of intrathymic precursors of donor origin were DN Pgp-1+IL-2R- and after a further 3 days in culture low levels of CD4 and CD8 molecules could be detected on three different Pgp-1, IL-2R subsets (Pgp-1+IL-2-, Pgp-1+IL-2R+ and Pgp-1-IL-2R+). However, the kinetics of detectable CD4 and CD8 expression differed among these
different populations suggesting a differentiation sequence of the DN cells from a Pgp-1+IL-2R- to Pgp-1+IL-2R+ cells followed by the subset of Pgp-1-IL-2R+ and, finally, a subset expressing neither of the markers, Pgp-1-IL-2R-. Further evidence of this phenotypic pattern of events among differentiating DN cells derived from the kinetics of host cells that had survived radiation. At day 6-7 when the majority of donor-derived cells were DN the radioresistant host cells displayed a DP phenotype with very few Pgp-1+ or IL-2R+ thymocytes indicating that they had already completed part of the differentiation between day 0-6. In a similar experiment where mice with different usage of the Thy-1 locus were reconstituted with adult DN thymocytes injected i.t a similar pathway of intrathymic DN cells was postulated (Pearse et al., 1989). This sequence in adult thymus has also been suggested by several other investigators in in vivo experiments (Nakano et al., 1987; Shimonkevitz et al., 1987; Scollay et al., 1988).

DNA labelling studies by (Egerton et al., 1990) using [6-3H] deoxythymidine ([3H]TdR) as an in vivo DNA precursor showed that HSA was expressed continuously by the majority of thymocytes along this differentiation pathway. Furthermore, the most mature DN population (HSA+Pgp-1-IL-2R-) contained cells with very high turnover which were capable of differentiating into DP cells and mature CD4 and CD8 single positive subsets. By correlating the number of cycling cells within this population to the number of DP cells produced it could be suggested that not all DN cells expressed CD8 transiently (or CD4) before becoming DP, indicating two pathways for DP production (1) DN to SP to DP and (2) DN to DP (Egerton et al., 1990). In addition, Penit et al., (1988), studied thymus regeneration by providing Bromodeoxyuridine (BrdUrd) as a DNA precursor, after cycling cells had been eliminated by administration of antimitotic drugs. In their studies they showed that cycling DN cells are responsible for the generation of DP cells while the CD4+CD8- subset can be produced from DP without proliferation. These differentiation sequences
in adult animals correlate very well with results obtained in foetal thymus (Husmann et al., 1988; Penit and Vasseur 1989). The earliest thymocytes were mostly Pgp-1+IL-2R- (and a few IL-2R+) at day 12-13 of gestation and at day 14 the majority of cells had progressed into Pgp-1+IL-2R+ cells and only one day later (day 15) two populations were found, Pgp-1+IL-2R- and Pgp-1-IL-2R+. The IL-2R expression that appears quite rapidly upon entry into the thymus is believed to be required for further differentiation into DP and SP cells (Shimonkevitz et al., 1987; Pearse et al., 1989). This finding was strengthened by Zuniga-Pflucker and Kruisbeek (1990) showing that radioresistant stem cells in the proliferative stage of thymus regeneration express IL-2R and that proliferation and differentiation is inhibited if exogenous mAb recognising the low affinity IL-2R is present.

The majority of thymocytes during late ontogeny and in adult mice are DP and reside in the cortex. This component of the thymus contains the greatest number of dividing cells which after an enormous expansion are believed to go through a fine selection when most cells die in situ and only ~3-4% mature into either CD4+CD8- or CD4-CD8+ single positive cells (Egerton et al., 1990) expressing functional T-cell receptors. This process of selection appears to involve cellular interactions between stromal components and thymocytes where the specificity of cells bearing TCRαβ receptors is either negatively selected (leading to death) or positively selected (leading to survival).
Figure 1.2 A schematic view of T-cell differentiation pathways

**BM**

BM-derived stem cells with thymus-homing capacity

CD3-

CD4+CD8-

CD4+CD8-

CD4+CD8+CD3low

CD4+CD8+CD3+

THYMUS

medulla

CD4+CD8+CD3+

CD4+CD8+CD3+

CD4+CD8+CD3+

CD4+CD8+CD3+

TCRB chain rearrangement

α-chain mRNA

recolonisation-capacity
A rare thymocyte population expressing the TCR but not the CD4, CD8 or HSA antigens, is present in the adult but not in the embryonic thymus (Egerton and Scollay 1990; Fowlkes et al., 1987). The majority of these cells are αβ+ (high Vβ8 usage) but also a few γδ+ cells (Fowlkes et al., 1987; Wilson et al., 1988b; Shortman et al., 1988; Pearse et al. 1988; Wu et al., 1990). These populations were classified as being mature because of its TCR expression (Crispe et al., 1987; Budd et al., 1987; Wilson et al., 1988b; Egerton and Scollay 1990). In the case of αβ+ cells their TCR was found to be functional since this population had the capacity to proliferate in response to mAbs recognising either CD3 or Vβ8 (Howe and MacDonald 1989). A number of explanations have been proposed to explain the origin of this cell type. One hypothesis is that the DN CD3- cells have progressed into DN CD3+ cells without a DP phase, another suggestion put forward by von Boehmer (1988) and Papiernik and Pontoux (1990) was that a down regulation of both CD4 and CD8 followed simultaneous interaction with class I and class II at the DP stage resulted in a mature DN CD3- phenotype.

1.2.7 TCR gene rearrangement and lineage relationship between TCRαβ and TCRγδ thymocytes in thymus ontogeny

Two different T-cell lineages bearing the CD3 antigen have been defined in terms of the heterodimeric receptor chains (αβ or γδ) expressed (Samelson et al., 1985; Brenner et al., 1988). In order to produce a diverse repertoire of functional TCRs, a series of rearrangements has to take place. Three sets of genes code for the TCR α and γ chains; Variable (V), Joining (J) and Constant (C) regions, while the β and δ chains contain additional Diversity (D) segments (Davis and Bjorkman 1988). V, D and J segments will rearrange and combine to a constant segment resulting in a gene assembly capable of encoding cell surface structures. The repertoire potential expands even further for γδ-receptors (particularly for the δ-chain) which contain nucleotide regions (N-region) at the junctional points and which participate in the final assembly of the
encoding receptor sequence by contributing with randomly added nucleotides (Davis and Bjorkman 1988; Clevers et al., 1988).

Through studies of rearrangement patterns of TCRγδ V segments, it became clear that they are combined and expressed in a regulated fashion during development and in adult life. Hence, a particular γ or δ segment(s) is rearranged predominantly at certain gestational days during foetal development or in adult life. By examining the expression of TCR γδ chains during thymus development, it appears that different VγVδ regions are produced in waves at certain days of gestation. Furthermore, particular V segment usage in a γδ receptor is often shown to be associated with a specific location in peripheral tissues in adult mice. For example, Thy-1+ dendritic epidermal cells of skin predominantly express Vγ5 and Vδ1 (Asarnow et al., 1988; Havran and Allison 1988). Moreover, in gut IEL of normal adult mice, the majority of γ-chains are encoded by the Vγ5 and Vγ7 genes while the δ-chain gene usage in these cells is diverse (Bonneville et al., 1990; Asarnow et al., 1989; Bonneville et al., 1988). γδ T-cells in intestinal mucosa and mesenteric lymph nodes preferentially use Vγ7 while γδ T-cells in other lymph nodes and spleen are predominantly Vγ4 (Itohara et al., 1990).

The δ-chain locus is located between the Vα and Jα genes. Therefore, in order to produce αβ receptors, this region has to be deleted (Chien et al., 1987; Fujimoto and Yamagishii 1987). During this process small circular DNA are excised which allows molecular analysis of the deleted segments where the status of the δ-gene can be determined by studying the recombination events which have occurred during TCR rearrangement. This method was employed by several investigators in an attempt to elucidate whether αβ and γδ T-cells originate from the same or separate lineages. Two contradictory models arose from these studies. Takeshita et al., (1989) and Okazaki and Sakano (1988) proposed a progressive rearrangement model for the generation of αβ and γδ receptors, suggesting one common lineage for the two separate receptor
chains. This was based on the fact that δ-genes on the deleted circles were rearranged and as a result of unsuccessful γδ rearrangement αβ-chains were produced. However, a similar experiment was performed where no rearrangement of the δ-gene could be found on the α-excisional circles indicating that αβ and γδ receptors originate from separate lineages (Winoto and Baltimore 1989b). This contention is in agreement with results obtained from γδ-TCR transgenic mice where αβ T-cells developed normally despite the fact that all cells contain rearranged γ- and δ-genes. In addition, αβ T-cells in these mice were not prevented from developing when self reactive γδ TCR thymocytes were deleted which suggests that they belong to two separate lineages (Dent et al., 1990).

A controversial finding of a human thymus-derived T-leukaemia cell line which expresses a βδ TCR (Hochstenbach and Brenner 1989) raises questions not only about sequential or separate lineages of αβ and γδ T-cells but also whether other means of pairing TCR chains can occur. Whatever the implication of this finding, it has to be established whether cells expressing βδ TCR are functional and furthermore if this is a rare case of a major fault in the regulation of gene expression which under normal conditions never take place.

Recent studies by Winoto and Baltimore (1989a) and Ishida et al., (1990) suggest that transcriptional events may be important in regulating the destiny of T-cell lineages. In addition, cellular interactions in the developing thymus between the immature thymocytes and the microenvironment may also play a role in producing two distinct lineages.

Before lymphoid colonisation of the thymus, rearranged Vγ7 genes have been found extrathymically in gut and liver while Vγ7 was not expressed in the thymus until day 12 of gestation (Carding et al., 1990). This finding suggests a thymic independent
pathway for Vγ7 gene rearrangement and expression. The presence of Vγ7 in athymic mice strengthens this proposal (Carding et al., 1990).

1.2.8 Function of γδ expressing cells

The two distinct CD3-associated TCR (αβ and γδ) are expressed at different time points and on cells of different phenotypes during thymic ontogeny. While there is clear evidence that CD8+ and CD4+ αβ TCR bearing T-cells recognise antigens in the context of MHC class I and class II, respectively, the precise function of γδ-receptors is yet unknown. Functional activities which have been reported include cytolytic activity and lymphokine production (interleukin-2 and 4 and interferon γ). In both man (Haregewoin et al., 1989; Holoshitz et al., 1989; Modlin et al., 1989; Porcelli et al., 1989) and mouse (Vidovic et al., 1989; Janis et al., 1989 and O'Brien et al., 1989) recent lines of evidence have suggested that γδ+ T-cells, which generally do not express CD4 or CD8, play an important role in defending the host against mycobacteria and other invading organisms. It has been shown that γδ lymphocytes (usually associated with epithelia) are increased in organs like lung, skin and gut which all have very close contact with the environment and are exposed to many foreign invaders. Contradictory results have emerged when trying to establish whether antigen-responding γδ cells require MHC-expressing presenting cells. Several investigators have isolated γδ+ lymphocyte cell lines reactive against mycobacterial antigens like a 65K heat shock protein (HSP65) (or other sources of mycobacterial antigens) and demonstrated proliferative responses of γδ without evidence of MHC restriction (Holoshitz et al., 1989; Porcelli et al., 1989; Janis et al., 1989; O'Brien et al., 1989) while others have shown a necessity for presentation by antigen presentation cells (APC) (Haregewoin et al., 1989; Modlin et al., 1989; Vidovic et al., 1989). These differences could depend on the pathogen and/or the specific tissue from which the cells were isolated. To date it is unclear whether the restricting elements are classical polymorphic MHC antigens. However, recent data indicate that non-polymorphic MHC-like molecules (CD1, Tl and Qa) could be involved in the recognition of antigen by γδ cells (Bluestone et al., 1988;
1.2.9 Positive and negative selection of immature thymocytes

The immune system is comprised of T-cells with a selected set of TCR specificities capable of recognising foreign antigens in the context of MHC class I and class II molecules. During thymic development the DP subset expressing low levels of αβ TCR is subjected to a selection process where the majority of cells die in situ and only ~1% mature into SP cells and emigrate from the thymus (Scollay et al., 1980). These selection processes are believed to be a result of interactions between the immature thymocytes and the stromal components in the thymus. Thymocytes within the DP subset which recognise self-MHC are selected to survive and mature (positive selection) while thymocytes which are self reactive are eliminated (negative selection).

In order to determine which molecules are involved and how they contribute to these events a number of approaches have been undertaken. First, the participation in antigen recognition of certain Vβ gene products has been investigated in a number of studies. Second, a body of data has been produced where monoclonal antibodies recognising the MHC class I and class II antigens, specific TCRs or CD4 were administered to animals in vivo or to thymic organ cultures in vitro. And third, the use of transgenic mouse models have given opportunities to elucidate and follow the fate of developing thymocytes in animals with a given TCR specificity and/or MHC restriction of the transgene introduced.

T-cells bearing the Vß6 (MacDonald et al., 1988b) or Vß8.1 (Kappler et al., 1988) TCR chain are deleted in mice that express the Mls^a allele of the Mls locus. Furthermore, mature SP cells expressing the Vß17 gene segment are absent in I-E^+ (a product of MHC class II) animals (Kappler et al., 1987). In experiments where antibodies recognising CD4 were administered to Mls^a neonatal mice, the clonal
deletion of self reactive class II specific TCR (Vβ6+) cells did not occur (MacDonald et al., 1988a). Instead this treatment lead to maturation of a CD4-CD8+ subset bearing Vβ6, indicating that the negative selection of cells expressing Vβ6 can be prevented by blocking the interaction of MHC and CD4. In addition, when BM-reconstituted mice expressing I-E were treated with anti-CD4 antibodies during the recovery period the negative selection (deletion) of Vβ17+ CD4-CD8+ was prevented (Fowlkes et al., 1988). Furthermore, Zuniga-Pflucker et al., (1989b) demonstrated that anti-CD4 antibody treatment could also inhibit positive selection of developing thymocytes. Antibody blocking studies directed against CD3/TCR (McDuffie et al., 1986; Born et al., 1987; Smith et al., 1989), class I (Zuniga et al., 1989a; Marusic-Galesic et al., 1988) and class II (Marrack et al., 1988; Kruisbeek et al., 1985) have shown similar results indicating that negative selection occurs at a CD4+CD8+ stage during thymus development. The destiny of a cell is determined through interactions between a number of specific cell surface molecules.

Using transgenic mouse models it has been possible to follow the maturation of individual T-cell specificities. In mice with transgene(s) encoding for a T-cell receptor with a specificity for MHC class I, the number of mature single CD8+ was increased (Kisielow et al., 1988; Sha et al., 1988; Teh et al., 1988) while expression of class II specific T-cell receptors resulted in more CD4+ cells (Berg et al., 1989; Kaye et al., 1989). To study the role of MHC class I antigen during thymic ontogeny a mutant mouse strain unable to express β2-microglobulin (β2-m), essential for class I expression, has been used. This system confirmed that class I is required for the production of mature CD4-CD8+ T-cells in the thymus since single CD8 positive cells expressing TCR were not present in the β2-m mutant mice (Zijlstra et al., 1990).

Taken together, these results and the findings that CD4 and CD8 molecules expressed on the surface of T-cells bind to non-polymorphic portions of class I and class II MHC molecules to assist binding (Dembic et al., 1987; Saito and Germain 1987; Gabert et
al., 1987) to antigen presenting cells suggest a direct involvement of the CD4 and CD8 molecules in actual signalling in the selection process.

The signals involved are as yet unknown but a tyrosine protein kinase (p56^{lck}) has been found in the membrane of mature human and mouse T-cells associated with CD4 and CD8 (Veillette et al., 1988). By cross-linking the CD4 molecule with anti-CD4 monoclonal antibodies an increased kinase activity of p56^{lck} was observed and accompanied by phosphorylation of the \( \zeta \)-subunit of the TCR (Veillette et al., 1989). This tyrosine phosphorylation activity of the zeta chain was also found in immature DP mouse thymocytes (Nakayama et al., 1989). Although the TCR-\( \zeta \) chain on CD4^{+}CD8^{+} cells was found to be already phosphorylated upon isolation, it could be dephosphorylated and rephosphorylated by stimulation with anti-CD3, anti-CD4 and anti-CD8 mAbs as well as by short-term co-culture with a cell line expressing class I and class II antigens. The reason for constitutive phosphorylation of TCR-\( \zeta \) is not resolved but it is suggested that p56^{lck} could function as a positive or negative (or both) signal transducing kinase which is involved in positive and/or negative selection of developing thymocytes. In addition, the haemopoietic cell surface molecule CD45 has a tyrosine phosphatase activity in its cytoplasmic domain (Thomas 1989; Tonks et al., 1988; Ostergaard et al., 1989) which appears to play an important role when T-lymphocytes are stimulated through their TCR (Koretzky et al., 1990).

An elegant model which has been used for studying both positive and negative selection is the \( \alpha \beta \) TCR transgenic mouse that expresses a TCR specific for the male H-Y antigen which is specific for class I (H-2D^{b}) MHC molecules. The proportion of CD4^{+}CD8^{+} cells is increased in female mice as a result of positive selection of CD4^{+}CD8^{+} thymocytes interacting with MHC molecules (encoded by H-2^{b}) without the H-Y antigen. In contrast, the population of thymocytes bearing the \( \alpha \beta \) transgene in male mice will be deleted when interacting with an H-2^{b} class I molecule presenting the H-Y antigen (Kisielow et al., 1988). The negative selection in this case can be looked upon
as a self-tolerance induction where a self antigen-reactive cell is deleted as a consequence of encountering a self peptide during development. Further discussion of clonal elimination and other ways of acquiring self tolerance will be presented below in the section 'T cell tolerance'.

Another issue important for understanding T-cell development is whether γδ T-cells are subjected to any type of selection process. To address this question γ- and δ-transgenes, specific for a Tla encoded determinant were inserted into the germ line of a mouse (Bonneville et al., 1990; Dent et al., 1990). Two different results were obtained. In one case, T-cells expressing transgenic γδ-receptors were eliminated in the thymus (Dent et al., 1990) while Bonneville et al., (1990) reported that self tolerance of self reactive T-cells was obtained by inactivation rather than deletion.

It is not clearly understood which thymic stromal cells are responsible for survival or death of thymocytes. So far the cortical epithelial cell has been the most favoured cell type to be connected with positive selection (Benoist and Mathis 1989a; Bill and Palmer 1989) while bone marrow derived stromal elements have been shown to be capable of eliminating immature DP thymocytes (von Boehmer 1990; Buckley et al., 1988).

In view of the different events which determine whether a developing thymocyte is going to live or die, it is of interest to establish how these effects are mediated. Is the signal dependent on the MHC molecule alone? This hypothesis is supported by data showing that the MHC molecules expressed by thymus cortical epithelial cells are different from those expressed by medullary stromal components (Murphy et al., 1989). Is the fate of a cell decided by which type of stromal component it encounters (thymic epithelial cell or bone marrow derived stromal cells)? Is the location in the thymus where the event takes place crucial for the outcome (cortex or medulla)? Does the affinity between the TCR and the MHC molecules dictate whether a cell is going to be deleted (high affinity) or positively selected (low affinity)? Alternatively, are the
developing thymocytes susceptible for positive and negative selection at different maturation stages during differentiation? Furthermore, it is not clear whether the MHC molecules responsible for positive selection is devoid of antigenic peptide as postulated in most reports or whether all MHC molecules involved in the selection processes contain peptides. Possible factors influencing positive and negative selection are listed in table 1.1.

1.2.10 T-cell tolerance

Burnet and Medawar were awarded the Nobel Prize for their discovery of immunological tolerance 1960 (Burnet 1959; Billingham et al., 1953). Ever since, it has been of great interest to determine how T-cells are induced to tolerate self in order to prevent autoimmunity. A number of pathways have been suggested for achieving tolerance. One type of induction is the elimination mechanism which removes self reactive cells (Lederberg 1959) while a second type of induction does not delete self reactive cells but makes them non-responding (anergic) (Jenkins and Schwartz 1987). A third pathway suggests that an anti-self T-cell which escapes the thymus will be suppressed by other cells (Gershon and Kondo 1971) although the existence of a distinct subset of suppressor cells has been much debated in recent years (Mitchison and Oliviera 1986).

Each T-cell bears on its surface only one antigen receptor specificity. Specific Vβ encoded gene products in the αβ TCR are required in order to recognise certain antigen-class II MHC complexes. These are called superantigens and can be of endogenous origin (like the minor lymphocyte stimulatory, Mls, encoded protein) or from an exogenous source (like bacterial toxins) (Marrack and Kappler 1990; Janeway et al., 1988; White et al., 1989). Animals which express a superantigen or are injected with one will delete the corresponding cell, bearing the Vβ specific for that particular antigen. T-cells expressing Vβ6, Vβ7, Vβ8.1 and Vβ9 contain Mlsα responding cells (MacDonald et al., 1988b; Kappler et al., 1988; Happ et al., 1989). Staphylococcal
enterotoxin B (SEB) when bound to class II will cause deletion of cells expressing the Vβ3, Vβ7 or Vβ8 chains and Vβ3 and Vβ11 respond to Staphylococcal enterotoxin A (SEA) on class II (White et al., 1989; Ferrick et al., 1989). Furthermore, T-cells bearing Vβ5 and Vβ11 domains are involved in the I-E-dependent deletion (Woodland et al., 1991; Dyson et al., 1991). Recent data show that certain Vβ13 products respond to toxins produced by Staphylococcus aureus (Choi et al., 1990). Another system used to study tolerance to self is the Vβ17a/I-E recognition which eliminates developing thymocytes with that particular β-chain in their TCR (Kappler et al., 1987). However, recent studies indicate that Vβ17a+ thymocytes can also be subjected to positive selection (Blackman et al., 1989).

The nature of the Mls gene product was puzzling, but recent findings by a number of groups present evidence that the ligands for a number of specific Vβ portions (Vβ3, Vβ5.1, Vβ5.2, Vβ11 and Vβ14) of the TCR are encoded by mouse mammary tumour virus (Mtv) genes (Marrack et al., 1991; Frankel et al., 1991; Woodland et al., 1991; Dyson et al., 1991). Another retrovirus, the murine leukaemia virus (MuLV) also expresses products which cause the Vβ element selection (Janeway 1991). These discoveries implicate a direct involvement of retroviral gene products in the selection of the Vβ repertoire.

Considering the selection of Vβ gene products which occurs in the thymus it seems reasonable to propose that the same kind of mechanisms exist for certain Vα encoded proteins. Nevertheless, since few mAb are available to identify specific α-chains expressed on the cell surface it has so far been difficult to prove such a mechanism. However, when studying the I-Ek restricted T-cell response to pigeon cytochrome c it appeared that Vα11 was often selected independently of the Vβ contribution, indicating that the overall receptor specificity may depend on individual participation of both chains (Winoto et al., 1986; Sorger et al., 1987; Matis et al., 1988).
Figure 1.3 The MHC antigens class I and class II, CD4, CD8 and the BCR/CD3 complex.

Some TCR/CD3 complexes contain a γδ heterodimer instead of an αβ. Others contain ζ-η or ζ-Fcγ heterodimers instead of the ζ-ζ homodimer.
Table 1.1 Factors influencing Positive and Negative selection

<table>
<thead>
<tr>
<th>Favoring positive selection</th>
<th>Favoring negative selection</th>
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<tr>
<td>low affinity TCR-MHC interaction</td>
<td>high affinity TCR-MHC interaction</td>
</tr>
<tr>
<td>location, cortex</td>
<td>location, cortico/medullary junction and medulla</td>
</tr>
<tr>
<td>cortical epithelial cell</td>
<td>BM-derived stromal cell</td>
</tr>
<tr>
<td>state of thymocyte, DP</td>
<td>state of thymocyte, DP/SP</td>
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<tr>
<td>MHC alone</td>
<td>MHC + antigen</td>
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Regarding the cell type responsible for tolerance induction, BM-derived components are the strongest candidate although certain experiments indicate that epithelial cells are also capable of deleting autoreactive cells. One approach when trying to find this cell has been to use 2'-deoxyguanosine (dGuo) treated foetal thymic lobes in transplantation experiments. A dGuo-treated lobe is devoid of lymphoid elements and also BM-derived dendritic cells which provides an opportunity to investigate the role of epithelial cells in these studies. In transplantation experiments, where cultured thymic fragments and dGuo-treated foetal thymic lobes were grafted into nude rats and allogeneic mice, respectively (Schuurman et al., 1986; Benson et al., 1987), it was shown that tolerance is probably not induced by epithelial cells. This finding is in agreement with in vitro recolonisation experiments where precursor cells were introduced into alymphoid lobes with a different haplotype (Jenkinson et al., 1985). In addition, when I-E+ dGuo-treated lobes were grafted into I-E− recipients no deletion of Vβ17a+ cells was observed, indicating that I-E expression on epithelial cells does not cause deletion (Marrack et al., 1988). It is interesting to note that von Boehmer and Schubiger (1984) and Suzuki et al., (1989) have suggested that tolerance cannot be obtained for the MHC class I expressed by the epithelial cells while alymphoid lobes can induce transplantation tolerance for thymic MHC class II. Furthermore, von Boehmer and Hafen (1986) reported that minor but not major MHC molecules can be presented by epithelial cells and induce tolerance. Recent studies by Inaba et al., (1991) and Mazda et al., (1991) suggest that dendritic cells and Mls-expressing B-cells are both necessary for the elimination of Mls reactive T cells.

Although the precise process is unknown by which self reactive cells are eliminated as a result of an interaction between the TCR and its ligand, several lines of data have suggested that cell death is apoptotic. Apoptosis is a programmed cell death in which there is chromatin condensation and DNA degradation (Wyllie et al., 1984). Immature DP thymocytes in thymic organ cultures were shown to die by this mechanism when
their TCR were engaged by exogenously added anti-CD3 mAbs (Smith et al., 1989). Mature autoreactive CD4+CD8- T-cells can be induced to undergo cell suicide in this way in short-term cultures (MacDonald and Lees 1990). Furthermore, a murine thymic epithelial cell line has been shown to induce apoptosis of thymocytes (Hiramine et al., 1990).

1.3 The thymic microenvironment

1.3.1 Embryonic origin and anatomy of the thymus

In vertebrates, gastrulation creates an embryo having an internal endodermal layer, an intermediate mesodermal layer and an external ectoderm. The endoderm's function is to form the lining of the two tubes within the body (the respiratory and the digestive tube). Between these two tubes there is a common region called the pharynx. In this region the mammalian embryo produces four pairs of pharyngeal pouches. The thymic epithelium is derived from the third pair of the pharyngeal pouches and the ectoderm of the branchial cleft (Cordier and Haumont 1980). Hence, the epithelial components originate from two distinct sources and later during ontogeny BM-derived stromal elements as well as T-cell precursors will seed the thymus and give rise to the final architecture of the organ.

The thymus consists of two lobes surrounded by a thin capsule of connective tissue. The capsule extends into the gland, forming septa which partially divide the lobes into lobules. Peripheral portions of the lobule (cortex) are heavily infiltrated with small CD4+CD8+TCRlow immature thymocytes and the epithelial cells in this compartment have long thin processes (van Ewijk 1984; van Ewijk 1988). The central part (medulla) contains fewer, but mainly CD4+CD8+ and CD4+CD8- mature T-cells, which express high levels of TCR. Both epithelial cells (with shorter processes than cortical epithelium) and bone marrow-derived dendritic cells reside in this area (Kaiserling et al., 1974). There is one compartment in the outer subcapsular area of the lobe, where
CD4^+CD8^+ immature thymocytes and reticular stromal cells can be found (van Ewijk 1984; van Ewijk 1988). Macrophages (which are of haemopoietic origin) exist throughout the tissue (Beller and Unanue 1980).

1.3.2 Stromal cells

The identity of phenotypically distinct epithelial and non-epithelial stromal components has been revealed by using monoclonal antibodies as well as by electron microscopy (EM). Epithelial cells constitute the major stromal compartment of the thymus and can be found in the medulla, cortex and in the subcapsular cortex (SCC) and are characterised by the presence of tonofilaments and desmosomes revealed by EM studies. Cortical epithelial cells interact with each other via their long thin processes giving rise to a fine meshwork. A different kind of epithelial cells also reside in the cortex, namely the thymic nurse cell (TNC). TNC are multicellular complexes consisting of an epithelial cell nursing between 20-400 enclosed thymocytes (Wekerle et al., 1980; Kyewski and Kaplan 1982). The precise function of TNC is not known but it has been suggested that they may provide a microenvironment for T-cell differentiation and maturation (Kyewski et al., 1986). In the medulla where the epithelial cells are more closely packed there is yet another epithelial structure present, the hassalls corpuscles. These aggregates are composed of hypertrophic epithelial cells shaped in rings (Lampert and Ritter 1988). A specific role of these clusters has not yet been defined. A schematic representation of the thymic microenvironment is shown in figure 1.4.

An antibody (A2B5) recognising a glycolipid of neuroendocrine cell types stains some epithelial cells in the medulla, SCC and TNC (Haynes et al., 1983). This reactivity might indicate neural crest derivation of certain epithelial cells, however, cells in the pancreas positive for A2B5 do not appear to be of neural origin and therefore this pattern of reactivity may reflect a common epitope or a common function rather than an identical embryonic origin. Three thymic hormones, thymulin (FTS-Zn), thymopoietin
and thymosin α1 have been shown by immunohistochemical studies to be present in the cytoplasm of mouse epithelial cells located in the medulla and SCC (Hirokawa et al., 1982; Jambon et al., 1981; Viamontes et al., 1986). Thymulin and thymopoietin appear to be of exclusive thymic origin (Bach and Dardenne 1973) while thymosin α1, which is present in athymic mice could be from another source as well (Safieh and Kendall 1988).

Of the various kinds of cell-type specific proteins and structures the intermediate sized filaments (IFs) represent a large family of proteins (Lazarides 1982). Among IFs cytokeratin is a hallmark of epithelial cell differentiation and has in humans been characterised as heteropolymers of two chains. Depending on the molecular weight (MW) of these chains they are components of either single-layered (simple) epithelia or groups of cells of stratified epithelia (Farr and Braddy 1989). A number of monoclonal antibodies raised against human cytokeratins, appear to divide cortical and medullary epithelial cells of the mouse thymus into two groups, depending on the isoform of keratin present. Hence, cortex-derived resembles simple epithelia expressing cytokeratin 8 and 18, while medullary-derived cells are comparable to stratified epithelium, containing a number of different MW forms (Cooper et al., 1985).

A number of antibodies have been produced which are useful for characterising thymic stromal components in the mouse. Two monoclonal antibodies differentially staining cortical and medullary epithelium are ER-TR4 and ER-TR5, respectively (van Vliet et al., 1984b). The TNC which is present in both the SCC and cortex also reacts with ER-TR4 (van Vliet et al., 1984a). Several other monoclonal antibodies in the ER-TR series have been very helpful in elucidating the location of various other stromal components as well. The ER-TR7 mAb recognises reticular fibroblasts and ER-TR6 identifies the BM-derived non-lymphoid cells (macrophages and interdigitating dendritic cells (IDC)) and also a subset of lymphoid cells (van Vliet et al., 1984b). Two recently produced mAbs MIDC-8 and NLDL-145 react with IDC in a number of
lymphoid tissues (Breel et al., 1987; Kraal et al., 1986). In the thymus, MIDC-8 stains the medullary IDC exclusively while NLDL-145 in addition to detecting IDC crossreacts with cortical epithelial cells. The epitope recognised by MIDC-8 is on the surface of the cell whereas the antigen recognised by NLDL-145 is a cytoplasmic component. Macrophages are stained with Mac-1 which reacts with a member of the integrin family of adhesion molecules (Springer et al., 1979; Springer 1990). Mac-1 along with a number of other mAb stain both macrophages and IDC (Brekelmans and van Ewijk 1990).

The major histocompatibility complex (MHC) molecules, class I and class II are expressed by the cells of the microenvironment, both on the fixed epithelial cells and the free BM-derived components (van Ewijk et al., 1980). IDC are characterized by their strong class I and class II expression and are very potent antigen presenting cells (Rouse et al., 1979; Kyewski et al., 1986). Macrophages on the other hand express class I constitutively but only a small subset are found to be class II positive. Epithelial cells of both the medulla and cortex express class I and class II and interestingly, TNC with their internal specialized microenvironment display very high density MHC class I and class II antigens (Wekerle et al., 1980; van Vliet et al., 1984a).
Figure 1.4 Schematic representation of the microenvironment in the thymus gland.
1.3.3 Stromal cell-thymocyte interactions

It is well documented that the stromal cells are required for the development of functionally mature T-cells. The large heterogeneity within individual cell types and between distinct lineages in the microenvironment has made it difficult to dissect out the precise role of each subset. Therefore it is important to define mechanisms by which stromal cells mediate their effects. The application of experimental techniques and reagents have allowed study of (i) what molecules are involved in stromal-lymphoid interactions; (ii) what kind of soluble stromal-derived mediators regulate the production of functional T-cells as well as identifying novel factors encoded by stromal elements and (iii) of how the extracellular matrix is involved in influencing the developing cells.

1.3.4 Adhesion molecules

There are at least three families of adhesion molecules involved in the immune system. First, the immunoglobulin superfamily which contains several adhesion molecules important in the education of T-cells. These are the lymphocyte function-related antigens: LFA-2 (CD2) and LFA-3, the intracellular adhesion molecule 1 and 2 (ICAM-1, ICAM-2), the CD3/TCR complex, CD4, CD8 and the MHC antigens class I and class II. Second, the integrin family including two adhesion receptors related to thymus function, LFA-1 and Mac-1. And third, the selectin family, important for homing of cells to the thymus, to which Mel-14 belongs (Springer 1990).

Mel-14 is a molecule expressed by some bone-marrow cells and peripheral circulating T-cells. In the thymus 1-3% of all thymocytes express Mel-14 and the small population of Mel-14hi cells represents thymus emigrants (Reichert et al., 1984). Furthermore, many cortisone-resistant thymocytes express high levels of Mel-14 (Reichert et al., 1984). Binding of circulating lymphocytes to high endothelial venules (HEV) of lymph nodes and other tissues is mediated through homing-receptors such as the Mel-14 antigen (Gallatin et al., 1983). Once a cell is activated the expression of Mel-14 is low
or negative (Springer 1990). From cloning studies of a murine cDNA it appears that Mel-14 mAb recognises a lectin-domain of the antigen involved in cellular adhesion (Lasky et al., 1989).

The T-cell antigen CD2 (T11, LFA-2) was first identified as the sheep erythrocyte receptor (Kamoun et al., 1981). CD2 appears early during foetal development (day 13) and is expressed on >95% of adult thymocytes and peripheral T-cells (Kyewski et al., 1989a; Altevogt et al., 1989). The role of CD2 is unknown but the fact that certain combinations of anti-CD2 antibodies can stimulate human thymocytes and resting T-cells indicate an important function in signalling during T-cell development and for mature T-cells (Meuer et al., 1984; Fox et al., 1985). The ligand for CD2 is LFA-3 (Vollger et al., 1987; Selvaraj et al., 1987) which has a wide cellular distribution. In the thymus one adhesion pathway between thymocytes and epithelial cells is mediated by CD2/LFA-3 (Denning et al., 1987; Singer and Haynes 1987). Another pathway by which thymic stromal cells probably interact with developing thymocytes is the binding of LFA-1 (present only on lymphoid cells) to its ligands ICAM-1 and ICAM-2 on the target cell (Staunton et al., 1988; Marlin and Springer 1987). These observations were originally made with human material where mAb are available for antibody blocking studies while similar experiments on mouse tissue have been hampered by lack of reagents. However, recently a mouse homologue of the human ICAM-1 molecule was identified by a rat mAb recognising a murine lymphocyte activation antigen 2 (MALA-2) (Prieto et al., 1989). The staining pattern in the thymus showed distribution on all epithelial components in the SCC, cortex and medulla and in addition dendritic cells and medullary macrophages were also positive for this antigen (Prieto et al., 1989).

The CD8+ cytotoxic and CD4+ helper T-cell subsets are stimulated by foreign antigen bound to a MHC class I and class II molecule respectively, on antigen-presenting cells. This recognition is mediated by the TCR/CD3 complex with either CD8 or CD4 on T-cells and the MHC molecule on the target cell (Doyle and Strominger 1987; Norment et
It is believed that CD4 and CD8 play a role in transmembrane signalling as well as increasing the affinity of the specific TCR recognising peptide plus MHC on the antigen presenting cell. Recently, the binding site for the CD8 molecule on the class I molecule was mapped which provided strong evidence that binding of TCR and CD8 to the same MHC molecule is necessary for T-cell activation (Salter et al., 1990). The association of these molecules is also crucial during thymic development. During an immature stage of the differentiating thymocytes, both CD4 and CD8 are expressed at the same time. It is at some point in this time-period that the decision is taken by the cell whether to differentiate into a cytotoxic or helper T-lymphocyte as a result of a recognition by the TCR of either class I or class II.

Resting lymphocytes which do not adhere to a target cell unless they are stimulated can be induced by phorbol esters to associate with target cells in a LFA-dependent adhesion (Patarroyo et al., 1985). It is interesting to note that anti-CD2 and anti-CD3 antibodies have also been shown to have an effect on the regulation of LFA-1/ICAM-1 adhesion by upregulating the avidity of LFA-1. This finding suggests that the recognition by TCR of MHC plus peptide in the thymus and in the periphery is facilitated by LFA-1-dependent cell-cell interactions and that the adhesiveness can be controlled by activation of intra-cellular second messenger through CD3/TCR and CD2 (Dustin and Springer 1989; van Kooyk et al., 1989). In vitro studies of thymic epithelial-thymocyte interactions using mouse epithelial cell lines supported the involvement of LFA-1 in an adhesion-de-adhesion mechanism (Lepesant et al., 1990). In these studies anti-CD3 mAbs were shown to induce both the adhesion and de-adhesion processes. This observation is thus in agreement with Dustin and Springer (1989 and van Kooyk et al., 1989). These data are strong indications that adhesion molecules like CD2, LFA-3, ICAM-1 and LFA-1 also cooperate in the association of TCR and MHC during thymocyte development in mouse (as have been shown for human mature T-cells).
A novel adhesion molecule has recently been reported to be expressed by a medullary thymic epithelial cell line (E-5) (Potworowski et al., 1986; Couture et al., 1990). This molecule was found to bind thymocytes in a rosette-assay and the majority (~94%) displayed a CD4+CD8+ phenotype, typical of an immature cortical-derived thymocyte (Hugo and Potworowski 1990).

Thy-1 which is expressed at high levels on thymocytes during early ontogeny, mature T-cells and also on stromal cells has been proposed to be yet another candidate involved in lympho-epithelial adhesion (He et al., 1991). Thy-1 is a member of the immunoglobulin superfamily and may, as do many other family members, have a role in cell recognition.

Several multicellular complexes composed of different stromal cells and thymocytes exist in the thymus, and can be isolated by a series of enzymatic digestions (Wekerle et al., 1980; Kyewski and Kaplan 1982). Among these complexes are the lympho-epithelial complexes (TNC) (Wekerle et al., 1980; Kyewski and Kaplan 1982) and the thymic rosettes (T-ROS) which consist either of a class II positive dendritic cell (DC-ROS) or a class II negative macrophage (MΦ-ROS) with bound thymocytes. The localisation of these stromal components in different compartments of the thymus indicate that they may assist differentiation of thymocytes at specific stages during development. One study which addressed this proposal, used congenic bone-marrow chimeras (Thy-1.1/Thy-1.2) and followed the appearance of stromal cell-thymocyte complexes with time in the recipients (Kyewski 1987a). The results demonstrated that MΦ-ROS (class II-) in the cortex were found first followed by TNC in the cortex and DC-ROS in the medulla. The dominant phenotype of associated thymocytes were CD4+CD8+ (which is an intermediate stage between immature CD4-CD8- and mature single positive cells). In another study by Shortman et al., (1989) T-ROS were isolated from normal adult thymuses and the distribution of all CD4, CD8 subsets among associated thymocytes were investigated. In this study, all four subpopulations could
be isolated from the rosettes, however, the DP phenotype was dominant. This result failed to prove a distinct phenotypic pattern of rosettes. Instead the bound population had a CD4 and CD8 distribution similar to the total thymocyte fraction. However, since the study was performed on 'bulk' rosettes (not taking into account that they include two different stromal cell types, IDC and MΦ), this may explain the CD4 and CD8 heterogeneity observed. The TCR/CD3 expression of rosette-bound thymocytes is low or negative (the majority CD3⁻) (Shortman et al., 1989; Kyewski et al., 1987b). This suggests that the interaction may not be TCR mediated. However, antibodies against the TCR/CD3 complex have been shown to interfere with the interactions between class II⁺ medullary dendritic cells as well as cortical epithelial cells and immature thymocytes but not with the adhesion of class II⁻ macrophages and immature thymocytes (Kyewski et al., 1989b).

Thymic nurse cells can be described as a specialised microenvironment which developing thymocytes may encounter during intrathymic differentiation. The TNC enclose thymocytes with a typical cortical phenotype (CD4⁺CD8⁺ with low CD3 expression) whereas no CD4⁻CD8⁻ have been isolated from these complexes (van Vliet et al., 1984a; Kyewski and Kaplan 1982). Interferon γ (IFNγ) and Tumour necrosis factor α (TNFα) can enhance reformation of TNC in vitro (Defresne et al., 1990). Furthermore, antibodies to class I and class II MHC antigens have been shown to block reformation of TNC in vitro, indicating that TCR/MHC interactions may mediate this process (Schuurman 1988). However, this inhibition was not seen using a human epithelial cell line and thymocytes (Singer et al., 1986; Vollger et al., 1987)). The finding that IFNγ can induce class II expression on thymic epithelial cells indicates that secretion of IFNγ can facilitate TNC formation directly. In contrast, no induction of class II could be observed with TNFα treatment, suggesting that TNFα might be indirectly involved in mediating epithelial-thymocyte adhesion or alternatively, induce other molecules important in these mechanisms, not yet identified (Defresne et al., 1990).
Most studies involving cell-cell interactions in T-ROS and TNC have so far excluded that adhesion molecules like CD2, LFA-3, ICAM-1 and LFA-1 may also cooperate in the association of TCR and MHC in developing thymocytes as have been shown in other systems. Therefore, in order to explore in what fashion these interactions occur and their function in vivo, more extensive studies have to take place.

1.3.5 Cell-matrix adhesion

In addition to cell-cell adhesive interactions leukocytes bear multiple receptors for cell-extracellular matrix adhesions. Within the integrin family, in addition to LFA-1 and Mac-1, there are other members which are involved in mediating attachment to extracellular matrix (ECM). One subgroup (named the β1 family), contains at least six very late antigen proteins (VLA 1-6) which in many cases represent receptors for matrix components like fibronectin, collagen and laminin and bind to the amino acid sequence Arg-Gly-Asp (RGD) on the ligand (Ruoslahti and Pierschbacher 1986; Ruoslahti and Pierschbacher 1987). VLA proteins are heterodimers with an α and a β chain and are expressed on both haemopoietic and non-haemopoietic cells and tissues (Hemler et al., 1987; Hemler 1988; Hemler 1990).

VLA-4 and VLA-5 are both receptors for fibronectin and are expressed in the thymus (Hemler 1990). It is interesting to note that fibronectin has proven to play an important role in the initial colonisation of the avian thymus (Savagner et al., 1986). In addition, both immature CD4-CD8- and CD4+CD8+ thymocytes bind to fibronectin (Cardarelli et al., 1988). Mature single CD4 positive cells can be stimulated to proliferate in a synergistic fashion by fibronectin and anti-CD3 antibodies and this activation was inhibited by anti-VLA 5 antibodies indicating a direct involvement of integrins in the immune responses (Matsuyama et al., 1989).
Another important function of the ECM, in addition to cell-adhesion, is the capacity to bind soluble factors (Gordon et al., 1987). The haematopoietic growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) was found to bind to glycosaminoglycans in the ECM which are produced by bone-marrow stromal cells. The glycosaminoglycan which appears to hold most of this capacity is heparan sulphate which could bind and present biologically active interleukin-3 (IL-3) and GM-CSF (Roberts et al., 1988). However, other distinct ECM components like hyaluronic acid and chondroitin sulphate found in bone marrow could also be important in the thymus. In fact, hyaluronate is the major glycosaminoglycan synthesized by thymic epithelial cells (Britz and Hart 1983). Furthermore, hyaluronate has recently been found to be the ligand for CD44 (Pgp-1) (Aruffo et al., 1990). This finding is important since expression of Pgp-1 appear to be crucial for precursors homing to the thymus. The implications of these results for the thymic microenvironment are many. First, different factors produced by specific stromal components may be localised by binding to the ECM in certain compartments of the lobe and thereby effect particular subsets of thymocytes. Second, growth factors and other soluble mediators may increase their lifespan by binding to ECM. And third, ECM may support thymocytes to move, in addition to maturing, during their transit through the thymus.

1.3.6 Soluble mediators

An increasing number of regulatory proteins have been identified and described to be produced by multiple cell types and act on specific target cells. This group of proteins are called cytokines and include interleukins of which some contain activity for cells of the lymphoid lineage like the interleukins 1, 2, 4, 6, 7. Four colony stimulating factors (CSF) act primarily on cells of the myeloid lineage: granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), macrophage CSF (M-CSF or CSF-1) and multipotential CSF (multi-CSF).
A large body of data has been accumulated over the last years where the role of different factors independently or in cooperation with other mediators in thymocyte activation and differentiation has been assessed. Most studies have examined these complex events by manipulating lymphoid and stromal elements outside the thymic environment. However, a number of studies have also been performed in intact thymic organ cultures in vitro or in normal or transgenic mice. In these studies the regulation, cellular specificity, action of specific factors and the possible relationship between several factors during thymic development have been examined and will be discussed.

Early immature CD4-CD8- thymocytes in embryonic thymus contain ~30% IL-2R positive cells and will proliferate in an IL-2 dependent fashion when stimulated in vitro with IL-2 plus the mitogen concanavalin A (Con A) (Raulet 1985; Ceredig et al., 1985). Foetal thymic organ cultures maintained in medium supplemented with IL-2 resulted in a reduced number of DP cells (Skinner et al., 1987; Plum et al., 1990). In addition, these culture conditions promoted growth of a DN subset with cytotoxic activity (Skinner et al., 1987; Plum et al., 1990; Leclercq et al., 1989). It has been proposed by Ceredig et al., (1989) and Plum et al., (1990) that this population is γδTCR+.

In experiments where anti-IL2R antibodies were added to organ cultures conflicting results have emerged considering the effect on the development of T-cells caused by antibody blocking (Jenkinson et al., 1987; Plum and De Smedt 1988). Since the IL-2R antibodies used in these studies recognise only the low affinity receptor chain one has to treat the results with some caution. However, in situ studies of IL-2 mRNA expression as well as cell-surface staining of the low affinity chain correlate well (Carding et al., 1989). Furthermore, the impaired growth and differentiation observed in organ cultures treated with anti-IL2R antibodies (Jenkinson et al., 1987) could be reversed by exogenous IL-2.
Interleukin 4 (IL-4) was first described as a B-cell growth factor (Howard et al., 1982). IL-4 has subsequently been shown to have pleiotropic effects on a variety of cell types (Paul and Ohara 1987). Normally, IL-4 is produced by CD4+ helper T-cells and mast cells (Mosmann and Coffman 1989; Conrad et al., 1990).

In the embryonic thymus IL-4 mRNA can be detected at day 13-14 of gestation, demonstrated by in situ hybridisation (Sideras et al., 1988; Carding et al., 1989). IL-4 induces proliferation of activated adult and foetal thymocytes (Zlotnik et al., 1987). The responding foetal thymocytes are DN or SP while the DP population is unaffected (Palacios et al., 1987; Zlotnik et al., 1987). The IL-4 response was confirmed to be IL-2 independent since anti-IL2 antibodies did not inhibit proliferation. In addition, to a proliferative response Palacios et al., (1987) reported that day 15 foetal thymocytes could differentiate into CD8+ cells after treatment with the phorbol ester PMA plus IL-4. This induced CD8 expression could not be seen after treatment with PMA+Ionophore+IL-2, although these agents induced mitosis. No differentiation was observed among adult DN thymocytes after growth induction by IL-4 and PMA (Lowenthal et al., 1988). It has, therefore, been proposed that the effect of IL-4 on adult immature thymocytes could be to promote self renewal.

In a comparison of the proliferative responses of adult and day 14 foetal DN thymocytes to a number of growth factors and mitogens it was suggested that the IL-4 pathway is used earlier than IL-2 during development (Takashi et al., 1989). In this study the response to IL-4 and PMA was greater among foetal thymocytes while PMA+IL-1+IL-2 or IL-2+IL-1 affected the growth of adult cells to a higher degree. The response to the latter agents of adult cells was the induction of CD3/TCR expression while no such induction could be shown in response to IL-4+PMA by foetal thymocytes. This indicates that the two different pathways could be important at different times during development. This result is in agreement with Carding et al., (1989), who demonstrated the highest IL-4 mRNA expression at day 15 of gestation.
when the majority of thymocytes are CD4-CD8- and CD3-, and no expression beyond day 18 when most cells express CD3. Differentiation of human thymocytes preferentially into γδTCR+ T-cells has been found to be promoted by IL-4 (Barcena et al., 1990).

Two transgenic mouse models have been used to address the role of IL-4 (Tepper et al., 1990; Lewis et al., 1991). Tepper et al., (1990) introduced the IL-4 transgene under the control of an immunoglobulin promoter/enhancer leading to expression of the transgene in both B- and T-cell compartments while Lewis et al., (1991) restricted the IL-4 expression to the thymus. Both groups reported a decrease of DP thymocytes and an intrathymic increase of mature single CD8+ cells. Surprisingly, very low numbers of CD4-CD8+ cells were found in the periphery, presumably as a result of cells failing to emigrate from the thymus. This significant increase of CD8+ cells in response to IL-4 is in agreement with previous findings by Palacios et al., (1987) and Zlotnik et al., (1987).

It is thought that during intrathymic differentiation DP thymocytes are selected to live and mature (positive selection) or die (negative selection). The decrease of DP cells in both transgenic mouse models could, therefore, be due to an increased cell death as a result of negative selection. The T-cell defect in transgenic thymuses of Tepper et al., (1990) was shown to be related to over expression of IL-4 in a BM-derived cell. This indication came from the observation that wild-type BM was able to reconstitute an irradiated mouse with abnormal thymic development and restore the thymus to a normal state. The BM-derived cell responsible for IL-4 secretion is most likely of lymphoid rather than stromal lineage. However the BM-derived dendritic cell may play a key role by engaging in negative selection. Dendritic cells express high levels of class II, which could be induced even further by increased IL-4 secretion, since B-cells when stimulated with IL-4 are found to enhance their class II levels (Noelle et al., 1984). Furthermore, DP cells in the transgenic mice have increased levels of CD3/TCR
expression which was shown in antibody blocking studies to be mediated by IL-4. The combination of high levels of class II and CD3/TCR may result in an efficient thymocyte-dendritic cell interaction with very high avidity between MHC and TCR leading to a rapid elimination of reactive DP cells. This deletion would affect the number of mature CD4+ cells since class II is its ligand and indeed the absolute number and percentage of CD4+CD8- cells are decreased in the transgenic thymus.

Interleukin 7 (IL-7) is another factor which is believed to play an important role for both T- and B-cell development. This factor was first described as a pre-B cell growth factor (named lymphopoietin-1, LP-1) and was secreted by a transfected BM stromal cell line (Namen et al., 1988b). The isolation of cDNA, encoding the growth factor resulted in the purification of both mouse and human IL-7 (Namen et al., 1988a; Goodwin et al., 1989). IL-7 mRNA has been isolated both from thymic tissue and a thymic epithelial cell line (Namen et al., 1988a; Murray et al., 1989; Sakata et al., 1990). Both adult and foetal thymocytes respond by proliferating in the presence of IL-7 (Conlon et al., 1989; Murray et al., 1989; Okazaki et al., 1989) and both DN and SP cells respond while DP are not affected. The proliferative IL-7 response can be augmented by mitogens like PHA and Con A and growth induction of immature thymocytes appears to be IL-2 and IL-4 independent (Conlon et al., 1989). In contrast, mature T-cells stimulated to proliferate by IL-7 and Con A were dependent on IL-2 production in order to proliferate. This was confirmed by growth inhibition by anti-IL2 antibodies (Morrissey et al., 1989).

Foetal thymic lobes, cultured in IL-7, showed increased cell recovery (Murray et al., 1989; Watson et al., 1989). The immature DN population can be kept viable for ~7 days outside the thymic microenvironment with IL-7 (Murray et al., 1989; Okazaki et al., 1989) and day 12 foetal thymocytes (CD4-CD8-CD3+) that have been kept in IL-7 can be induced by IL-2 to differentiate and express CD3 and CD8 (Watson et al., 1989). Neither pro-T clones of BM origin or foetal thymocyte clones responded to IL-
7 supplied either exogenously or by an IL-7 secreting cell line. This suggests that IL-7 may act later in development than the stages these clones represent (Takeda et al., 1989).

In the immune system the interleukin 1 molecules (IL-1α and IL-1β) have mainly been characterised as mediators released by macrophages and other cell types during an inflammatory response (di Giovine and Duff 1990; Oppenheim et al., 1986). In the thymus, IL-1 is secreted by stromal cells and has been shown to have an effect on thymocyte growth (Farr et al., 1989). Thymic organ cultures treated with anti-Ia antibodies showed disturbed development of functional T-cells (DeLuca et al., 1985). This inhibition could be restored by addition of IL-1 (DeLuca and Mizel 1986). The addition of anti-IL-1 antibodies to organ cultures resulted in reduced thymocyte growth and inhibition of CD4 and CD8 expression (DeLuca and Mizel 1986; Crosier et al., 1989). Furthermore, in vitro studies of thymic dendritic cells has shown that IL-1 can enhance the accessory function of dendritic cells resulting in an increased proliferation of immature DN thymocytes (Inaba et al., 1988). This effect could be inhibited by anti-IL-1 antibodies. In situ hybridisation demonstrated IL-1 mRNA expression of a few cells (~0.1% of total) at the cortical-medullary junction and in the medulla of mouse adult thymus (Takacs et al., 1988). The location of IL-1 producing cells resembles the distribution of IDC cells. Taken together, these findings indicate that IL-1 plays an important role in the thymus by inducing developing thymocytes to proliferate and mature and that the BM-derived dendritic cells are likely to mediate these events. Furthermore, the distribution of IL-1 mRNA expressing cells in the compartment between the cortex and the medulla suggest an important role at the crucial time during differentiation when thymocytes are selected for further maturation.

In addition to influencing developing thymocytes, IL-1 secretion has been shown to induce production of interleukin 6 (IL-6) by human thymic epithelial cells (Galy et al., 1990). In mouse thymus, IL-6 is produced by epithelial cells and macrophages
IL-6 contains CSF-like activities and in mouse can support the proliferation of some granulocyte/macrophage progenitors (Wong et al., 1988).

As already described, the CSFs have the capacity to induce myeloid differentiation. In addition, a number of CSFs have been shown to be produced by thymic stromal cells. Fibroblasts and epithelial cells expressing IL-4R can be stimulated by IL-4 to produce CSFs (Park et al., 1988). GM-CSF treatment of organ cultures in vitro resulted in an increased number of Mac-1+ cells, in agreement with the reported target of GM-CSF in the granulocyte-macrophage lineage (Crosier et al., 1989). Furthermore, GM-CSF and multi-CSF (interleukin-3, IL-3) induce a small population of accessory precursor cells within the DN thymocyte subset, to mature into MΦ and DC in vitro (Papiernik et al., 1988). The maturation of accessory cells enhances thymocyte proliferation in response to IL-2. It has been suggested that the effect of GM-CSF is mediated by IL-1, which perhaps is produced by the accessory cells. In contrast to the in vitro experiments, long-term in vivo administration of GM-CSF to adult mice did not influence either growth or maturation of the thymus (Pojda et al., 1990).

A novel T-cell growth factor with a proliferative effect on T helper cells has recently been described in mouse (Ogata et al., 1987; Sato et al., 1988). This factor was shown to be secreted by an epithelial cell line and primary cultures of thymic stromal cells and is named Thymic-stroma-derived T-cell growth factor (TSTGF). Further studies indicated that TSTGF in combination with PMA could act on immature DN thymocytes and enhance growth (Mizushima et al., 1989).

Proliferation assays in vitro where different growth factors are used in combination has given an opportunity to investigate the network of communication within the thymus. Suda et al., (1990) have shown that the effect of IL-4 induced proliferation of single positive T-cells can be enhanced by IL-6. IL-1 and IL-6 synergise with IL-7 in the
proliferative response of unfractionated and SP (CD4⁻CD8⁺ and CD4⁺CD8⁻) adult T-cells, respectively, (Murray et al., 1989; Chantry et al., 1989). When TSTGF was used in combination with IL-1 and IL-4 or IL-1 and IL-2 a synergistic growth promoting effect on CD4⁻CD8⁻ thymocytes could be observed (Mizushima et al., 1989).

Evaluating the effects of soluble factors on T-cell differentiation reveals that they act on early cells (at the DN stage) or on late cells (at the SP stage) in development, whereas it appears that the intermediate stage, when the cells express both CD4 and CD8, may be dependent primarily on direct contact with stromal cells. A possible cytokine network in the thymus is shown in figure 1.5.

Described here are a number of cytokines important in thymic development. Most studies have focused on one or a few factors and their effects on a limited number of selected populations and obviously the situation in vivo is much more complex. However, the foetal thymic organ culture provides a system in which the action of a given cytokine can be studied in the context of a whole organ. The response of a particular factor can be assessed taking into account all cell types in the thymus which will respond. Furthermore, the effect of a factor in conjunction with other cell regulators expressed simultaneously can be evaluated. Although, this in vitro assay offers a model system to study the biological function of secreted proteins where the cellular interactions involved can be easily monitored and manipulated, it has some disadvantages. First, cytokines interact in a complex network where they induce other factors to be secreted which will contribute to the effect by antagonistic, additive or perhaps synergistic functions. The interpretation of results from these kinds of experiments must, therefore, be treated with caution when considering whether the response is directly due to a particular cytokine, or whether the influence of other factors play a crucial role in the response. Second, cytokines are usually secreted locally and transiently in response to a particular signal. A constitutive supply of one
molecule to a whole organ for a long period will probably disturb the regulation of locally secreted proteins and result in a non-physiological situation. Third, it is believed that cytokines mediate their effect locally at very low concentrations. Hence, the situation where cells suddenly are subjected to large quantities of growth factor(s) most likely does not occur in vivo and raises the question of the usefulness of these kinds of experiments. In this respect, experiments where the influence of a certain factor and/or its receptor(s) is studied by antibody blocking may elucidate more specifically how and at what stage(s) the action of a particular factor is important during thymocyte development.

The complexity of the organ culture system can be avoided by studying the effect of a given factor on a single cell type in vitro. This offers a simple method where both dose dependent responses and antibody blocking studies can be employed. However, cells in culture may not behave as their counterpart in vivo. First, the response to a particular cytokine in vivo maybe dependent on the presence of other cell types. Second, in order to mediate its full effect other cellular regulators may be recquired and third, the in vitro milieu could lack certain ECMs necessary for the binding and utilisation of a certain factor.

Injection of cytokines and/or corresponding antibodies into whole animals can have a very broad effect due to their distribution or it may not give any effect at all in the organ of interest because some soluble mediators may need to be presented through cell-cell contact. To circumvent this problem, transgenes linked to a tissue(s) specific regulatory sequence can bias the expression to the organ of interest. One example is the IL-4 transgenic mice generated by Lewis et al., (1991), here the transgenic expression of IL-4 was limited to the thymus. Studying the secretion of IL-4 and its effect it appeared that this factor influenced its targets locally since no effect was observed on non-T-cell lineages in these mice, perhaps indicating that this cytokine acts through cell-cell interactions or alternatively as suggested by the authors, formation of IL-4-IL-4R
complexes might limit the migration of IL-4 to other sites. Notwithstanding the usefulness of transgenic mouse models it should be mentioned that an over production of a single gene will no doubt lead to non-physiological levels of expression. This will probably disturb a possible steady-state condition with drastic effects on the system studied and must therefore be analysed carefully.

Although, simplified in vitro assays and in vivo mouse models contain problems they are neccessary to establish the role of specific factors in lymphopoiesis.
Figure 1.5 Soluble mediators in the thymus
P indicates proliferation, D indicates differentiation
CHAPTER 2

MATERIALS AND METHODS
2.1 General materials

2.1.1 Plastics

Disposable polystyrene and polyvinyl materials were used throughout this study and standard tissue culture grade plastics were used for all cell culture.

<table>
<thead>
<tr>
<th>Item</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bijou bottle (7ml)</td>
<td>Sterilin Ltd., Hounslow, U.K.</td>
</tr>
<tr>
<td>Conical test tubes (110x16mm)</td>
<td></td>
</tr>
<tr>
<td>Universal container (30ml)</td>
<td></td>
</tr>
<tr>
<td>Flexible Microtiter plates (96-well)</td>
<td>Dynatech Laboratories Inc., Chantilly, Virginia, U.S.A.</td>
</tr>
<tr>
<td>Tissue culture dishes (35, 60, 100mm)</td>
<td>NUNC, Kamstrup, Roskilde, Denmark.</td>
</tr>
<tr>
<td>Cryotubes</td>
<td></td>
</tr>
<tr>
<td>Tissue culture plates (24 and 48-well)</td>
<td>Becton Dickinson Labware., New Jersey, U.S.A.</td>
</tr>
<tr>
<td>Tissue culture flasks (25, 75 and 175cm²)</td>
<td>(Falcon)</td>
</tr>
<tr>
<td>Pipettes (2, 5, 10ml) (Nalgene)</td>
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<td>Cellscraper (Falcon)</td>
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<tr>
<td>Syringes (1, 5, 10 and 20ml)</td>
<td>Sabre International Product Ltd., Reading, England.</td>
</tr>
</tbody>
</table>

2.1.2 Glassware

All glassware was washed with detergent, rinsed in distilled water and autoclaved at 121°C for 15 minutes.
2.2 Reagents

2.2.1 Antibodies
Monoclonal antibodies (mAbs) and antisera used in this study are listed in tables 2.1 (a), (b) and (c).

2.2.2 Other reagents
Trypsin and Versene (PBS-A, EDTA) were supplied by ICRF Service department (Clare Hall laboratories). A stock of 2'-deoxyguanosine (dGuo) (Sigma) was prepared in PBS-A to a concentration of 9mM and stored at -20°C until use. Ficoll Hypaque and Percoll were purchased from Pharmacia (Uppsala, Sweden). Baby rabbit complement was obtained from Seralab (Crawley Down, G.B). Mouse Interferon γ was purchased from Genzyme (Boston, MA, U.S.A).

2.3 Murine foetal organ culture system

2.3.1 Mice
BALB/c and AKR/lcrf mice were obtained from the ICRF breeding unit. Foetuses were removed 14 days after observation of a vaginal plug (day 0).

2.3.2 Reagents
A stock solution of 9 mM 2'-deoxyguanosine (dGuo) (Sigma) was prepared as described in section 2.2.3 and used at a final concentration of 1.35mM. Biotinylated Ulex europeus agglutinin (UEA) and FITC-conjugated Tetragonolobus purpureas agglutinin (TPA) were purchased from Sigma.

2.3.3 Mouse foetal thymus organ culture
Thymic lobes were isolated from embryos of normal mice at the fourteenth day of gestation by dissection. The embryonic lobes were placed individually on Nuclepore polycarbonate filters (Sterilin. Feltham, U.K.) (pore size 0.8μm) which were floated in
petri-dishes containing Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal calf serum (FCS) and antibiotics (IMDM/FCS). The lobes were cultured at 37°C with 5% CO₂ in a humidified atmosphere for various lengths of time or depleted of lymphoid elements by deoxyguanosine treatment for 5 days (Jenkinson et al., 1982).

2.3.4 Preparation of donor cell populations from embryonic thymus, liver and peripheral blood and from adult thymus.

Donor cell populations were prepared as follows; liver (day 14 of gestation) were dissected from mouse embryos and disrupted by passing through a 25 gauge needle to obtain a single cell suspension. These preparations were centrifuged over Ficoll Hypaque (Pharmacia) at 450g for 20 minutes at room temperature to enrich for mononuclear cells, washed and used as candidate populations for seeding dGuo-depleted alymphoid thymic rudiments. Individual thymus lobes from embryos at 14 days of gestation were removed and teased apart using fine cataract knives to obtain a single cell suspension. Peripheral blood mononuclear cells were collected from sacrificed pregnant mice, separated using a Ficoll gradient and washed three times in IMDM/FCS. Adult thymuses were obtained from normal and pregnant mice, single cell suspensions prepared as described previously and mononuclear fractions separated on a Ficoll gradient.

2.3.5 Isolation of the CD4⁺CD8⁺ subpopulation from adult thymus by complement dependent cytotoxicity

A single cell suspension of adult thymocytes was prepared as described in materials and methods, section 2.3.4. Thymocytes (7.5x10⁷/ml) were incubated with saturating amounts of antibody to CD4 (GK 1.5 and YTS 191.1.2) and CD8 (YTS 169.4.2) and polyclonal rabbit anti-mouse Ig (0.1 mg/ml) for 30 min. at 4°C and then washed twice with cold medium. The antibody coated cells were then incubated with rabbit complement (Seralab, Crawley Down, GB), diluted 1:10 in serum free medium for 1h
at 37°C in 100mm petri dishes in order to kill antigen positive cells. Viability was assessed by trypan blue exclusion and cells were layered onto a Ficoll Hypaque gradient (δ=1.006g/ml) centrifuged for 20 min. at 750xg and live cells were recovered at the interphase and washed three times in medium. To confirm depletion of antigen positive cells, cell preparations were restained with (1) anti-rat FITC second layer alone and (2) directly conjugated primary antibodies against CD4 and CD8 and the number of CD4+ and CD8+ cells were assessed by flow cytometry.

2.3.6 Isolation of different subpopulations from adult thymus by panning
This was carried out according to the method of Moore and Nesbitt (1986). A single cell suspension of adult thymocytes was prepared and coated with anti-CD4, anti-CD8 and anti-mouse Ig as already described in section 2.3.5. Petri dishes were coated with polyclonal rabbit anti-rat Ig and rabbit anti-mouse Ig (20μg/ml in bicarbonate buffer pH 9.5) by incubation at 37°C for 1h. The plates were rinsed with cold PBS-A and cells were added and brought into contact with the antibody coated dish by gentle centrifugation (40xg) of the plate at 4°C for 3 min followed by an incubation at 4°C for 30 min. After the incubation period non-adherent cells were removed by careful washes and the adherent population (CD4+CD8-,CD4-CD8+ and CD4+CD8+) was recovered by scraping the bound cells off with the rubber tip of a sterile syringe plunger. Bound and unbound cells were washed and the purity of the cells tested by flow cytometry. In some cases when antibody coated cells remained in the unbound fraction the panning procedure was repeated to ensure depletion of antigen positive cells.

2.3.7 Recolonisation assay
Alymphoid lobes were prepared by culturing thymic lobes isolated from embryos at the fourteenth day of gestation in IMDM/FCS with 1.35mM dGuo for 5 days (Jenkinson et al., 1982). After extensive washing in a large volume
of medium, lobes were used as targets for recolonisation using a hanging drop method. Donor cells were titrated (in general, five fold dilutions) in a total volume of 25μl, placed in individual wells of a Terasaki plate, into which single alymphoid lobes were added. Cells and lobes were allowed to make contact by inverting the plate and incubated at 37°C for 2 days. Subsequently, the lobes were washed and transferred to culture on floating filters for a further 7-12 days before harvesting. Individual lobes were harvested by teasing apart, using fine cataract knives to prepare a single cell suspension. In each experiment unseeded lobes were used as controls and cell viability assessed using trypan blue exclusion.

2.3.8 Endpoint titration and serial passage
Seeded thymic lobes were cultured for 7-12 days, harvested individually and the number of viable cells recovered per lobe estimated. Thymocytes from lobes seeded with the lowest titration point capable of recolonising an empty lobe (≥10⁴ viable cells recovered, a cut-off point defined by a number of criteria, section 3.2.3), were passaged by titration into new dGuo-treated lobes in the manner described in 2.3.7. This procedure was repeated for up to 6 passages.

2.4 Cells and cell culture

2.4.1 Media and sera
IMDM (Gibco) was obtained as powder and prepared by adding distilled water and sodium bicarbonate (NaHCO₃) (1.023g/l) according to the manufacturers instructions. RPMI 1640, containing L-glutamine and HEPES, was prepared and supplied by ICRF Service department (Clare Hall laboratories). Fischers medium, Ham's F12 nutrient mixture, Foetal calf serum (FCS) and Horse serum (HS) were purchased from Gibco. FCS and HS were routinely heat inactivated by incubation at 56°C for 45 minutes prior to use to minimise complement activity and stored as aliquots at -20°C before use.
2.4.2 Cell lines and their culture conditions
Virus producing cell lines ($\Psi_2$), established mouse thymic stromal cell lines and NIH 3T3 cells were grown in IMDM/FCS (section 2.3). Wehi-3b cells were maintained in Fischers medium supplemented with 20% horse serum (HS) (Gibco) and antibiotics (Fischers/HS). The IL-3 dependent mouse cell line A4 (FDCP mix) established from a long term bone-marrow culture was grown in Fischers/HS supplemented with 10% Wehi 3B supernatant (containing IL-3) (Spooncer et al., 1986).

2.4.3 Establishment of embryonic thymic stromal cell cultures
Primary embryonic stromal cultures were established by covering disrupted foetal thymic lobes with a small glass coverslip (13mm in diameter) in a 60mm petri dish to which IMDM/FCS was added. This approach allowed the stromal cells to grow out and form a monolayer (Singer et al., 1985).

2.4.4 Isolation of thymocyte rosettes and thymic nurse cells from adult thymus by enzyme digestions
Thymocyte rosette/thymic nurse cell (T-ROS/TNC) cultures were prepared by enzyme digestions of adult thymuses, as described by Kyewski and Kaplan (1982) and Wekerle et al., (1980). Adult thymuses (15-20), obtained from BALB/c mice, were minced with sharp scissors and incubated in 10mls of RPMI-1640 with 25mM Hepes (pH 7.3), supplemented with 3% FCS for 15 minutes at room temperature (RT) during which time the fragments were gently agitated and free thymocytes were removed and discarded. The remaining thymic fragments were then incubated in 7mls of the same medium containing collagenase (Worthington Biochemical corporation, New Jersey, U.S.A.) (0.5mg/ml) at 25°C for three successive periods of 15 minutes each and subsequently digested with 7mls of collagenase/dispase (dispase was obtained from Boeringer Mannheim, Germany) (0.5mg/ml in phosphate buffered saline, PBS, supplemented with DNase I, 4µg/ml) with agitation at 37°C for four successive periods
of 20 minutes each until all tissues were completely digested. The collagenase and collagenase/dispase fractions were then layered on to a 30% FCS in PBS cushion and were allowed to sediment through this for 30 minutes at 4°C. The top layer (containing single cells) was aspirated and discarded and the lower layer centrifuged to recover sedimented T-ROS and TNC (Wekerle et al., 1980). Prepared cells were plated onto 60mm dishes and left 1-3 days before infection.

2.4.5 Passaging of adherent cells

Adherent cell lines were routinely passaged by incubating the cells with serum free medium for 10 min. followed by an incubation with versene (PBS-A, EDTA) and trypsin (3:1) until cells detached. Subsequently cells were washed in medium containing serum, diluted and plated out in new flasks at subconfluence.

2.4.6 Freezing of cells

Cell lines were washed in medium and resuspended in 0.5-1.0 ml of freezing mix, 95% FCS and 5% dimethylsulphoxide (DMSO) (Fisons), transferred to cryo tubes (NUNC) and incubated at -20°C for 30 min, followed by an overnight incubation at -70°C before transfer to liquid nitrogen (N₂(i)) for preservation.

2.4.7 Thawing of cells

Cell lines preserved in N₂(i) were thawed by incubating the cryo tubes at 37°C. When the cells were completely thawed, 10mls of medium was added slowly during a 5 min period and the cells centrifuged and washed twice before culture.
2.5 Retroviral infection

2.5.1 Construction of recombinant retroviruses and preparation of virus producing cell lines

The recombinant retroviruses ZipSVtsA58 and ZipElal2S were constructed as previously described (Tegtmeyer 1975; Mann et al., 1983; Cepko et al., 1984; Roberts et al., 1985; Jat and Sharp 1989). Briefly, the recombinant plasmids were inserted into the unique Bam HI site of the pZipneoSV(x)1 shuttle vector. Producer cell lines were made by transfection of plasmid DNA into \( \Psi_2 \) cells. These constructs were prepared by P. Jat at the Ludwig Institute of Cancer Research, London and the virus producing \( \Psi_2 \) cell lines were obtained from P. Jat. Supernatants containing virus were collected from confluent flasks of virus producing cells. The virus stocks were filtered through 0.45\( \mu \)m filters and stored at -70°C until use.

2.5.2 Titration of virus containing supernatants

Virus-containing supernatants were titrated to measure their infectivity using NIH 3T3 cells. NIH 3T3 cells were plated at a density of 5x10^5 cells/100 mm petri dish and infected with 1 ml of dilutions of virus supernatant (four tenfold dilutions), in the presence of 0.5ml of medium and 0.5ml of polybrene (8\( \mu \)g/ml) (Aldrich Chemical Co: Inc.) for 2 hours at 37°C, with rocking every 15 min. Complete medium was added and the cells were cultured for 48 hours, after which time the cultures were trypsinised and split into five large petri dishes (100mm) (Jat and Sharp 1989). Once the cells had adhered, medium containing G418 (Davies and Jimenez 1980) (500\( \mu \)g/ml) (Gibco) was added and exchanged every 4-5 days, for about 10-14 days. NIH 3T3 cultures infected with the temperature sensitive variant SV40tsA58 were cultured at 33°C after the infection at 37°C. After this period colonies resistant to G418 were visualised by staining the dishes with 2% methylene blue (Sigma) in 50% ethanol, and the number of colonies counted.
2.5.3 Infection and isolation of cell lines

Primary stromal or T-ROS/TNC cultures were infected with virus containing supernatants (diluted 1/2) as described in 2.5.2. Colonies resistant to G418 were isolated by using cloning cylinders (made from plastic pipette tips), transferred to a 24-well plate and expanded.

2.6 Immunofluorescence staining and FACS analysis

All immunofluorescence staining was performed with serum to reduce non-specific binding. Hybridoma supernatants contained 10% FCS and 0.1% sodium azide and all other antibodies were diluted in PBS-A, 0.2% bovine serum albumin and 0.1% sodium azide.

Cells to be tested for expression of cytoplasmic antigens were grown on glass cover slips in 24 well plates. Subconfluent cultures were washed in PBS-A buffer, fixed in acetone:methanol (3:1), air dried, incubated with primary antibodies for 30 minutes at RT, washed in PBS-A buffer and incubated with Fluorescein Isothiocyanate (FITC) conjugated second layer antibodies for 30 minutes at RT. After washing (one hour at 4°C), staining was visualised by fluorescence microscopy.

Cells to be assayed for expression of surface antigens were stained in suspension in 96 well flexible plates (Dynatech Laboratories Inc.). For indirect staining approximately 1x10^5 cells were incubated with primary antibody for 30 minutes on ice, washed three times in cold buffer (PBS-A, 0.2% bovine serum albumin and 0.1% sodium azide) and incubated with FITC-conjugated second layer reagents (30 minutes on ice). Cells were washed three times, and analysed on a FACScan (Becton Dickinson). For direct immunofluorescence, cells were incubated with mAb directly conjugated with phycoerythrin (PE) or FITC for 30 min., washed and staining visualised as described above. Between 5-10x10^3 events were acquired per sample depending on the number of cells available.
2.7 Immunoperoxidase staining

2.7.1 Buffers and Solutions

Tris buffered saline (TBS) pH 7.6

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Tris/HCl buffer pH 7.6=DAB substrate solution

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DAB solution

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Mayers Haematoxylin

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<td>1g</td>
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<td>50g</td>
</tr>
<tr>
<td>Water</td>
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</table>
The haematoxylin, alum and sodium iodate is left to dissolve overnight. The following day chloral hydrate and citric acid are added and the mixture boiled for 5 min and thereafter cooled. The Solution is filtered before use.

1% Acid alcohol
1% conc. HCl in 70% ethanol

Phosphate Buffered Saline (PBS):
PBS A tablets were obtained from Oxoid Ltd., U.K. and dissolved in distilled water, according to the manufacturers instructions.

2.7.2 Staining procedure
Frozen sections (6-8 μm) or cytocentrifuged cells were thawed (30 minutes at RT), air dried and fixed in acetone (frozen sections 30 min. and cells 10 min) at RT and washed in TBS-buffer (2.7.1) for 10 min. to remove residual fixative. The slides were then incubated with saturating amount of primary antibody for 1 hour at RT in humidified boxes. Sections/cells incubated with rat antibodies were preincubated with normal mouse serum 1/20 for 20 min. prior to primary antibody. Next, slides were rinsed twice and washed with TBS for 15 min followed by an incubation of optimally diluted peroxidase conjugated second layer antibody (rabbit anti-rat Ig was preincubated with normal mouse serum 1/20) for 30 min. at RT and then rinsed and washed with TBS. The staining of peroxidase labelled antibody was visualised by adding DAB-substrate solution (materials and methods section 2.7.1) to the slides and incubating for 10 min. after which time slides were rinsed and washed with TBS-buffer. Frozen sections and cells were counterstained with Mayers Haematoxylin (2.7.1) for 30 sec-1.5 min depending on the age of the solution. The slides were then rinsed in running tap water, dipped in 1% acid alcohol (2.7.1), rinsed in tap water for 3 min and thereafter sequentially dehydrated in 70% and 100% ethanol and transferred to CNP
(trichloroethane & tetrachloroethane) (Penetone, Northumberland, U.K) and mounted in Ralmount containing Xylene (BDH Ltd, Poole, U.K).

In each experiment negative controls were included where the primary antibody was omitted in the staining procedure.

2.8 Cytochemical Staining

2.8.1 Buffers and Solutions

May-Grunwald solution

A 0.3% stock solution is prepared by dissolving dry dye in absolute methanol. The working concentration is prepared by adding an equal volume of buffered water at pH 6.8.

Giemsa Solution

Giemsa solution was purchased from Sigma Diagnostics and used undiluted.

Non specific esterase incubation solution

(1) 1.5ml 4% NaNO₂ + 1.5ml Pararosanilin (stock) are mixed and left for 2 min.
(2) 4.5ml phosphate buffer is diluted to 45ml in distilled water.
(3) 50mg alpha naphthyl acetate is dissolved in 2.5ml 2methoxy ethanol
(4) Mix (1), (2) and (3) and adjust the pH to 6.1 with 1M NaOH and filter the solution.

Phosphate buffer

Potassium dihydrogen phosphate (0.912g/100ml) 1.3ml
Disodium hydrogen phosphate (0.951g/100ml) 8.7ml

Pararosanilin stock solution

Pararosanilin hydrochloride 2g
HCl (2M) 50ml
The Pararosanilin is dissolved by gentle heating, cooled and filtered.

2.8.2 May-Grunwald and Giemsa
Cells cytocentrifuged onto glass slides were air dried and fixed in methanol for 5 min at RT. May Grunwald solution was layered on the slides and incubated for 15 min at RT after which period the Giemsa solution was added on top and left for 20 min. Next, water was added to produce a film on the surface of the slide and left for 5 min. The solution was then poured off and the slides dehydrated sequentially in 70% and 100% ethanol, transferred to CNP and mounted in Ralmount (Xylene).

2.8.3 Non-specific esterase staining
Cells cytocentrifuged onto a glass slide were air dried and fixed in formalin vapour for 5 min and immersed in incubation solution at 37°C for 45 min. (2.8.1) (Gomori 1950). Thereafter the slides were washed in tap water and blotted on 3MM paper and Xyelene mounted with Ralmount (BDH).

2.9 Electron Microscopy
All electron microscopy studies were done in collaboration with Kenneth Blight at the Electron Microscopy unit, ICRF, London.

Stromal cells were grown to confluence in 60mm petri dishes, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through graded ethanol and embedded in resin. Sections were cut (90 nm) and visualised in a Zeiss EM10 electron microscope.

2.10 Growth kinetics of stromal cell lines
SV40 large T (ts) and E1a clones were plated in 60mm dishes at a density of 2 x 10⁴ cells/dish. Cells were allowed to attach overnight, at 33°C (ts-clones) and at 37°C (E1a clones). Next day, some ts-cultures were switched to 39°C and some E1a-cultures to
33°C and 39°C, and incubated for 8-10 days. At different time points individual plates were trypsinised and the number of viable cells estimated by trypan blue exclusion.

2.11 Induction of MHC antigens by Interferon γ

Cells were plated at a density of 2x10⁴ cells/60mm petri dish and cultured in IMDM/FCS supplemented with 0, 5, 50 and 500U mouse Interferon γ/ml. At different time points cells were harvested with versene (EDTA-PBSA), washed and counted. Cell viability was assessed by trypan blue exclusion.

2.12 Rosette assay

2.12.1 Rosette assay

Rosetting was performed at 4°C using a cell suspension assay. Briefly, stromal cells (harvested with versene) and unfractionated adult thymocytes (from BALB/c mice) were mixed 1:12 in a total volume of 200μl and incubated on ice for one hour. The mixture was centrifuged at 200g for 5 minutes, the pellet gently resuspended and the number of rosettes counted using a haemocytometer (≥3 thymocytes bound to a stromal cell was scored as a rosette).

2.12.2 Antibody blocking studies

Unfractionated thymocytes and/or stromal cells were incubated with the appropriate antibody for 30 min on ice, washed three times and thereafter mixed and assessed for the number of rosettes formed (as in section 2.12.1).
2.13 Co-culture assays

2.13.1 Co-culture of A4 cells and stromal cells through a semi-solid layer of soft agar

Thymic stromal cell lines were plated at $1 \times 10^5$ in 35mm petridishes and allowed to adhere overnight and subsequently overlaid with 1ml of semi-solid agar (0.3%) in Fischers medium. After 24 hours $1 \times 10^5$ A4 cells in 2mls of medium (without IL-3) was added and the number of viable A4 cells was estimated 7 days later.

2.13.2 Co-culture of A4 cells and irradiated stromal cells

Stromal cells were plated to form a monolayer in 48 well plates (~1x10^4cells/well). Confluent cultures were irradiated (3000rad) and $5 \times 10^4$ A4 cells in 1ml of Fischers medium (-IL-3) was added. Medium was changed every 4 days by adding 0.5ml of new Fischers medium.

2.13.3 Co-culture of thymocytes and stromal cells

The CD4^−CD8^− subset of adult thymus was prepared from BALB/c mice as described in sections 2.3.4 and 2.3.5. Freshly prepared CD4^−CD8^− adult thymocytes ($2 \times 10^4$cells/well) were co-cultured with stromal cell lines ($5 \times 10^3$/well) in 48-well plates.

Foetal thymic lobes were removed from BALB/c embryos at day 14 of gestation and cultured in organ culture (section 2.3.3) for 1 to 2 days. These lobes corresponding to day 15 and day 16 of gestation, respectively, were homogenised in a 0.1ml homogeniser (Jencons, England) to obtain a single cell suspension. Foetal thymocytes ($2 \times 10^4$ cells/well) were co-cultured with stromal cells ($1 \times 10^4$ cells/well) in 24-well plates.

Co-cultures of adult and foetal thymocytes were maintained in IMDM/FCS. Culture supernatant (1:100) from COS-7 cells transfected with a cDNA of an IL-7 coding
sequence (Murray et al., 1989) was used as a source of IL-7 (final concentration 15.5U/ml). The COS-7 supernatant was supplied by Dr. A. Zlotnik, DNAX, Palo Alto, U.S.A.

2.14 Yeast-uptake assay

Prepared yeast cells and normal human AB serum were kindly provided by Dr. Chris Bunce, Birmingham University.

The percentage of macrophages was assessed by determining the capacity of cells to phagocytose complement-coated yeast cells. A cell sample of 15μl (10⁶ cells/ml) diluted in 10% FCS and 10% normal human AB serum was mixed with 15μl of complement coated procion red dyed yeast particles. The mixture was incubated in 96-well flexiplates at 37°C for 30 minutes. After this period each sample was resuspended and viewed on a haemocytometer. The number of cells which had phagocytosed 3 or more yeast cells was determined by phase contrast microscopy.

Complement and dyed yeast cells were prepared as follows. Briefly, glutaraldehyde fixed yeast cells (Saccharomyces cerevisiae) were mixed in 0.2% procion rubine in 0.2M Na₂HPO₄ for 2 hours at RT. Dyed yeasts were washed in complement fixation buffer (CFT) (Oxoid, London, U.K.) and stored at 4°C in CFT containing 0.1% sodium azide. To complement coat, yeast cells were washed and resuspended in 800μl CFT and 200μl of normal sheep serum was added as a source of complement. This mixture was incubated for 30 minutes at 37°C and shaken at 5 minutes intervals. Subsequently, yeast cells were washed and resuspended in 20mls CFT and stored as 0.5ml aliquots at -20°C.
2.15 Preparation of tissues

2.15.1 Snap-freezing of foetal thymic lobes
A drop of 5% eosin (in methanol) was added on top of each thymic lobe cultured on Nuclepore filters before mounting onto a drop of OCT compound gel (BDH, Pool, U.K.) placed on a piece of card. The eosin is absorbed by the connective tissue around the lobe and will consequently make the tissue visible. The lobe was then snap frozen in $N_2(0)$ and stored in a cryo vial at -70°C until use.

2.15.2 Cutting of frozen sections
A cryostat microtome (Bright Ltd.) was used to cut frozen sections at 6-8 μm. The sections were picked up on a clean glass slide, air dried and stored at -20°C until use.
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<td>A. Cooke</td>
<td>Cobbold et al., 1984</td>
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<tr>
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<td>A. Cooke</td>
<td>Galfre et al., 1979</td>
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<td>Lyt2 (CD8)</td>
<td>E. Simpson</td>
<td>Saramiento et al., 1980</td>
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<td>RL172.4</td>
<td>rat IgM</td>
<td>L3T4 (CD4)</td>
<td>E. Simpson</td>
<td>Ceredig et al., 1985</td>
</tr>
</tbody>
</table>
(b) Conjugated Antibodies

**Antibody** | **Source**
--- | ---
L3T4-phycoerythrin (clone GK1.5) (CD4-PE) | Becton Dickinson
Lyt-2-fluorescein isothiocyanate (clone 53-6.7) (CD8-FITC) | Becton Dickinson
Goat anti-Rat Ig-fluorescein isothiocyanate (GARat-FITC) | Nordic Laboratories
Swine anti-Hamster Ig-fluorescein isothiocyanate (SwAH-FITC) | Nordic Laboratories
Sheep anti-Mouse Ig-fluorescein isothiocyanate (ShAM-FITC) | Sigma

(c) Antisera

**Serum** | **Raised against** | **Source**
--- | --- | ---
Goat serum | Vimentin | ICN Immuno Biological
Rabbit serum | Keratin | Sigma
Rabbit Ig | Mouse | Sigma
Normal mouse serum | - | Dako Patts
CHAPTER 3

THE MURINE THYMIC ORGAN
CULTURE SYSTEM
3.1 INTRODUCTION

During embryonic life the thymic rudiment becomes colonised with stem cells immigrating from foetal liver (Moore and Owen 1967). The precise phenotype of these cells and how they migrate to and enter the thymus is still unclear. However, these events are required for intrathymic differentiation which leads to the production of functionally mature, immunocompetent T-cells.

Kinetics studies of stem cell migration during embryonic life, using the chick/quail chimeric system, have suggested that colonisation occurs in cyclic waves (Jotereau et al., 1980; Jotereau and Le Douarin 1982). Colonisation of the mouse thymus occurs at day 10-11 of gestation (Owen and Ritter 1969; Fontaine-Perus et al., 1981) and has shown similar 'waves' of colonisation (Jotereau et al., 1987). Transplantation of foetal thymuses under the kidney capsule of adult mice indicated that initial colonisation occurs during a short period at day 10-11 of gestation and that cells entering the lobe after 12 days generate a pool of thymocytes which does not appear in the periphery until 1-2 weeks after birth (Jotereau et al., 1987). Stem cell migration, proliferation and differentiation in adult life have mostly been studied in vivo by examining the capacity of bone marrow derived donor cells to reconstitute untreated or irradiated recipients. The earliest stage of T-cell development is thought to be represented by a minor cell population lacking the T-cell differentiation markers CD4 and CD8 (Ceredig et al., 1983a). Further analyses of this subset have indicated that this population is quite heterogeneous with respect to other cell surface molecules including CD3, HSA, Thy-1, Pgp-1 and IL-2R (reviewed in the introduction section 1.2.5). Upon in vivo transfer double negatives give rise to three other thymocyte populations, cells with the phenotype CD4+CD8+, and cells expressing single-positive phenotypes similar to peripheral T-cells, the CD4+CD8- and CD4-CD8+ subsets (Fowlkes et al., 1985; Crispe et al., 1987).
One in vitro model that has been used for studying T-cell development is the mouse foetal thymus organ culture system. In this system embryonic thymic lobes are removed from the mouse embryo at day 14 of gestation and placed on polycarbonate filters floating in medium. In this organ culture T-cells are able to be maintained and develop in the intact thymic microenvironment similar to an in vivo situation. The differentiation can be documented by the appearance of the two functional phenotypes of T-cells, CD4+ CD8- (L3T4+Lyt2-) and CD4-CD8+ (L3T4- Lyt2+).

In an in vitro co-culture transfilter system of day 10 foetal thymic lobe and embryonic BM or already colonised thymus it was shown that the older tissue could seed day 10 thymic rudiment with lymphoid cells (Fontaine-Perus et al., 1981). This co-culture technique was further developed by Jenkinson et al., (1982) where they used foetal thymic lobes which had been treated with 2'deoxyguanosine (dGuo). The exposure of dGuo depletes dividing lymphoid elements and the remaining thymic rudiment is capable of supporting thymocyte development and maturation. Another in vitro recolonisation system is the hanging drop method where alymphoid lobes are prepared by dGuo-treatment and seeded with a selected donor population as a single cell suspension (Kingston et al., 1985).

By seeding populations in limiting numbers it is possible to determine the recolonisation capacity within different sources and estimate the relative abundance of precursor cells from both foetal and adult tissues. In addition, this in vitro model enables the production of chimeric thymuses where stromal cells and lymphoid cells of different haplotype can be studied. It also allows one to ask questions about the differentiation potential of precursor cells from different sources. The disadvantage on the other hand, is the difficulty to study the emigration from the thymus which can be followed in an in vivo reconstitution experiment. Another limiting factor might be that certain stromal elements in embryonic lobes do change during culture and no longer resemble the cellular composition found in vivo.
Using this organ culture system the following questions have been investigated (i) the ability of precursor cells to recolonise an alymphoid thymic lobe (ii) the frequency of precursors among different donor populations (iii) the proliferative capacity of cells capable of recolonising dGuo-treated lobes (iv) phenotypic changes occurring during differentiation in this system and (v) the phenotype of precursors present in adult thymus capable of repopulating a lobe in vitro.
3.2 RESULTS

3.2.1 Kinetics of cell growth of foetal thymic lobes maintained in vitro

The purpose of this study was to compare the in vitro model with published in vivo data on T-cell development in embryonic life and thus to find out whether the model is accurate and could be used for answering questions about T-cell ontogeny.

Thymic organ cultures were established from day 14 foetuses of BALB/c mice. The cell growth kinetics of foetal thymocytes were analysed by harvesting individual lobes at different time points during a 20 day culture period. As shown in figure 3.1, during the first nine days of culture the number of viable cells recovered/lobe increased significantly after which time the cell numbers decreased slightly. At day 0 (day 14 of gestation) approximately $1.2 \times 10^4$ viable cells/lobe were recovered and after 9 days in culture the mean recovery per lobe was about $6 \times 10^5$. After 20 days in culture fewer than $6 \times 10^4$ viable cells remain in each lobe. In a comparison with the in vivo situation, day 6-7 in our system would resemble the time of birth (day 20-21). Up to this time in culture the cell recovery/lobe is very similar to that which can be recovered in vivo. However, during the following 13 days in culture no significant proliferation is seen and the recovery is no longer comparable with the number of thymocytes present in postnatal life (in a 0-13 day old mouse). The results shown here, are similar to those of Kisielow et al., (1984) who reported an expansion of thymocytes in culture from $2.5 \times 10^4$ (the day of explantation) to $5 \times 10^5$ after 5 days in culture, followed by a constant recovery thereafter.

3.2.2 Phenotypic analysis of CD4 and CD8 expression of foetal thymocytes maintained in organ culture

To document T-cell differentiation in thymic organ cultures phenotypic analyses of CD4 and CD8 expression by thymocytes in organ culture during a 3 week period were
studied. Both the percentage and absolute numbers of cells expressing CD4 and CD8 were examined. CD4+CD8* or CD4-CD8+ (single positive, SP) and cells expressing both CD4 and CD8, CD4+CD8+ (double positive, DP) and cells negative for both markers, CD4-CD8- (double negatives, DN) were observed in culture. Table 3.1 shows data from individual lobes (which are representative of the mean values from figure 3.2), and indicate the variability that exists in the system. As shown in figure 3.2(a)-(d), the majority of cells (>99%) recovered at day 14 of gestation (day 0) display an immature (double negative) phenotype (figure 3.2(d)). During culture, cell proliferation was accompanied by maturation of the thymocytes, as indicated by the acquisition of differentiation antigens, CD4 and CD8. The percentage of DN thymocytes dropped rapidly during the first 6 days in culture and this decrease (from 100% to 43%) seemed to be related to a rapid increase of DP cells, from 0% (day 0) to 37.5% at day 6 (figure 3.2(c) and (d)). The DP subpopulation is the dominant phenotype in normal thymus and many reports suggest that this population contains precursors for the single positive, mature thymocyte (Fowlkes et al., 1988; Smith 1987; Guidos et al., 1989b). As shown in figures 3.2(a) and (b), single CD4 and CD8 positive cells were absent from day 14 thymus but increased during the first 6 days in culture; CD4 expressing cells from 0% to 10.5% (figure 3.2(a)) and CD8+ cells from 0% to 8.5% (figure 3.2(b)). Later, between days 9 and 16, single CD8 positive cells expand further from 10.5% to 24.5%, CD4 bearing cells on the other hand seem to increase in one of two lobes (table 3.2), but the mean values remain similar at both timepoints. This increase of mature single CD8 positive cells (and in one lobe of CD4+ cells) is accompanied by a decrease of DP cells which suggest that thymocytes in this organ culture have differentiated from an intermediate DP stage to a mature single positive stage. The DN population after an initial drop (from >99% to 43%) during the first 6 days remains at 40-60% during the next 2 weeks in culture. However, although the percentage of DN thymocytes decreases during the first 6 days, the absolute number of this phenotype does increase up to 9 days. This result could indicate that some cells within this population do proliferate without maturing (figure 3.2(d)) or that they are
'mature' CD4-CD8-TCRαβ+ and/or CD4-CD8-TCRγδ+ cells. In a comparison of all four subpopulations in terms of absolute numbers (filled squares), the data indicate that all cell types do increase in numbers during the first 9 days in culture after which time they decrease slightly. The differentiation exhibited during the first week in this in vitro model, shows quite a similar pattern to studies performed in vivo (Ceredig et al., 1983a; Ceredig et al., 1983b; Kisielow et al., 1984), but after that period the differences are significant. In these studies the authors reported a much greater percentage of CD4+CD8+ (~85%) cells and fewer CD4-CD8- (~10%) cells at day 27 of gestation in vivo than was observed in our in vitro organ cultures after 14 days in culture (~55% DN and ~15% DP cells). However, the number of single positive cells recovered in vivo and in vitro at these time points were quite similar.

These discrepancies between the staining pattern obtained in vivo in comparison to that obtained in vitro after long term culture could probably be a result of a number of reasons; (i) failure to migrate in the in vitro system, (ii) death of crucial cell populations necessary for maintaining thymocytes, (iii) not sufficient or appropriate growth factors available in vitro, and (iv) necrosis at the centre of the lobe due to lack of O2.

Despite these problems, the organ culture is still the only in vitro system in which heterogenous T-cell development can take place. Several other in vitro systems have been employed for this purpose, such as addition of growth factors and co-culture with stromal components, although none has, as yet, been shown to support differentiation of T-precursors to mature T-cell.
Figure 3.1 Kinetics of cell growth in thymic organ culture. Foetal thymic lobes grown in organ culture were harvested at different timepoints and the number of viable cells recovered per lobe was estimated by trypan blue exclusion. Each point represents an individual lobe.
Figure 3.2  Phenotypic analysis of foetal thymic lobes cultured in vitro.
Foetal thymic lobes cultured in vitro were harvested at different time points and cells stained in cell suspension according to section 2.6 for the expression of CD4 and CD8 using directly conjugated mAb L3T4-PE and Lyt2-FITC (Becton Dickinson). The different populations are shown as (a) CD4+CD8", (b) CD4+CD8+, (c) DP and (d) DN. Shown are positive cells in percentage (□-□) and absolute number (■-■). The staining was visualised on a FACScan (Becton Dickinson).
These are the mean values from one experiment and the variability is demonstrated in table 3.1.
Table 3.1 CD4 and CD8 expression by thymocytes maintained in thymic organ culture.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>cell recovery/lobe (a)</th>
<th>CD4⁺CD8⁻ (%) (b)</th>
<th>CD4⁺CD8⁺ (%) (b)</th>
<th>CD4⁻CD8⁻ (%) (b)</th>
<th>CD4⁺CD8⁺ (%) (b)</th>
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(a) The number of viable cells recovered from individual lobes.
(b) The percentage of cells expressing CD4, CD8, both and neither of these antigens.
3.2.3 Frequency analysis of the capacity of different cell populations to recolonise 2'deoxyguanosine treated thymic lobes

To identify cells capable of recolonising thymic rudiments the assay described by Kingston et al., (1985) (materials and methods, section 2.3.7) was used. Using this system, the abundance of pro-T cells among different donor populations was estimated. Decreasing numbers of cells were seeded into dGuo-treated, alymphoid lobes and individual lobes analysed for reconstitution. Using this approach an endpoint was chosen to judge recolonisation since several variables were usually included in the experiments such as (i) the origin of precursor cells (ii) the phenotype of precursor cells and (iii) the length of time of each experiment. In our attempt to quantify recolonisation in individual lobes it was difficult to estimate the number of cells present/lobe at the end of each culture period in cases where less than ~20 cells (in ~50μl) were counted. Furthermore, when the number of cells recovered/lobe was low the viability of cells was usually very poor. Therefore we judged a recolonisation to have occurred when at least 1x10^4 viable cells could be recovered/lobe. This number of cells (10^4) also represents three times the mean number of cells recovered from unseeded dGuo-treated lobes. A second criterion for true recolonisation was that a titration effect could be seen since we found that the cell recovery after recolonisation was related to the number of precursors present within a population. In other words, when empty lobes were mixed with decreasing number of cells, a reduction in recovery was observed.

The recolonisation potential of cells from foetal liver, foetal thymus, adult thymus and peripheral blood mononuclear cells was investigated. As shown in figure 3.3, day 14 foetal liver cells, a rich source of haemopoietic precursors, could successfully repopulate a dGuo-treated lobe when as few as 100 cells were added. The number of cells recovered after 10-12 days in culture from lobes seeded with 100 foetal liver cells was 3.2x10^4 and increased about 8-fold to 2.6x10^5 when 10^5 cells were seeded/lobe. As demonstrated in figure 3.3, recolonising cells were not detected among foetal thymocytes when less than ~5x10^3 cells were seeded. In contrast to foetal liver and
thymus very few precursor cells were present among unfractionated adult thymocytes, consistent with the low precursor activity detected in vivo (Scollay et al., 1986). Successful recolonisation was only obtained when $10^5$ adult thymocytes were seeded ($\geq 10^4$ cells recovered/lobe). From lobes seeded with PBM less than 5000 cells could be regained in all cases and no titrating effect was seen, when decreasing number of cells were seeded. This result might be due to lack of T-cell precursors or indicate that the frequency of precursor cells within this population is too low to give rise to a population that can be measured in this system. One limitation in this model is that the lobe and donor cells are mixed for 2 days in a very small volume (25μl) and the maximum number of donor cells that can be added without decreasing the viability of the lobe is $\sim 10^6$.

3.2.4 Kinetics of cell growth of alymphoid lobes recolonised with foetal thymocytes and foetal liver cells

In order to study the rate of thymocyte proliferation after recolonisation in vitro, the kinetics of cell growth of foetal lobes seeded with defined number of precursor cells from mouse foetal liver and thymus was measured. Lobes recovered from the hanging drop cultures, were washed in a large volume of medium to reduce the number of cells sticking to the lobes. The growth curves (figure 3.4(a)-(c)) show results using foetal liver cells, thymocytes and unseeded control, respectively. Five days after seeding with $1-2 \times 10^5$ day14 foetal liver cells between $1-2 \times 10^4$ cells could be recovered/lobe and after about 9 days $\sim 2 \times 10^5$ cells/lobe. Similar to untreated lobes grown in culture, an extensive proliferation of thymocytes was seen in the first 9 days in culture after which time the growth curves reach a plateau. Comparable results were obtained when day 14 foetal thymocytes were used as donor cells. These experiments demonstrated that precursor cells entering a lobe will proliferate considerably in vitro.
Figure 3.3 Frequency of cells capable of recolonising dGuo-treated lobes among different cell populations. Donor populations used for in vitro reconstitution of alymphoid lobes, using the hanging drop method, are shown as unfractionated adult thymocytes (□-□), unfractionated foetal thymocytes (▲-▲), foetal liver cells (day 14 of gestation) (○-○) and peripheral blood mononuclear cells (×-×). Lobes subjected to recolonisation were harvested after 10-12 days in culture. The results shown for each titration are mean values from 2-3 separate experiments where 2-3 lobes were harvested at each time point and assessed individually.
3.2.5 Changes in CD4 and CD8 expression of cells capable of recolonising thymic lobes in vitro.

To investigate whether differentiation occurs in parallel with proliferation of thymocytes in a recolonised thymic lobe, the expression of the T-cell differentiation markers CD4 and CD8 was examined at various time points after seeding. In a comparative study, equal numbers (5x10^4) of foetal liver cells and thymocytes from day 14 of gestation, both rich sources of pro-T cells, were seeded into individual dGuo-treated lobes and after 3, 6, 9, 13 and 16 days (post mixing) individual lobes were harvested. At each time-point the number of viable cells was estimated and the recovered cells from individual lobes were subjected to double simultaneous staining using directly conjugated anti-CD4 (L3T4-PE) and anti-CD8 (Lyt2-FITC) to calculate the abundance of the four major subpopulations (CD4⁺CD8⁻, CD4⁻CD8⁺, DN and DP). Figure 3.5 demonstrates the results obtained from FACS analysis. The left panel shows data from lobes recolonised with foetal liver cells and the right panel shows the phenotype of cells recovered from lobes seeded with foetal thymocytes. Three days after seeding, 87% of the cells recovered from lobes recolonised with liver cells (figure 3.5(d), left panel) still display a double negative phenotype, 3.5% were double positive (figure 3.5(c)), 9% CD4⁺CD8⁻ but no single CD8⁺ could be found (figure 3.5(a) and (b)). After a further 2 days in culture (day 5) the percentages of each cell type were unchanged but a significant expansion in absolute numbers was observed among the DN cells and the SP cells. The DN population increased tenfold; from ~2x10³/lobe (day 3) to ~2x10⁴/lobe (day 5) and the number of single positive cells (CD4⁺ and CD8⁺) also increased considerably (10 fold and 20 fold, respectively). The DP subset increased approximately 1000 fold in numbers between day 5 and 9 and at day 9 about 50% of the cells were DP (figure 3.5(c)), while the DN population decreased ~55% during this period (figure 3.5(d)). These results suggest that seeded cells matured and that the majority of the cells where now at an intermediate stage of development. During this period between day 5 and 9 there was a 20 and 70 fold increase in number of CD4⁺ and CD8⁺, respectively, suggesting that some cells had matured completely into SP. After
9 days in culture there was a decline in proliferation and the only population that expanded was cells expressing CD8, from 4% day 9 to 16.5% day 16, while the other subpopulations, either in percentage or in numbers, were unchanged.

In parallel experiments, in which day 14 thymocytes were used as donor cells, a very similar pattern was observed, except that the DN population does increase in percentage terms between day 9 and 16 (figure 3.5(h)), although the actual number of cells with that particular phenotype do not change. An explanation of this result could be that the percentage of DP during the same period decreased by 30% (figure 3.5(g)), which indicates that some of these cells could have converted to single positive (CD8 expressing cells increase by 10%) or that the DP cells have died in situ and therefore made the DN subset the most abundant (62%).

Day 9 in this in vitro assay can be compared with the 20th day of gestation (9 days after in vivo colonisation of the thymus), which is the time of birth. In a comparison of these results with similar studies performed in vivo (Kisielow et al., 1984; Ceredig et al., 1983a), it appears that the data presented here resemble quite well the differentiation pattern seen in vivo. The similarities are apparent up to 9 days in culture (day 20 in mice) but after that period, up to day 16 in this assay which could be compared with a 7-day old mouse, the cellular composition show clear differences.

3.2.6 The CD4⁻CD8⁻ thymocyte population is enriched for cells capable of reconstituting alymphoid lobes

Precursor cells capable of recolonisation in vivo and in vitro reside in the CD4⁻CD8⁻ compartment (Scollay et al., 1984; Kisielow et al., 1984; Fowlkes et al., 1985). In order to establish whether any cells within the single positive or double positive subsets are able to recolonise an 'empty' lobe, adult and foetal thymocytes were fractionated using a panning technique (materials and methods section 2.3.6). Figures 3.6 (a) and (b) show two representative experiments using adult thymocytes (a) and foetal
thymocytes (b). As shown in (a) unfractionated adult thymocytes seeded in numbers greater than 10⁵ are able to colonise alymphoid lobes and give rise to ~1-2x10⁴ cells after 7-9 days in culture (open squares). A comparison of the number of seeded cells required for recolonisation shows that populations depleted of CD4 and CD8 (DN) (open circles) are enriched for precursors compared with whole thymocyte fractions. In contrast, cells expressing one or both of the markers CD4 and CD8 (filled circles) grow very poorly in dGuo-treated lobes. The results shown in figure 3.6(b), using different fractions of foetal thymocytes confirm that the fraction containing cells expressing CD4 and/or CD8 (filled circles) was unable to repopulate the alymphoid thymus while the DN population (open circles), in comparison with unfractionated cells, show considerably higher colonisation capacity. From these data it appears that cells expressing CD4 and/or CD8 do not include precursors or that the frequency of colonising cells of this phenotype is too low to be detected in this assay. Based on these results we performed similar experiments in which adult thymocytes were depleted of CD4 and CD8 expressing cells by treatment with antibodies to CD4 and CD8 plus complement (materials and method section 2.3.5). A representative experiment using this method is shown in figure 3.6(c)). These data confirm that thymocyte populations lacking CD4 and CD8 display an enhanced recolonisation potential.
Figure 3.4 Proliferation kinetics of foetal thymic lobes recolonised with foetal liver cells and foetal thymocytes. A lymphoid lobes which had been pretreated with dGuo were recolonised using the hanging drop method (section 2.3.8). In (a) $5 \times 10^4$ (■-■) and $1 \times 10^5$ (□-□) foetal liver cells (day 14 of gestation) were used as seeds and in (b) three separate experiments with $1 \times 10^4$ (○-○) and one experiment with $5 \times 10^4$ (●-●) foetal thymocytes (day 14 of gestation) were used as donors. Unseeded controls are shown in (c). Each point represents a mean value of 2-3 lobes, harvested and counted separately using trypan blue exclusion.
Figure 3.5

(a) CD4

(b) CD8

(c) DP

(d) DN

(e) CD4

(f) CD8

(g) DP

(h) DN

Time/days in culture

Absolute number

%
Figure 3.5  CD4 and CD8 expression of lobes recolonised in vitro with foetal liver cells and foetal thymocytes. dGuo-treated thymic lobes were recolonised with $5 \times 10^4$ foetal liver cells (a-d) and $5 \times 10^4$ foetal thymocytes (e-h) (both day 14 of gestation) in separate experiments. At different time points individual lobes were harvested, counted and subjected to double staining for CD4 and CD8 using directly conjugated L3T4-PE and Lyt2-FITC mAbs and the staining visualised on a FACSscan. The results are shown as percentages of antigen expressing cells (□-□) and the absolute number of cells with a particular phenotype (■-■). Each point represents a mean value of 2-3 individually harvested, counted and stained lobes.
Figure 3.6 Analysis of the surface expression of CD4 and CD8 molecules by cells capable of recolonisation in vitro. Adult and foetal thymocytes (foetal thymocytes were obtained from embryonic thymuses grown 1 week in organ culture) were separated into DN and positive (DP and SP) fractions and titrated into lymphoid depleted lobes. The results in (a) show adult thymocytes and in (b) foetal thymocytes fractionated using a panning technique (section 2.3.6). In (c) adult thymocytes were separated using a complement cytotoxicity assay (described in section 2.3.5). The different separated donor populations are shown as unfractionated cells (□-□), CD4 and CD8 expressing cells (●-●) and CD4CD8 depleted cells (○-○). The results shown are mean values of 2 lobes in (a), (b) and (c).
3.3 DISCUSSION

A series of events including migration to and colonisation of the thymus followed by intrathymic differentiation has to occur in order to produce a pool of immunocompetent T-cells later found in peripheral lymphoid tissues. Using the mouse foetal thymic organ culture system as a model for studying T-cell development, we sought to examine the nature of cells capable of colonisation and proliferation in this system and the phenotypic changes they undergo while maturing in vitro.

We found that cells derived from the liver and thymus of day 14 embryos are capable of recolonisation and extensive proliferation in such cultures whereas cells derived from adult sources (blood and thymus) display a more restricted potential for seeding and division in this organ culture. This could be due to low numbers or lack of precursor cells in these adult populations or absence of an additional cell type (perhaps a stromal element) among the cells required for repopulation. Another explanation could be a difference in the proliferation capacity of adult and embryonic cells within the rudiments where embryonic stromal cells may not provide the right microenvironment for adult cells to proliferate. Alternatively, cells from adult thymus may lack homing receptors necessary for recolonisation. This hypothesis could be tested by microinjecting selected populations into alymphoid lobes and then examining whether a recolonisation event occurs.

In the kinetics experiment where the rate of proliferation was investigated in lobes seeded with foetal thymocytes and foetal liver cells (figure 3.4) it could be demonstrated that the cell recovery/lobe was very similar between each experiment and both donor populations although the number of seeded cells differed 5-fold in some cases. Furthermore, in lobes recolonised with foetal thymocytes about $2 \times 10^3$ cells could be recovered after 1 day in culture when the initial seeding was about $1 \times 10^4$. In addition, lobes recolonised with $5 \times 10^4$ foetal liver cells contained only $\sim 1-2 \times 10^3$ cells on day 3 in culture. It is worth discussing whether the number of cells that can be
seeded into a lobe during a 2 day incubation period in a hanging drop culture is restricted or if the actual number of cells capable of colonisation among $1 \times 10^4$-$1 \times 10^5$ cells is the limiting factor. Our results favour the first explanation that the number of cells colonising the lobes at one time are few and not necessary dependent on the actual number of cells seeded but rather the source of T-cell precursors and the receptiveness of the lobe. This could maybe be tested in an alternative way, where fixed numbers of cells are microinjected into dGuo-treated recipients and cell recovery compared with each donor population. Kinetics studies in vivo by (Kadish and Basch 1976) indicated that only a small number (less than 100) cells are required to repopulate the thymus of an irradiated mouse when a rich source of precursors, like bone marrow is used as donor. Wallis et al., (1975) and Ezine et al., (1984) have also observed that in radiation chimeras only a few cells were needed for thymus reconstitution.

Changes in the expression of the CD4 and CD8 molecules are very similar among untreated lobes in culture and dGuo-treated lobes recolonised with foetal liver or thymus. In an untreated lobe proliferation and differentiation of cells already residing in the lobe at the day of removal (day 14 of gestation) can be observed. The most dramatic increase in cell numbers and the appearance of CD4 and CD8 occur during the first six days in culture. This culture period can be compared with the last six days of gestation in vivo, before birth (day 20). However, in a recolonised lobe in which we are observing the events from the initial day of seeding (day 10-11 in vivo), the first nine days resemble the time before birth and indeed during this time we see proliferation and the appearance of all four CD4/CD8 subpopulations found in the normal thymus.

We have shown that cells capable of seeding and proliferating in mouse foetal and adult thymus lie within the DN population in experiments enriching for this phenotype by panning and antibody plus complement treatment. By separating cells with panning it was shown that cells expressing CD4 and/or CD8 did not contain repopulation
potential. However, recent data by Wu et al., (1991) showed that early intrathymic precursor cells in *in vivo* reconstitution experiments express low levels of CD4. Kinetics studies revealed that this population, CD4<sup>low</sup>CD8<sup>-</sup> is a precursor to CD4<sup>-</sup>CD8<sup>-</sup> thymocytes and represents only 0.05% of the total thymocyte population, which may explain why it has not been recognised previously.

The CD4 and CD8 expression alone is insufficient to define mature and immature SP cells without knowing the status of CD3 expression on these cells. Another important discussion point is that the foetal organ culture system only allows to assay those cells able to migrate into the thymus in contrast to the system using intrathymic injected cells.
CHAPTER 4

IDENTIFICATION OF MURINE THYMOCYTE POPULATIONS CAPABLE OF EXTENSIVE PROLIFERATION IN SERIALLY PASSAGED THYMIC ORGAN CULTURES
4.1 INTRODUCTION

In chapter 3 we showed that alymphoid lobes, prepared by dGuo-treatment, can be used as recipients in a recolonisation assay in vitro in which T-cell precursors from various sources can serve as donor cells. As an extension of this study we sought to examine the proliferative capacities of cells derived from adult and embryonic tissues and to investigate the lifespan of cells with high proliferative potential.

From the eleventh day of gestation, stem cells derived from the liver of embryonic mice colonise the thymic rudiment and establish a pool of lymphocyte precursors (Moore and Owen 1967). These cells do not initially express CD4 or CD8 molecules but, if introduced into thymuses in vitro or in vivo, can differentiate to establish populations of cells bearing either or both these markers (Ceredig et al., 1983a; Ceredig et al., 1983b; Mathieson and Fowlkes 1984; Kingston et al., 1985). In adult life, a small fraction (0.03-0.25%) of bone marrow cells provide a multipotent source from which progenitor cells are dispatched to the thymus (Basch et al., 1978). Numerous converging lines of evidence, from studies using CBA/H, CBA/H-T6T6 parabiotic mice (Harris et al., 1964), from adoptive transfer into corticosteroid-depleted (Scollay et al., 1986), irradiated (Ford et al., 1966; Micklem et al., 1966) and normal congenic recipients (Scollay et al., 1986), and from intrathymic transfer experiments (Goldschneider et al., 1986), have suggested that there is a continual influx of precursor cells into the adult thymus, that the daily input is low, and that once a stem cell enters the thymus its proliferation is limited to a couple of weeks (for a critical review of the experimental evidence see Scollay et al., 1986). More recently, these issues have been addressed using the approach of specifically labelling thymocytes which are synthesizing DNA with bromodeoxyuridine (a non-reutilised analogue of thymidine which can be recognised by specific antibody) (Penit and Vasseur 1988). This has allowed the proliferative status of different thymocyte subsets to be evaluated in situ (Penit and Ezine 1989; Penit and Vasseur 1989). The results of these studies suggest that whilst the majority of CD4⁺CD8⁻ thymocytes in the adult are either resting
or engaged in differentiation-linked proliferation (leading to a loss in autorenewal capacity) between day 14-17 of embryonic development, a massive expansion of the immature CD4⁻CD8⁻ compartment occurs. Whether such an expansion reflects an intrinsic property of the thymic immigrants which colonise during that time, or an enhanced ability of stromal components to sustain CD4⁻CD8⁻ renewal, is not resolved.

In this chapter an alternative strategy for investigating the properties and lifespan of T cell precursors in the embryonic thymus and liver of mice is described. The approach involves titrating decreasing numbers of precursor cells into alymphoid embryonic thymuses. After 10-14 days in culture, seeded lobes are examined to see if they have been successfully recolonised so that the abundance of functional 'stem' cells, present in the initial seed populations can be estimated. The progeny of lobes successfully recolonised with the minimum number of seed cells are then re-titrated into fresh alymphoid thymic lobes and the process repeated for a further 4-6 passages. Using the approach of end point titration and sequential passage we have sought to investigate the self renewal and proliferative capacities of T cell precursors from different sources.
4.2 RESULTS

4.2.1 Frequency analysis of recolonisation capacity of different thymocyte populations during serial passage in thymic organ culture

A titration approach in which decreasing numbers of candidate cells were introduced into dGuo-treated thymic rudiments (from BALB/c embryos), was used to estimate the abundance of functional T-stem cells among thymocyte populations obtained from five BALB/c foetuses (day14). After 10-14 days in culture the seeded lobes were harvested and recolonisation judged on the basis of recovering at least $10^4$ viable cells per lobe (this value represents greater than three times the maximum number of cells recovered from an unseeded control lobe). As shown in the top left hand panel of figure 4.1, using five foetal thymus populations, successful recolonisation was established in four cases. Between $10^3$ and $10^4$ cells were required to be seeded into lobes in order to guarantee reconstitution, consistent with previous analyses of T cell precursor activity in whole fractions of BALB/c foetal thymocytes (Goff et al., 1990). Approximately $10^5$ cells were recovered from successfully seeded lobes, suggesting that a ten to one hundred fold expansion in total cell numbers had occurred in several of the lobes during the 10-14 day culture period.

Lobes successfully recolonised with minimal numbers of seed cells (the end points of each titration series), were used to prepare single cell suspensions and re-introduced into fresh dGuo-treated thymic rudiments (passage 2), and this process repeated (passages 3 and 4). As shown in figure 4.1, the remaining four thymocyte samples maintained re-colonising capacity throughout four passages (●, passage 2) (○, passage 3), and in several cases the abundance of cells capable of recolonisation appeared to increase with serial passage. These observations suggest that thymocytes resident within an in vitro organ culture may be capable of extensive proliferation.
To exclude the possibility that addition of donor thymocytes into dGuo-treated recipient rudiments may rescue recipient cells which would otherwise die (i.e., the recolonising cells are not of donor origin), these experiments were repeated using AKR/Icrf foetal thymocytes as donor cells. This allowed to distinguish between donor (Thy-1.1) and recipient (Thy-1.2) cells at each stage of passage. As shown in the right hand panel of figure 4.1, of five AKR/Icrf samples tested, four successfully recolonised (passage 1), requiring between $10^3$ and $10^4$ candidate cells to be seeded. Fewer cells were recovered from these lobes ($10^4 - 4 \times 10^4$), and these proved somewhat more difficult to passage serially. After four passages in culture, three AKR/Icrf samples (Δ, ○, ●) retained low but detectable recolonising capacity but none survived subsequent passage (passage 5). At all stages of the experiment the majority of cells (>96%) were of the Thy-1.1 phenotype clearly proving their donor (AKR/Icrf) origin.

The loss of recolonising activity among AKR/Icrf samples could be a result from at least two causes; (i) thymocytes proliferating in organ culture were undergoing differentiation-linked senescence leading to a loss in self renewal and/or (ii) the number of cells recovered from BALB/c -AKR/Icrf lobes may be too small to allow rare cells to be expanded. With regard to the latter point, we and others have noted that although dGuo-treated thymuses can be reconstituted with cells from mice with very different genetic backgrounds (Ready et al., 1984), certain combinations (such as AKR/Icrf and BALB/c appear less compatible than others (such as CBA and BALB/c), and that developing thymocytes display a clear syngeneic preference both in organ cultures (Eren et al., 1989) and in vivo (Stutman 1986).

4.2.2 Comparison of the capacity of embryonic and adult precursor populations to recolonise and be serially passaged in vitro by limiting dilution

In subsequent studies the AKR/Icrf donor, BALB/c recipient system was continued to be used since it had been shown to be an easy and clear way of identifying Thy-1.1
donor cells. In an attempt to discover whether the extensive thymocyte proliferation occurring in sequential organ cultures was a property contingent on the embryonic stromal environment or an intrinsic feature of the foetal T-progenitor cells themselves, the recolonising potential of cells from adult peripheral blood and thymus as well as embryonic tissues (liver and thymus) was investigated. As shown in table 4.1, peripheral blood mononuclear cell fractions from adult mice failed to recolonise thymic rudiments in vitro. This result suggests that precursor cells are absent or too rare to be detected in this assay system. Thymocytes from adult mice established recolonisation in four of five attempts although it was not possible to passage them at limiting dilution for more than two rounds. Under the same conditions samples of foetal thymocytes and foetal liver cells survived and proliferated for between 2 and 5 passages (see table 1). The superior ability of embryonic cells to expand in long-term organ cultures suggests that there are differences in T cell precursor functions during ontogeny, although from this data it is not clear whether these discrepancies lie at the level of single cells or at a population level. Data from Eren and colleagues appear to support this contention since they report that T cell precursors from the bone marrow of older mice are less able to recolonise thymic rudiments and suggested that cells display a developmental inferiority with increasing age of donor (Eren et al., 1989; Eren et al., 1988).

4.2.3 Thymocytes lacking CD4 and CD8 and a subset of CD4+CD8+ cells are selectively expanded after serial passage in organ cultures

The phenotype of murine cells surviving serial passage in organ culture was investigated by staining with antibodies to CD4 and CD8. As shown in table 4.2, CD4 or CD8 expressing cells were not detected in liver samples from day 14 AKR/Icrf embryos. After passage in thymic rudiments 40% of cells expressed both these markers (CD4+CD8+) and 15% and 23% of cells expressed either CD4 or CD8, respectively. This result is consistent with foetal liver containing haemopoietic precursor cells capable of generating T cells in an appropriate thymic environment.
Figure 4.1: Neonatal thymocytes (14 days of gestation) were obtained from...
Figure 4.1  Murine foetal thymocytes (14 days of gestation) were obtained from
embryos of pregnant BALB/c (left hand panel) and AKR/Icrf (right hand panel) mice
and seeded in decreasing numbers into dGuo pre-treated BALB/c foetal thymic lobes.
After 10-14 days seeded lobes were harvested, counted, and the cells from lobes
seeded with the least number of donor cells able to recolonise recipient lobes were
recovered and titrated into a fresh set of dGuo pre-treated BALB/c lobes. Experiments
using cells from 5 different mice are indicated as □-□, ○-○, △-△, ■-■ and ●-●
and are shown at each stage of passage. Results from passages 1-4 and 1-5 are shown,
in which AKR/Icrf donor cells (right hand panel) were discriminated by Thy-1.1
reactivity.
Table 4.1. Recolonising potential of murine precursor cells as judged by their capacity to be serially passaged in thymus organ cultures.

<table>
<thead>
<tr>
<th>Source of candidate recolonising cells</th>
<th>Number of cultures established</th>
<th>Passage number at which recolonising capacity was lost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AKR peripheral blood mononuclear cells</td>
<td>3</td>
<td>t</td>
</tr>
<tr>
<td>AKR adult thymocytes</td>
<td>5</td>
<td>t</td>
</tr>
<tr>
<td>AKR foetal thymocytes</td>
<td>5</td>
<td>t†</td>
</tr>
<tr>
<td>AKR foetal liver</td>
<td>5</td>
<td>t†</td>
</tr>
</tbody>
</table>

Table 4.1 Candidate cells from AKR/Icrf (Thy-1.1) mice were titrated into embryonic BALB/c (Thy-1.2) thymic lobes which had been pre-treated with dGuo to remove lymphoid cells. After 10-14 days the lobes were harvested. Cells from lobes seeded with the lowest number of cells that gave a successful recolonisation were re-titrated into fresh dGuo-treated lobes. Recolonisation was judged successful if ≥10^4 viable cells were recovered from an individual lobe and if the cells expressed the donor (Thy-1.1) marker.

The symbol † marks the point in each experiment where recolonising cells were no longer detected.
With subsequent passage in culture the proportion of double positive cells contained within lobes fell dramatically (from 40% to 3-7%) and the majority of surviving cells appeared to be double negatives (74%, 67% and 71% CD4⁺CD8⁻ cells, passages 3, 4 and 5, respectively). A similar reduction in CD4⁺CD8⁺ with passage in culture was observed when AKR/Icrf or BALB/c foetal thymocytes (from day 14 embryos) were used as seeds. Up to 81% (mean of four experiments) of cells contained within lobes (passage 3, BALB/c foetal thymocytes) were double negative (see table 4.2). When BALB/c thymocytes from adult mice were used to initiate parallel cultures loss of double positives with passaged number was again noted, as was a slight increase in the fraction of CD4⁻CD8⁻ cells. However, in contrast to cultures derived from foetal sources (in which the CD4⁺CD8⁻ to CD4⁺CD8⁺ ratios tended to favour CD8 single positives), the ratios of CD4⁺CD8⁻, CD4⁺CD8⁺ and CD4⁺CD8⁻ were approximately equivalent. These data collectively suggest that the sequential passage of murine T-cell precursors in organ cultures leads to the selection of double negative cells and the rapid loss of CD4⁺CD8⁺ intermediates. Loss of CD4⁺CD8⁺ cells may conceivably result from their conversion into more mature cell types, their susceptibility to death (perhaps by apoptosis) or outgrowth by an active proliferating population of CD4⁻CD8⁻ cells. In addition, these results suggest that precursor cells derived from foetal sources may have different properties from those obtained from adults in terms of lifespan/generative capacity. The tendency for foetal pro-T cells to generate a long-lived population of predominantly CD4⁻CD8⁻ cells has been documented by intra-thymic transfer studies in mice (Guidos et al., 1989a), as has the superior ability of human CD4⁻CD8⁻CD3⁻ thymocyte populations to proliferate in culture (Vives et al., 1987), suggesting that sequential passage may expand selectively these populations.

4.2.4 Phenotypic analysis of cell types in late passaged organ cultures
Since CD4⁻CD8⁻ thymocytes represent an extremely heterogenous subset comprising thymic precursors as well as more mature CD4⁻CD8⁻CD3⁺ (TCRαβ⁺ or γδ⁺) cells, a more detailed analysis of cell types in late passaged cultures was attempted (see figure...
4.2). Since we wished to examine the progeny of individual lobes, such analyses were limited to small numbers of cells (<2 x 10^5). Figure 4.2 shows data from cultures seeded with BALB/c foetal thymocytes (passage 4, left hand panels) and AKR/Icrf foetal liver cells (passage 3, right hand panels). As shown in figure 3.2(a), 46% and 50% of cells had a CD4⁻CD8⁻ and CD4⁻CD8⁺ phenotype, respectively. To exclude that the CD4⁻CD8⁻ cells were epithelial or other stromal contaminants, cells were stained with anti-CD45 and >97% were found to be leukocytes. Staining with anti-CD3 revealed that 82% of cells were CD3⁺ and comparable reactivity (76%) was seen with anti-TCRαβ. A small proportion of cells (<12%) were stained with anti-TCRγδ (data not shown), showing heterogeneity of TCR expression within these cultures. The profiles seen with the foetal liver (passage 3) cultures were 60% and 33% CD4⁻CD8⁻ and CD4⁻CD8⁺ respectively, where 54%, 37% and 15% of cells were reactive with antibody to CD3, TCRαβ and TCRγδ respectively (not shown). These data suggest that the cell types present in long term organ cultures are heterogeneous. While it is not possible to identify the precise phenotypes of cells present in these lobes (since additional double staining was precluded by the small numbers of cells obtained from individual lobes), at least four different cell types can be inferred from our results. These include CD4⁻CD8⁺, CD4⁻CD8⁻CD3⁺αβ⁺, CD4⁻CD8⁻CD3⁺γδ⁺ as well as a minor population of CD4⁻CD8⁻ cells lacking CD3. Conceivably these populations may also include NK cells, since such cells are reported to be of a CD4⁻8⁻3⁺ (CD5⁺ CD44⁺ TCR-Vβ8⁺) phenotype in adult mice (Ballas and Rasmussen 1990) and postulated to arise from a NK/T bipotential progenitor cell.
Table 4.2. Phenotype of thymocytes after serial passage in organ culture

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>Percentage of cells at each passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>(a) AKR/Icrf foetal liver</td>
<td></td>
</tr>
<tr>
<td>CD4^+8^-</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^-8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^+8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^-8^-</td>
<td>99(0.5)</td>
</tr>
<tr>
<td>(b) AKR/Icrf foetal thymocytes</td>
<td></td>
</tr>
<tr>
<td>CD4^+8^-</td>
<td>2(0.6)</td>
</tr>
<tr>
<td>CD4^-8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^+8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^-8^-</td>
<td>97(0.9)</td>
</tr>
<tr>
<td>(c) BALB/c foetal thymocytes</td>
<td></td>
</tr>
<tr>
<td>CD4^+8^-</td>
<td>7(0.6)</td>
</tr>
<tr>
<td>CD4^-8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^+8^+</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD4^-8^-</td>
<td>92(0.6)</td>
</tr>
<tr>
<td>(d) BALB/c adult thymocytes</td>
<td></td>
</tr>
<tr>
<td>CD4^+8^-</td>
<td>15(0.3)</td>
</tr>
<tr>
<td>CD4^-8^+</td>
<td>10(2.9)</td>
</tr>
<tr>
<td>CD4^+8^+</td>
<td>70(2.9)</td>
</tr>
<tr>
<td>CD4^-8^-</td>
<td>5(0.3)</td>
</tr>
</tbody>
</table>
Table 4.2 Cells from murine foetal (day 14 of gestation) and adult thymus and from foetal liver (day 14 of gestation) were used to seed dGuo pre-treated BALB/c thymic lobes. The phenotype of cells harvested from successfully seeded lobes, were examined 10-14 days later, using L3T4-PE (anti-CD4) and Lyt 2-FITC (anti-CD8) reagents. The results shown represent the mean and standard error of 3 to 8 independent experiments. Values shown in brackets are results from single experiments.
Figure 4.2

AKR foetal liver cells  BALB/c foetal thymocytes

(a) L3T4-PE (CD4)  (f) L3T4-PE (CD4)
Ly-2-FITC (CD8)  Ly-2-FITC (CD8)

(b) MI/9.3 (CD45)  (g) MI/9.3 (CD45)

(c) 145-2C11 (CD3)  (h) KT3-1.1 (CD3)

(d) H57-597 (TCR αβ)  (i) H57-597 (TCR αβ)

(e) anti-hamster Ig-FITC  (j) anti-rat Ig-FITC
Figure 4.2 Phenotypic analysis of cell types maintained by serial passage in organ culture. Cultures were initiated by seeding either foetal liver cells (from day 14 embryos of AKR mice) or foetal thymocytes (from day 14 embryos of BALB/c mice), into dGuo-treated BALB/c thymic lobes. These cells were passaged by end point titration into BALB/c thymus organ cultures as described previously (figure 4.1, legend). Cells recovered from a lobe after 4 passages (initially seeded with AKR foetal liver cells) and after 3 passages (initially seeded with BALB/c foetal thymocytes) are illustrated in panels (a) to (e) and (f) to (j), respectively. Cells expressing mouse CD4 and CD8 (revealed using L3T4-PE and Ly2-FITC) panels (a) and (f), CD45 (revealed using M1/9.3) panels (b) and (g), CD3 (revealed using 145-2C11 or KT3-1.1) panels (c) and (h) respectively, and T cell receptor alpha-beta polypeptides (revealed using H57-597) panels (d) and (i), are shown. Staining of cells with second layers only (FITC labelled anti-hamster or anti-rat immunoglobulins) are shown as controls in panels (e) and (j).
4.3 DISCUSSION

Using the approach of titrating precursor cells into mouse thymus organ cultures and serial passage provide evidence that murine foetal thymocytes are capable of considerable proliferation in organ culture for extended periods (10-12 weeks). During this time a tendency for CD4⁻CD8⁻ (double negative) leukocytes to expand and accumulate in organ culture (45-81% of cells), and selective loss of CD4⁺CD8⁺ cells (3%) was found. A preliminary examination of 'late passage' cells indicates that these comprise of a heterogenous population of cells in which representatives of mature (CD4⁻CD8⁻CD3⁺TCRαβ⁺) and immature (CD4⁻CD8⁻CD3⁻) double negative thymocytes are abundant, and where a subpopulation of CD4⁻CD8⁺ cells are reproducibly recovered. Since these distinct subsets arise from lobes in which minimal numbers of cells were seeded (titration endpoints) this resulting heterogeneity is noteworthy and at least two explanations could be postulated. Firstly, there may be a direct precursor-progeny relationship between the populations recovered. A CD4⁻CD8⁻CD3⁻ cell type may give rise to more mature cell types (CD4⁻CD8⁻CD3⁺) expressing either TCR αβ or γδ and also CD4⁻CD8⁺ cells. Conceivably this latter cell type could represent either a mature (post-double positive) cell or the postulated CD8⁺ (CD3⁻ or dim), intermediate stage between double-negative (CD4⁻CD8⁻) and double positive (CD4⁺CD8⁺) stages (Paterson and Williams 1987). If the CD4⁻CD8⁺ cells that were recovered are at a pre-DP stage in the development they appear to be cells that have lost their capacity to mature further to DP cells and may represent an abnormal population that has lost its capacity to respond to normal differentiation signals. Another explanation could be that cell-cell interactions are required for normal differentiation and that one of the populations is lost on serial passage. This seems unlikely since single cell reconstitution experiments by (Kingston et al., 1985) showed that single precursor cells was able to colonise alymphoid lobes in vitro at a frequency of 2/100 (2%).
In support of a precursor-progeny interpretation of these results, there is considerable evidence that double negative αβ+ and double negative γδ+ cells are derived from CD4-CD8-CD3- precursors (Guidos et al., 1989b; Denning et al., 1989; Miescher et al., 1988; Egerton and Scollay 1990), although the sequential developmental relationship of these cell types remain controversial (Egerton and Scollay 1990; Wu et al., 1990; Papiernik and Pontoux 1990). An alternative explanation for this finding of heterogeneity among cells from serial organ cultures is that the cell types may be interdependent. If such is the case then this may resolve the finding that CD4-CD8+ and CD4-CD8-CD3+ cells almost invariably coexist in lobes at late passage seeded at limiting dilution, and would suggest that at least one of the partners were capable of supporting the proliferation of the others.

Although it is not clear whether the outgrowth of these cells types indicates their direct descendency from a common precursor or whether interactive events between these populations may be promoting their combined survival, it can be proposed that serial passage of cells at limiting numbers may provide a novel means for the expansion and study of normally rare or elusive cell types within the thymus which may have considerable generative capacity.

Furthermore, these results provide evidence that the capability of cells to survive sequential passage in organ culture differs according to whether they originate from adult or embryonic sources. The superior proliferative potential of foetal pro-T cells as compared with counterparts isolated from adult mice, is compatible with the hypothesis that the developmental potential of haemopoietic stem cells changes during ontogeny (Ikuta et al., 1990) (as suggested by Weissman and colleagues) and that foetal tissues such as thymus and liver may contain populations of precursors which are absent or undetectable in the adult (Havran and Allison 1990).
These data suggest that sequential passage of thymocytes in organ culture may be a useful alternative strategy for characterising cells with high proliferative potential, resident in the thymus and also for probing their lineage relationships.
CHAPTER 5

ESTABLISHMENT AND INITIAL CHARACTERISATION OF MURINE THYMIC STROMAL CELL LINES IMMORTALISED WITH RECOMBINANT RETROVIRUSES
5.1 INTRODUCTION

The thymic microenvironment is composed of multiple cell types of non haemopoietic and haemopoietic origin which support stem cell proliferation and T-cell differentiation (Moore and Owen 1967). Direct contacts between stromal elements and thymocytes are believed to be crucial for the development of functionally mature, antigen specific and MHC-restricted T-lymphocytes (Owen and Ritter 1969; Stutman 1978). The nature of these cell-cell interactions, and also the effect of soluble factors known to be involved at different stages of T-cell development, are not fully understood.

The foetal thymic organ culture system has proved to be very useful for studying lymphopoiesis, but due to its complexity it has been difficult to assess the specific roles of the various types of cells in the thymic microenvironment. In an attempt to understand the possible role of different microenvironments within the thymus we have attempted to immortalise different stromal elements using recombinant retroviral constructs containing a temperature sensitive simian virus 40 (SV40tsA58) large T antigen gene (Tegtmeyer 1975; Jat and Sharp 1989) or the adenovirus 5 E1a (Roberts et al., 1985) region linked to the gene coding for resistance to G418. Cell lines containing the thermolabile large T antigen encoded by SV40 proliferate at the permissive temperature of 33°C and arrest growth when transferred to the non-permissive temperature of 39°C. In this chapter we describe the isolation of eighty four cell lines following retroviral infections of primary embryonic stromal cultures and thymocyte rosette/thymic nurse cell (T-ROS/TNC) cultures isolated from adult thymuses.
5.2 RESULTS

5.2.1 Titration of recombinant retroviruses
The retroviral recombinants SV40tsA58 and E1a12S were constructed by inserting the appropriate DNA segments into the *BamH1* site of the pZipNeoSV(X)1 shuttle vector, containing the sequence encoding G418 resistance (Mann et al., 1983; Cepko et al., 1984; Roberts et al., 1985; Jat and Sharp 1989). Plasmid DNA was transfected into \( \Psi_2 \) cells in order to establish virus producing cell lines. The virus-containing supernatants from confluent cultures of transfected \( \Psi_2 \) cells were collected and NIH 3T3 cells were infected and subjected to selection of G418. Subsequently, the number of resistant colony forming units (CFU) obtained were counted and the virus titre determined. The titration curves of SVtsA58 and E1a are shown in figure 5.1 (a) and figure 5.1 (b). A titre of \( \sim 1-4 \times 10^4 \) CFU/ml was observed on 3T3 cells after infection with E1a and the titres observed in two different samples from cell lines producing SVtsA58 were \( \sim 5 \times 10^3-1 \times 10^4 \) and \( \sim 10^2-2 \times 10^2 \) CFU/ml respectively. The low titre observed in the latter tsA58 sample is probably a result of multiple freezing and thawing of that particular aliquot, since it was used for infection several consecutive times.

5.2.2 Immortalisation of thymic stromal cell lines
Eighty four thymic stromal cell lines were isolated by infecting primary cultures of foetal thymus with recombinant retroviruses SV40tsA58 (Tegtmeyer 1975; Jat and Sharp 1989) or E1a 12S (Roberts et al., 1985), and selecting colonies resistant to G418 (500ug/ml) (Davies and Jimenez 1980). In the first set of experiments (see table 5.1) most clones isolated by infection with SV40tsA58 (49 of 72) had a fibroblastic appearance, judged by morphology and strong vimentin expression. Two such cell lines (designated 5.10 and 7.5) were cloned and selected for further study as representatives of this large stromal cell group. Approximately one third of cell lines obtained from Sv40tsA58 infections (23 of 72) had a markedly different morphology, with a polygonal outline. Among this cell type, clone 6.10 was selected for further
Figure 5.1

(a) Graph showing the number of colony forming units (CFU/dish) plotted against titration.

(b) Images of colonies for different samples: E1a12S, SV40tsA58, SV40tsA58.
Figure 5.1  Titration of virus containing supernatants on 3T3 cells. Shown in (a) are the titration curves obtained after infection with two different samples of ZipSVtsA58 (□-□) and one sample of ZipElal2S (■-■). Shown in (b) is one set of petri dishes from each virus titration representative for the mean values displayed in (a). The dishes have been stained with methylene blue to make the CFU visible.
study and a subclone which displayed a mosaic-like morphology (6.10 subclone 13) was also isolated. Infection with E1a 12S virus yielded a more limited panel of seven cell lines in which three morphological cell types could be distinguished. Cell lines with a fibroblastic appearance (3 of 7) were not studied further. Two clones (8.40 and 14.5), morphologically distinct from fibroblasts were selected for detailed characterisation.

A second series of experiments were performed in an attempt to generate stromal cell lines representing epithelial, dendritic and macrophage elements. To avoid the repeated generation of fibroblast-like cell lines, thymocyte rosettes (T-ROS; medullary dendritic or cortical macrophages with thymocytes bound) and thymic nurse cells (TNC; lymphoepithelial complexes containing cortical epithelium and fused thymocytes) were isolated from adult thymus by a series of enzymatic digests and separations (Wekerle et al., 1980; Kyewski and Kaplan 1982) (materials and methods, section 2.4.4). These enriched populations of cells were used as targets for virus infection, and five additional polygonal cell lines were obtained with E1a 12S of which two (15.5 and 15.18) were expanded, cloned and retained for further study. Three representative morphologies are shown in figure 5.2; in (a) 6.1013 with its mosaic arrangement, (b) 14.5 cells forming a fried egg shape and (c) 15.5 that has a polygonal shape.

5.2.3 Temperature dependent growth and differentiation of cell lines derived from SV40tsA58 infection

The rationale for using SV40tsA58 as an immortalising agent to generate thymic stromal cell lines was that cells may be clonally expanded at 33°C (SV40 large T permissive temperature) and yet stop proliferating and adopt a more 'normal', differentiated phenotype at 39°C (at which temperature SV40 large T is non-functional). In view of this, the growth of cell lines 5.10, 7.5 and 6.10 at 33°C and 39°C was studied in detail (see figure 5.3). A well characterised rat fibroblastic line (tsa 8) obtained from an infection of primary skin cultures with SV40tsA58 (Jat and
Sharp 1989) was used as a control in these studies, as were two E1a 12S derived clones 8.40 and 14.5. Cells were initially plated at a density of 2x10^4 cells/60mm dish and grown either at 33°C or 39°C (plus 37°C for clones 8.40 and 14.5). As shown in figure 5.3., clones tsa 8, 5.10, 7.5 and 6.10 grew well at 33°C (with a doubling time of approximately 30-36 hours), but grew poorly or not at all when maintained at the higher temperature (39°C). In contrast, clones obtained from E1a 12S infection (8.40 and 14.5) grew extremely slowly at 33°C, but grew well at 37°C and 39°C. These data suggest that the immortalisation of clones 5.10, 7.5 and 6.10 is dependent on a temperature sensitive element (SV40 large T). This was verified by direct staining of cells at 33°C and 39°C with a monoclonal antibody (mAb) to SV40 large T product (PAb 412). As shown in figure 5.4 (lower panels), strong nuclear staining was observed when these cells were grown at 33°C but not after maintainance at the nonpermissive temperature of 39°C. Concomittant with the change in SV40 large T expression at 39°C, clones 5.10, 7.5 and 6.10 were also observed to undergo a number of other changes. Most notably, the cytoplasm to nucleus ratio increased, many cells adopted a multinucleated appearance (perhaps indicating a general problem in successfully completing cell division) and the cells became contact inhibited (see upper right panel of figure 5.4). Taken together, these findings suggest that clones 5.10, 7.5 and 6.10 are temperature sensitive and that switching to a nonpermissive temperature not only leads to cessation of growth but also to alterations in their properties consistent with a more 'normal', less transformed phenotype.
Table 5.1 Murine thymic stromal cell lines isolated by retroviral infection

<table>
<thead>
<tr>
<th>Retrovirus used for infection</th>
<th>Type of culture infected</th>
<th>Morphology of cells derived</th>
<th>Number of clones/total number isolated</th>
<th>Designation of clones selected for further study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zip SVtsa58</td>
<td>primary embryonic thymic stromal</td>
<td>fibroblastoid</td>
<td>49/72</td>
<td>5.10, 7.5</td>
</tr>
<tr>
<td>Zip SVtsa58</td>
<td>primary embryonic thymic stromal</td>
<td>polygonal</td>
<td>23/72</td>
<td>6.10a</td>
</tr>
<tr>
<td>Zip E1a 12S</td>
<td>primary embryonic thymic stromal</td>
<td>fibroblastoid</td>
<td>3/7</td>
<td>none</td>
</tr>
<tr>
<td>Zip E1a 12S</td>
<td>primary embryonic thymic stromal</td>
<td>cobblestone</td>
<td>1/7</td>
<td>8.40b</td>
</tr>
<tr>
<td>Zip E1a 12S</td>
<td>primary embryonic thymic stromal</td>
<td>&quot;fried-egg&quot;</td>
<td>3/7</td>
<td>14.5</td>
</tr>
<tr>
<td>Zip E1a 12S</td>
<td>adult thymic T-ROS / TNC fractions</td>
<td>polygonal</td>
<td>2/5</td>
<td>15.5, 15.18</td>
</tr>
</tbody>
</table>
Table 5.1 Murine thymic stromal cell lines isolated by retroviral infection. (a) A subclone of 6.10, designated 6.1013, in which cells displayed a uniform mosaic appearance was also derived and selected for subsequent examination. (b) derived from mouse strain AKR/Icrf, all other selected clones from BALB/c. (c) thymocyte rosettes/thymic nurse cell.
Figure 5.2. Cell morphology of three selected clones.

(a-c) Cell morphology. Cells were photographed on an inverted microscope. (a) subclone 6.10^{13} (SV40-ts-derived), (b) clone 14.5 (E1a-derived) and (c) clone 15.5 (E1a-derived).
Figure 5.3 Growth kinetics at permissive and non-permissive temperatures. Three SV40ts clones (5.10, 7.5 and 6.10), two E1a clones (8.40 and 14.5) and the established ts-clone tsa 8, (used as a control), were examined. Cells were harvested and counted at different timepoints. Values are means of two independent experiments where each point represents counts on duplicate cultures, 33°C (○), 37°C (□) and 39°C (●).
Figure 5.4 Temperature dependence of n-clones grown at 33°C and 39°C. Examination of vimentin and SV40 large T expression in SV40tsA58 clones immunofluorescence labeling on acetic acid ethanol fixed cells.
Figure 5.4 Temperature dependence of ts-clones grown at 33°C and 39°C. Examination of vimentin and SV40 large T expression on SV40tsA58 clones by immunofluorescence staining on acetone:methanol fixed cells.
5.3 DISCUSSION

The heterogeneity of thymic stromal cells is probably related to their role in providing different microenvironments where molecular events that have been triggered by interactions between stromal cells and thymocytes, as well as released soluble factors, can induce T-cell development. To study this developmental process and many other tissue-specific functions several investigators have tried to establish in vitro cell models that can simulate in vivo events.

Cell lines from various sources of many mammalian species have been established by transformation with oncogenes (Yoakum et al., 1985), tumour viruses (Isom et al., 1980) and chemical carcinogens (Stampfer and Bartley 1985). One virus widely used is SV40 which has been shown to immortalise a variety of cell types such as rat and human fibroblasts (Jat and Sharp 1986; Radna et al., 1989), mouse and human bone-marrow stromal cells (Williams et al., 1988; Slack et al., 1990), mouse endothelial cells (O'Connell and Edidin 1990) and mammary epithelial cells (Rudland et al., 1989). Cell lines initiated in this way will exhibit a transformed phenotype, which may not resemble the cell type found in vivo. The use of a temperature sensitive (ts) SV40 large T mutant can circumvent these problems since it enables a controlled inactivation of large T by switching the immortalised cells to the non-permissive temperature. By using this system a cell line can be studied both at the permissive temperature when the cells are transformed and at the non-permissive temperature where cells revert to a non-immortal state with a more differentiated phenotype. The tsA mutant of SV40 have successfully immortalised many different cell types and made it possible to study differentiated cells in a controlled system. Examples of established cell lines are mouse hepatocytes (Zaret et al., 1988), mouse macrophages (Takayama et al., 1986), human thyroid epithelial cells (Wynford-Thomas et al., 1990), human epidermal cells (Banks and Howley 1983) and rat skeletal muscle cells (Iujvidin et al., 1990). Another transforming agent that has also been shown to immortalise successfully mammalian cells is the adenovirus, a DNA tumour virus. In fact, adenovirus has been shown to
successfully infect and transform primary epithelial cells from both human and rodent cultures (Dutt et al., 1990; Lynch and Trainer 1989). Furthermore, the gene product of E1a 12S can induce the production of a growth factor that is required for immortalisation of primary epithelial cells (Quinlan et al., 1987; Quinlan et al., 1988). Thus, in our attempt to establish what part each stromal element plays in the thymus we immortalised different stromal cells using a temperature sensitive mutant A of SV40 large T antigen or adenovirus E1a 12S.

By studying growth kinetics at 33°C and 39°C we have shown that ts-isolates will proliferate at 33°C and are inhibited at 39°C. The cessation of growth found at the non-permissive temperature was accompanied by loss of SV40 large T expression as well as a morphological change resembling a more differentiated cell. These results demonstrate that cell lines established from infection by the SV40ts mutant are dependent on SV40 large T for their growth and the maintenance of their immortal phenotype.

By careful examination of the morphology of clones isolated from E1a and SV40ts infections we could demonstrate a wide variety of cell shapes among isolates (but with a dominant shape resembling a fibroblastoid cell in 49/84 cases), which indicates that the use of recombinant retroviruses as immortalising agents is a very efficient way of generating different stromal cell lines.

When examining primary stromal cultures that were used for infections the majority of cells appeared epithelial in terms of morphology and keratin expression (data not shown). Despite this, the majority of lines (49/72) generated from SV40tsA appeared fibroblastoid. It is unclear whether this is an effect of SV40 or if the number of fibroblasts in the population infected was larger than predicted. Perhaps SV40 immortalises a minor population of fibroblasts very efficiently and/or that fibroblasts proliferate faster and therefore are more readily infected. Interestingly, E1a does not
seem to immortalise fibroblasts preferentially to the same extent as SV40 (table 5.1). Perhaps SV40 transformation is insufficient, as a single event, to immortalise thymic epithelial cells and requires a second event, while E1a may be sufficient alone.

Table 5.2 shows eight published stromal cell lines from mouse thymus in terms of their origin (adult or embryonic) and how they were established. All cell lines (8/8) listed in table 5.2 have been established by continuous culture and/or cloning without the use of immortalising agents and 7 of the 8 lines have been assigned an epithelial lineage. In addition, the majority of cell lines (7/8) originate from adult tissue. Comparison of our 84 cell lines with these single isolated cell lines may provide an indication of the most useful way of generating different cell types in the future.

It seems that continuous culture and cloning is sufficient for generating epithelial cell lines but these lines will most likely have a limited lifespan. In addition, certain cell populations within the thymic microenvironment may have a limited proliferation capacity and can therefore not be generated in this way. To overcome these limitations it seems attractive to use transforming agents in order to obtain immortalised cell lines with different characteristics. Our data show that embryonic fibroblasts are the predominant target for SV40 transformation and perhaps in order to obtain more diverse cell types it will be necessary to pre-select for certain cell types prior to infection. One method is to enrich for cells depending on their functional capacity like the TNC/T-ROS enrichment performed with adult thymus prior to E1a infection. Another approach could be to use the wide variety of monoclonal antibodies that are available and isolate different populations prior to immortalisation. At present a limitation is the range of cell types which can be immortalised by available constructs (e.g. SV40 predominantly transform fibroblasts in the thymus). However, a combination of methods including cell fractionation and different constructs may help to establish a variety of stromal cell types.
Table 5.2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Method of establishment</th>
<th>Reference</th>
<th>Proposed lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEPI</td>
<td>primary culture of adult thymus</td>
<td>continuous culture and cloning</td>
<td>Beardsley et al., 1983</td>
<td>epithelial&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E-5</td>
<td>adult thymus</td>
<td>continuous culture and cloning</td>
<td>Potworowski et al., 1986</td>
<td>epithelial</td>
</tr>
<tr>
<td>TE-71</td>
<td>adult thymus</td>
<td>continuous culture and cloning</td>
<td>Farr et al., 1989</td>
<td>epithelial</td>
</tr>
<tr>
<td>TE-75</td>
<td>adult thymus</td>
<td>continuous culture and cloning</td>
<td>1989</td>
<td>epithelial</td>
</tr>
<tr>
<td>St3</td>
<td>tumour cell culture of adult thymus</td>
<td>continuous culture and cloning</td>
<td>Brightman et al., 1989</td>
<td>epithelial</td>
</tr>
<tr>
<td>ET</td>
<td>embryonic thymus, day 16</td>
<td>cloning&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Palacios et al., 1989</td>
<td>epithelial</td>
</tr>
<tr>
<td>MRL 104.8a</td>
<td>long-term liquid culture of adult thymic stromal cells</td>
<td>continuous culture and cloning</td>
<td>Kosaka et al., 1989</td>
<td>none</td>
</tr>
<tr>
<td>B/c TEC-LI</td>
<td>stromal culture of adult thymus</td>
<td>continuous culture</td>
<td>Hiramine et al., 1990</td>
<td>epithelial</td>
</tr>
</tbody>
</table>

<sup>a</sup> TEPI was initially characterised as an epithelial cell line but was further analysed by Savino et al. (1986) and was assigned to a non-epithelial lineage.

<sup>b</sup> Thymic adherent cells to which a foetal thymocyte clone bound were selected and transferred to a new dish. The normal medium was replaced with medium containing dGuo and the clone isolated after continuous culture.
CHAPTER 6

CHARACTERISATION OF SELECTED STROMAL CELL LINES
6.1 INTRODUCTION

Chapter 5 described the generation of thymic stromal cell lines using recombinant adenovirus E1a and a temperature sensitive SV40 large T construct. This chapter describes the further characterisation of selected clones in terms of their phenotype, function and possible origin.

The non-lymphoid elements in the thymus play an important role during early differentiation of T-lymphocytes. Previous studies have suggested that (i) contact between epithelial cells and progenitors involves specific cell surface interactions (such as CD2 and its natural ligand LFA-3) (Selvaraj et al., 1987; Vollger et al., 1987) (ii) inductive interactions are probably mediated by physiological factors/receptors (such as IL-2 and its receptors) (Ceredig et al., 1985; Jenkinson et al., 1987) (iii) expression of class I and class II MHC antigens on the thymic stroma leads to the acquisition of MHC restriction and self tolerance by maturing T-cells (Zinkernagel 1982; Lo and Sprent 1986) and (iv) these interactions can ultimately lead to the expression of a more differentiated phenotype by progeny cells (such as the progressive rearrangement of T-cell receptor genes (TCR) (Davis and Bjorkman 1988) and/or the expression of Thy-1, CD3, CD4 and CD8 (Borst et al., 1983; Fowlkes et al., 1985; Smith 1987). These findings as well as the lack of an in vitro system to study stromal cell-thymocyte interactions led us to establish thymic stromal cell lines of adult and embryonic origin to be used for studying selective interactions with distinct subtypes of the thymic stroma.

The epithelial cells contribute a large part of the thymic stroma and are located in the cortex (the peripheral part of the thymus), in the medulla (the central part) and in the subcapsular region (the area outside the cortex). Cortical and medullary epithelial cells have been described to have different morphology as well as a different reactivity pattern with a panel of monoclonal antibodies. In addition, cortical epithelial cells can form lympho-epithelial complexes enclosing up to 30 thymocytes (Kyewski and Kaplan 1982). The two BM-derived stromal elements, macrophages and dendritic cells
have also been shown to form complexes with thymocytes in the form of rosettes, with a central stromal cell (Wekerle et al., 1980). Specific mAbs for BM-derived cells residing in the thymus are, as yet, not available but Mac-1 and antibodies to the leukocyte common antigen (CD45) recognise both cell types. Dendritic cells, only found in the medulla have been described to express high levels of MHC classII (Rouse et al., 1979; Kyewski et al., 1986).

By studying the morphology, phenotype and some functional properties of established cell lines we have tried to determine the origin of a few selected clones. Furthermore, we have examined the ts-derived clones at the permissive and non-permissive temperature. At the non-permissive temperature there are no interfering effects of large T and the clones generated by the ts-mutant of SV40 alter their phenotype but remain metabolically active. In addition, to document the diversity found in the thymic microenvironment, we explore the feasibility of using these lines for studying T-cell development in vitro, providing a model system in which stromal-thymocyte interactions can be examined.
6.2 RESULTS

6.2.1 Properties and lineages of isolated stromal cell lines

To determine whether thymus derived stromal cells generated in this study were of epithelial, fibroblastoid, dendritic or macrophage origin, a series of experiments were carried out. In the first study all cell lines were tested for their expression of the intermediate filaments (IF) keratin and vimentin. Intermediate filaments are a heterogenous group of cytoskeletal proteins whose expression and function is dependent on the type and differentiated state of the cell (Lazarides 1982). All cell lines expressed detectable levels of vimentin and some variation of both intensity and pattern of intracellular staining, was observed between cells grown at different temperatures (see table 6.1 and figure 5.3). No staining was observed using two different antibodies to keratin (the mAb; LE61 and a polyclonal antiserum raised in rabbit). This lack of reactivity did not rule out the possibility that some of our stromal cell panel may be epithelial since epithelial cells undergoing differentiation and epithelial cells in primary culture have been shown to alter their IF expression in some cases (Ben Ze'ev 1984a; Ben Ze'ev 1984b; Kim et al., 1987).

In a second set of experiments, electron microscope studies were performed to look for desmosomal bodies, which are characteristic of epithelial cells (Farquhar and Palade 1963). As illustrated in table 6.1 and figure 6.1, desmosomal junctions were readily identified in preparations from four cell lines; 6.10, 8.40, 15.5 and 15.18. EM-images of whole 15.5 and 15.18 cells demonstrated that fine tonofilament fibres along the cell membrane, as well as bundles of tonofilaments in the cytoplasm, were present. Furthermore, cell organelles such as mitochondria, lysosomes, endoplasmic reticulum and microtubules were also present at abundant levels (data not shown). These results together with their morphological appearance which differs from putative fibroblast lines (5.10 and 7.5) suggest that these cell types could be of epithelial origin.
Table 6.1 Characterisation of selected clones.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Vimentin&lt;sup&gt;a&lt;/sup&gt; expression</th>
<th>Keratin&lt;sup&gt;b&lt;/sup&gt; expression</th>
<th>Desmosome&lt;sup&gt;c&lt;/sup&gt; formation</th>
<th>Non-specific esterase activity</th>
<th>Rosette formation (%)</th>
<th>Proposed cell lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.10</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 (1)</td>
<td>fibroblast</td>
</tr>
<tr>
<td>6.10</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>14 (4)</td>
<td>epithelial</td>
</tr>
<tr>
<td>7.5</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16 (2)</td>
<td>fibroblast</td>
</tr>
<tr>
<td>8.40</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4 (1)</td>
<td>uncertain</td>
</tr>
<tr>
<td>14.5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>5 (2)</td>
<td>uncertain</td>
</tr>
<tr>
<td>15.5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>84 (4)</td>
<td>epithelial</td>
</tr>
<tr>
<td>15.18</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>72 (4)</td>
<td>epithelial</td>
</tr>
<tr>
<td>tsa 8</td>
<td>+</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>2</td>
<td>rat embryonic fibroblast&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caski</td>
<td>+</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>human epithelial&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expression levels: +, ++, +++
<sup>b</sup> Expression levels: -, +, +/
<sup>c</sup> Formation levels: -, +, +/
<sup>d</sup> Activity levels: -, +, +/
<sup>e</sup> Rosette formation levels: (x)
Table 6.1 (a) Vimentin expression was tested using a goat anti-mouse vimentin antibody (ICN) and FITC-conjugated second layer antibody. (b) Keratin expression revealed by immunofluorescence staining on acetone:methanol fixed cells using the antibody LE61 and FITC-conjugated anti-mouse immunoglobulin antibody. (c) Investigated by electron microscopy of 2.5% glutaraldehyde fixed, confluent cultures. (d) Tested using standard techniques (Gomori 1950), monocytoid cells were used as positive control for staining. (e) Stromal cells and unfractionated adult thymocytes (1:12) were mixed, centrifuged at 4°C and the number of rosettes counted using a haemocytometer (>3 thymocytes bound was scored as a rosette). Values are means of 2-5 independent experiments. (f) An established cell line from primary skin cultures, generated from a SVtA58 infection (Jat and Sharp 1989) (g) A human squamous epithelial cell line derived from a carcinoma of the cervix (Pattilo et al., 1977). nt = not tested.
In addition to these studies a third set of experiments was carried out, in which cell lines were stained with a panel of antibodies and assessed for non-specific esterase activity. Two mAbs to mouse thymic epithelial cells (TEC) (ER-TR4; recognising cortical epithelium and ER-TR5; recognising medullary epithelium) (van Vliet et al., 1984b), were unreactive in all cases tested. Epithelial heterogeneity has been documented within the medulla from the staining pattern obtained by two L-fucose binding lectins (Farr and Anderson 1985). Ulex europeus agglutinin (UEA) and Tetragonolobus purpureas agglutinin (TPA) have been shown to have specificity for reticular epithelial cells and Hassall's corpuscles in the medulla, respectively. All cell lines were negative for these lectins (data not shown) and this result, in conjunction with the lack of reactivity with the ER-TR mAbs, therefore failed to clarify a possible epithelial origin. Next, a number of antibodies to haemopoietic stromal components were tested, NLDL-145 and MIDC-8 (Kraal et al., 1986; Breel et al., 1987) (recognising dendritic cells) Mac-1 (reacting with cells of monocyte/macrophage lineage) (Springer et al., 1979) and M193 (Springer et al., 1978) (an antibody to the leukocyte common antigen (LCA, CD45). These antibodies were also unreactive with all cell lines tested (data not shown). The T lymphocyte marker Thy-1 has been shown to be present on a variety of stromal cell types of mouse including fibroblasts and neural cells (Mc Kenzie and Potter 1979), epidermal cells (Koning et al., 1987), stromal cell lines of bone-marrow and spleen (Pietrangeli et al., 1988) and thymic epithelium (Tucek and Boyd 1990). To examine whether our clones express this antigen, cells were stained in suspension using antibodies to Thy-1 (Ledbetter and Herzenberg 1979; Marshak-Rothstein et al., 1979) and analysed on a FACScan. One line, 15.5 contained 50-60 % Thy-1+ cells and the positive population exhibited high levels of expression (data not shown). Non-specific esterase activity, which appears to be associated particularly with cells of monocyte/macrophage lineage, was clearly demonstrated with clone 14.5 (see table 6.1 and figure 6.1(i)), which is somewhat puzzling in view of our failure to identify Mac-1 or CD45 positive cells.
Figure 6.1 Characteristics of two selected clones.

Electron micrographs showing the occurrence of desmosomes. Stromal cell-thymocyte rosettes formed in suspension at 4°C and cytocentrifuged (May-Grunwald and Giemsa stain). Nonspecific esterase staining of cytocentrifuged cells. Shown are the results obtained with clones 14.5 and 15.5 (E1a-derived).
Since cell lines 15.5 and 15.18 (tentatively assigned to an epithelial lineage) were originally isolated from T-ROS/TNC cultures, containing multicellular complexes between stromal cells and thymocytes, it was of interest to assess whether these cells still retain this functional capacity after immortalisation and cloning. In a fourth set of experiments, unfractionated adult thymocytes, which consist largely of immature double positive (CD4^+CD8^+) T-cells (data not shown), were used as targets in a rosette assay. As shown in table 6.1 and illustrated in figure 6.1, five of eight stromal lines showed little or no rosetting capacity (above that of the fibroblast line tsa 8, used as a control). E1a derived lines (15.5 and 15.18) formed approximately 80% and 70% rosettes respectively. Since both 15.5 and 15.18 have been shown to form desmosomes these data suggest that these cells may represent cortical epithelial cells, which are believed to form complexes with thymocytes in vivo.

6.2.2 Expression of MHC class I and class II antigens on murine thymic stromal cell lines.

Expression of MHC class I and II is believed to be crucial for the generation of mature CD4^+ and CD8^+ T-cells in the thymus (Doyle and Strominger 1987; Marusic-Galesic et al., 1988). Therefore, it was of interest to examine the level of expression of these molecules on our stromal lines and to determine whether this could be influenced by IFNγ and/or by maintaining the cells at 33°C or 39°C. In these studies, stromal cells were cultured in IMDM/FCS supplemented with 5, 50 or 500 U/ml IFNγ and class I and II expression was monitored using the antibodies HB24 and TIB120, respectively. All three cell lines derived from infection with SVtsA58 (5.10, 6.10 and 7.5), express MHC class I in the presence of IFNγ (results of clone 5.10 and 6.10, shown in figure 6.2, top panels). However, their response to changing the temperature differs, class I expression on 5.10 in the absence of IFNγ, is unaffected by temperature while 6.10 more readily expresses class I when maintained at 39°C, when SV40 large T is non-functional. In our experiments clone 5.10 did not express class II antigens at either 33°C or 39°C and was not sensitive to induction by IFNγ. On the other hand, clone
6.10 which also failed to express class II at the cell surface at the permissive temperature of 33°C could be induced with IFNγ to express class II at the non permissive temperature of 39°C. These variations in MHC class I and class II expression and inducibility of ts-clones were also reflected in the panel of cells generated by infection with retroviruses containing E1a (see lower panels of figure 6.2). Cell line 14.5 expressed class I poorly and required activation by IFNγ. In contrast, cell line 15.5 expressed class I constitutively at high levels (cell line 8.40 and 15.18 showed similiar staining patterns to 15.5, data not shown). Both cell types (14.5 and 15.5) appear equally dependent on induction by IFNγ for the expression of class II at the cell surface (figure 6.2 bottom right panels). Clone 15.18 expressed class II after induction by IFNγ, at levels similar to 15.5, in contrast, clone 8.40 was unable to express class II under any conditions tested (data not shown).

6.2.3 Molecules involved in thymocyte-stromal cell adhesion
Phenotyping of adult thymocytes bound to clones 15.5 and 15.18 showed that the majority of the cells (>90%) belonged to the CD4+CD8+ subset and expressed CD3. Less than 1% showed no surface expression of CD4 and CD8 (data not shown). To investigate the nature of these interactions mAbs recognising thymocytes were used to see whether they would have an effect on the adhesion process at 4°C. Thymocytes were pre-incubated with appropriate mAb for 30 minutes prior to the rosette assay. As shown in table 6.2, adhesion of adult thymocytes to clone 15.5 was not affected by mAbs recognising the conventional adhesion molecules, CD2 (Altevogt et al., 1989) and LFA-1 (α-chain) (Pont et al., 1986), on the lymphoid cells. Similarly, pre-incubation of anti-Thy-1 and anti-IL-2R antibodies with the thymocytes did not interfere with the adhesion process. On the other hand, the inhibition pattern observed with clone 15.18 showed some differences. Antibodies recognising Thy-1 and CD2 inhibited rosette formation by 30% and 35%, repectively. These results suggest that the binding with 15.18 may involve CD2 and its ligand LFA-3 on stromal cells (Vollger et al., 1987; Springer et al., 1987).
Figure 6.2: Expression of MHC class I and II antigens in cell lines cultured under the influence of temperature switching and/or IFNγ. The percentage of cells expressing MHC class I and II is shown at different temperatures and time points in days of culture.
Figure 6.2. Expression of MHC class I and II antigens on stromal cell lines under the influence of temperature switching and/or IFNγ. The percentage of cells expressing class I and II histocompatibility is shown for SV40ts clones (5.10 and 6.10) and E1a clones (14.5 and 15.5). Expression was measured following culture at 33°C or 39°C and monitored at different days after treatment with IFNγ (500U/ml) (□) or medium alone (■). Antibodies HB24 and TIB120 (and FITC-conjugated second step reagents) were used to measure MHC class I and class II expression respectively. Results from a single experiment are shown, which are representative of multiple (3) independent experiments.
Furthermore, Thy-1 which is a member of the Ig-superfamily has recently been shown to have an adhesive function on thymic epithelial cells (He et al., 1991), and may also play an important role in adhesion to clone 15.18. Since Thy-1 is expressed by 15.5 cells it will be of interest to elucidate whether blocking with anti-Thy-1 antibodies on these non-lymphoid cells could have an effect on thymocyte-15.5 cell adhesion.

The strongest inhibition of cell adhesion with clones 15.5 and 15.18 was observed with a CD8 specific mAb (table 6.2). A similar result was obtained with clone 15.5 using an antibody recognising CD4 (15.18 not tested). Since class I is expressed by both 15.5 and 15.18, one may speculate that the adhesion of the CD4+CD8+ subset could be a consequence of the recognition of MHC class I molecules on the stromal cells and CD8 on the thymocytes. However, no class II is expressed on untreated 15.5 cells which makes it difficult to propose that the inhibition with anti-CD4 of adhesion to 15.5 is by interference with the class II and CD4 interaction. Since these experiments are rather preliminary there is a possibility that the effect is Fc-mediated and in order to confirm these results other anti-CD4 and anti-CD8 antibodies and Fab or F(ab')2 fragments would need to be tested for inhibition.

The failure to inhibit adhesion with anti-LFA-1 antibodies may be because the LFA-1/ICAM-1 interaction is temperature dependent (Martz et al., 1983; Rothlein and Springer 1986). However, the rosette assay used here, in which the stromal monolayer is disrupted by versene treatment, must be carried out at 4°C to avoid stromal cell aggregation. In order to investigate the role of LFA-1 and ICAM-1 in adhesion with these cell lines, binding may have to be evaluated on monolayers at 37°C.

Treatment with IFNγ has been shown to cause strong induction of ICAM-1 on a variety of stromal cell types and increase binding of lymphocytes through LFA-1 (Dustin et al., 1988). As described in section 6.2.2, IFNγ can induce class I and in some cases class II on established SV40tsA and E1a-derived stromal cell lines. To investigate whether
induction of expression of these molecules would have an effect on the adhesion pattern, selected clones were stimulated with IFNγ (100U/ml) for 5 days. As shown in figure 6.3 (a and b) adhesion was significantly enhanced as a result of IFNγ stimulation. It is interesting to note that tsA-clones 5.10 and 7.5 cultured at 39°C (their non-permissive temperature) in the presence of IFNγ showed a greater capacity to form rosettes than cells cultured at 33°C (their permissive temperature). Another temperature dependent effect was observed with clone 6.10, where the percentage of rosetting cells increased from 3% at 33°C to 20% at 39°C in the absence of IFNγ (figure 6.4). These results indicate that the tsA-derived cells are more receptive to stimulation at the non-permissive temperature when the SV40 large T is not expressed. This finding is in agreement with the results obtained from IFNγ induction of MHC antigens (section 6.2.2). The E1a-derived clones 8.40 and 14.5 which without stimulation showed limited capacity to form multicellular complexes, responded strongly to IFNγ and formed 70% and 100% rosettes respectively. In the case of clone 15.5 and 15.18 which already formed rosettes there was a slight increase of adhesion in the presence of IFNγ.

In addition to a quantitative effect of IFNγ on the number of rosettes formed, the quality of adhesion was also enhanced. In other words, instead of scoring ~4-5 thymocytes bound/stromal cell, ≥8 cells usually adhered as a result of IFNγ treatment. This finding are demonstrated in figure 6.4.

From these studies it is not possible to determine whether the enhanced adhesion observed after IFNγ stimulation and/or after culture at 39°C is a result of newly expressed adhesion molecules and/or increased affinity of the molecules already expressed by the cell. However, staining and inhibition studies should elucidate which ligands participate in the formation of these complexes. Furthermore, although the CD4+CD8+ thymocyte population was found to bind unstimulated 15.5 and 15.18 cells, the cell-cell interactions post-IFNγ treatment may have involved other populations as well and this should be investigated.
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Table 6.2  Blocking of thymocyte binding to clones 15.5 and 15.18

<table>
<thead>
<tr>
<th>Antibody</th>
<th>15.5 Rosettes (%)</th>
<th>15.18 Rosettes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>91, 91</td>
<td>75, 66</td>
</tr>
<tr>
<td>CD2 (12-15)</td>
<td>98, 94</td>
<td>31, 50</td>
</tr>
<tr>
<td>LFA-1 (H35-89)</td>
<td>87, 88</td>
<td>61, 81</td>
</tr>
<tr>
<td>CD2+LFA-1</td>
<td>80, 86</td>
<td>65, 73</td>
</tr>
<tr>
<td>IL-2R (7D4)</td>
<td>94, 87</td>
<td>57, 77</td>
</tr>
<tr>
<td>Thy-1 (HO-22.1)</td>
<td>80, 84</td>
<td>36, 34</td>
</tr>
<tr>
<td>CD8 (YTS169.4.2)</td>
<td>8, 22</td>
<td>16, 15</td>
</tr>
<tr>
<td>CD4 (GK 1.5)</td>
<td>49, 21</td>
<td>nt</td>
</tr>
</tbody>
</table>

Table 6.2 Inhibitory effect of anti-lymphoid mAbs on the adhesion of adult thymocytes to clones 15.5 and 15.18 at 4°C. The antibody blocking and rosette assay were carried out as described in materials and methods (section 2.12.1 and 2.12.2). The results are shown as percentages of binding (≥ 3 thymocytes bound/stromal cell) and represent the values of two independent experiments.
Figure 6.3 Effect of IFNγ and temperature switching on stromal cell-thymocyte adhesion. The results are shown as percentages of binding (≥ 3 thymocytes bound/stromal cell) and represents ± variance of two independent experiments. (a) shows the results obtained with E1a-derived clones, -IFNγ and +IFNγ. (b) shows the results obtained with SV40tsA-derived clones at 33°C -IFNγ and +IFNγ and at 39°C -IFNγ and +IFNγ.
Figure 6.4 Induction of stromal cell-thymocyte adhesion by IFNγ.

Shown are the results with clones 6.10 and 15.5.

6.10

33°C -IFNγ 33°C +IFNγ 39°C -IFNγ 39°C +IFNγ

15.5

-IFNγ +IFNγ
6.3 DISCUSSION

The thymus is composed of a variety of stromal elements including cells of non-haemopoietic origin (fibroblasts and epithelial cells) and cells of haemopoietic lineage (macrophages and dendritic cells). These stromal cells provide different microenvironments in which T-cell precursors can develop into functionally mature T-cells. Therefore, in order to understand how T-cell lymphopoiesis is regulated it is important to document the specific role of each cell type. One approach for studying the complex architecture of the microenvironment has been to raise mAb to different stromal elements. A large panel of reagents have been produced and catalogued into groups recognising distinct areas of the thymus (Brekelmans and van Ewijk 1990).

Another approach to study the function of the microenvironment has been to isolate stromal cells and generate corresponding cell lines. This has been used in human, rat and mouse systems (Itoh et al., 1982; Beardsley et al., 1983; Mizutani et al., 1987). A problem with this approach is obtaining cell lines with functional properties that reflect their normal counterparts in vivo, for example stromal cells which assist T-cells to mature and differentiate. At present, the number of functional stromal cell lines is small. To circumvent such limitations we tried to generate cell lines using a temperature sensitive mutant of SV40 large T. In addition, the transforming agent E1a was also employed to overcome a possible limitation in cell populations susceptible to the immortalising effects of SV40 large T. Moreover, to increase the possibility of generating cell lines useful for in vitro reconstitution of in vivo events, cells were pre-selected prior to immortalisation, on the basis of their capacity to form multicellular complexes in vivo.

From several retroviral infections 84 lines were generated, maintained and characterised in terms of their morphology and intermediate filament expression. From that large group of cell lines, a panel of seven clones were characterised in more detail and found to resemble stromal cells of different origins judged by their morphology and
phenotype. One Ela-derived line, 14.5, had a distinct morphology; the cells appeared very flat in culture ('fried-egg shape') and had strong non-specific esterase activity (indicative of cells of the monocyte/macrophage lineage). That we were unsuccessful in finding Mac-1- or CD45-expressing cells requires further investigation in order to identify the origin of 14.5. Clones 5.10 and 7.5 (derived from an SV40 tsA58 infection) appeared fibroblastoid according to their elongated morphology, the absence of desmosomes and their lack of reactivity with antibodies recognising cells of macrophage, dendritic or epithelial origin. Another ts-derived cell line, 6.10, also failed to stain with any of the lineage specific markers tested, but in contrast to 5.10 and 7.5 formed desmosomes, suggesting it may be an epithelial cell type. Desmosomes were also found among three Ela-derived lines, 8.40, 15.5 and 15.18. Clones 15.5 and 15.18 (isolated from a T-ROS/TNC culture) retained the capacity to form multicellular complexes with adult unfractionated thymocytes in vitro. In contrast, clones 6.10 and 8.40 (derived from primary culture of embryonic thymus) did not show the same capacity, suggesting that the origin (embryonic or adult) and/or the type of culture they were isolated from (primary stromal or T-ROS/TNC) may influence the phenotypic range of cells that are immortalised.

TEC can be divided into different subpopulations depending on location in the thymus and reactivity with defined monoclonal antibodies. Therefore, it was of interest to establish whether clones that have been shown to form desmosomes (6.10, 8.40, 15.5 and 15.18) but with different morphology and functional ability, resembled epithelial cells of different origin. In order to do so we adopted the criteria for TEC lines proposed by Brekelmans and van Ewijk (Brekelmans and van Ewijk 1990); (1) presence of desmosomes and tonofilaments, studied by EM; (2) detection of cytokeratin with mAbs; (3) detection of TEC-specific antigens with mAbs; (4) absence of antigens specific for macrophages, dendritic cells and fibroblasts, indentified with mAbs. Our isolated clones tentatively proposed to be of epithelial origin fulfil only two of these four requirements (1) and (4). However, both 15.5 and 15.18 form rosettes with
thymocytes in which the majority of bound cells express both CD4 and CD8 (>93%) (data not shown). This phenotype is typical of cortical thymocytes (Fink et al., 1984; Kyewski et al., 1987b), which may suggest that these cell lines (15.5 and 15.18) resemble cortical epithelial cells. The failure to demonstrate ER-TR4+ ER-TR5- cells (a mAb defined phenotype, indicative of cortex-derived epithelial cells) and cytokeratin expression by 15.5 and 15.18 needs clarification. It is perhaps worth noting that cytokeratin expression is known to be heterogeneous and dependent on the differentiated state of the cell (Ben Ze'ev 1984b; Ben Ze'ev 1984a; Lazarides 1982; Kim et al., 1987), which may in part explain our results. Furthermore, long-term culturing may also result in decreased or lost expression of certain antigens as has been reported by Cattermole et al., (1989). In order to establish whether the lack of reactivity with a number of stromal cell specific markers is a result of immortalisation and/or long-term culturing, conventional lines could perhaps be used as targets for infection. This kind of study would be valuable for investigating what phenotypic changes occur after a retrovirus has been introduced into the cell and in addition, how well the immortalised and non-immortalised cells represent their counterparts in vivo.

Several investigators have reported the generation of murine stromal cell lines including those of epithelial origin: TEPI (Beardsley et al., 1983), E5 (Potworowski et al., 1986), TE-71 and TE-75 (Farr et al., 1989) and St3 (Brightman et al., 1989). Many of these lines display a medullary phenotype according to their reactivity with ER-TR5 (and failure to react with ER-TR4). All these cell lines have been established by continuous culture and cloning without the use of immortalising agents. It seems that this method is sufficient for generating epithelial cell lines. However, certain cell populations within the thymic microenvironment may have a limited proliferation capacity and can therefore not be generated in this way. To overcome these limitations it seems attractive to use transforming agents in order to obtain cell lines with different characteristics. Our data show that embryonic fibroblasts are the predominant target for SV40 transformation in our system while E1a appears to contain the capacity to
immortalise epithelial- and macrophage-like cell types. In this context it is interesting to note that recombinant retroviruses containing the v-myc and v-Ha-ras oncogenes have been shown to generate a different panel of stromal cell lines from embryonic thymus (Cattermole et al., 1989). Here, a large group of adherent cell lines, most likely representing cells from the macrophage/dendritic cell compartment (concluded by the presence of Mac-1, Fc receptors and class II) was established. A second group of adherent stromal lines was also established. This group showed no expression of macrophage/dendritic cell and epithelial cell markers tested. Taken together, in order to completely reconstruct the thymic microenvironment in vitro it may be necessary to use a variety of immortalising agents.

Few stromal cell lines with functional capacity, allowing T-cell differentiation have been reported. Recently, Palacios et al., (1989) reported a mouse TEC line capable of mediating differentiation of Pro-T lymphocyte clones into TCR/CD3 expressing cells. Another report by Brightman et al., (1989) shows that the stromal cell line St3 can induce expression of Thy-1 and CD4 by a T lymphoid cell line. Two mouse thymic stromal cell lines (MRL-104.8a and TEL-2) have been described which have been claimed to induce selective elimination of immature double positive (CD4+CD8+) thymocytes and a T-cell clone, respectively (Kosaka et al., 1989; Nakashima et al., 1990). No functional properties of the possible epithelial lines, designated TG, produced by v-myc, v-Ha-ras transformation have been reported (Cattermole et al., 1989).

It is believed that cortical epithelial cells are involved in positive selection for MHC following contact with immature thymocytes (Benoist and Mathis 1989; Berg et al., 1989). Since clones 15.5 and 15.18 express MHC class I and will express class II after induction by IFNγ, these cell lines may prove useful for studying the requirements for positive selection and the signals required for differentiation or cell death in the thymus. Furthermore, these cells will allow us to investigate in more detail how
different cell surface antigens are involved during multicellular complex formation. In addition, the inducible adhesion pattern (with IFN\(\gamma\) and shift in temperature) found with all clones tested, provides an additional tool for studying other mechanisms possibly involved in cell-cell adhesion.

In this study 72 SV40tsA58-derived cell lines were isolated. By switching the cells to the non-permissive temperature of 39\(^\circ\)C, we hoped to observe a change in phenotype to a more 'normal differentiated' cell. Our findings, that clone 6.10 will only express MHC class I after culture at the non-permissive temperature of 39\(^\circ\)C, (unless supplemented with IFN\(\gamma\)), and that class II is only inducible at 39\(^\circ\)C (after addition of IFN\(\gamma\)) suggest that cells cultured without the influence of large T do adopt a more normal phenotype. The ts-lines described here also provide an opportunity to search for genes corresponding to novel thymic growth factors, (by subtractive cDNA approaches at 33\(^\circ\)C versus 39\(^\circ\)C). An extension of this approach has been the introduction of the ts-mutant into the germline of mice. This transgenic model system, pioneered by Jat and colleagues (Jat et al., 1991) yields a variety of interesting conditionally immortalised stromal cells. These combined approaches suggest that the use of SV40ts large T and E1a as immortalising agents may have widespread applications for studying T-cell differentiation.
CHAPTER 7

THE ABILITY OF STROMAL CELL LINES TO SUPPORT PROLIFERATION AND DIFFERENTIATION OF PRECURSOR CELLS
7.1 Introduction

Haemopoietic precursor cells have the capacity to self-renew and differentiate. These processes are affected by external stimuli. Different microenvironments (e.g. the bone marrow and the thymus) promote generation of different progeny by influencing precursor cells through cell-cell contact and production of factors. To simulate in vivo lymphohaemopoietic microenvironments a number of culture conditions have been used in vitro. A long-term bone marrow culture system was developed by Dexter et al., (1977) which allowed myeloid commitment processes to be characterised. Furthermore, murine bone marrow cultures, originally described by Whitlock and Witte (Whitlock et al., 1984) have been used to study B-lymphocyte development in vitro. With regard to T-cell development, the mouse foetal thymic organ culture system (described in detail in chapter 3) is the most widely used in vitro model for studying differentiation of T-cells. Although it is well established that stromal cells are responsible for mediating some developmental events in these organ cultures, the specific function of each cell-type remains uncertain. Consequently, mouse thymic stromal cell lines were established for the purpose of devising a simple assay system, in which different cell-types could be assessed for their capacity to support lymphopoiesis.

In chapter 5 and 6 the generation and characterisation of 84 stromal cell lines was described. This chapter describes the ability of some of these clones to promote growth and/or differentiation of precursor cells.

An IL-3-dependent murine multipotent stem-cell clone (FDCP-mix or A4) was isolated from a long-term bone marrow culture infected with the src oncogene of the Moloney sarcoma virus (Spooncer et al., 1986). This cell line will alter its capacity to self-renew and differentiate when cultured with haemopoietic stromal cells or recombinant growth factors. Under these circumstances cells of the myeloid and the erythroid lineages can develop (Spooncer et al., 1986). The possibility of modifying A4 cells by changing the
culture conditions created an opportunity to investigate whether SV40- and E1a-derived stromal clones could influence growth and/or differentiation of these cells. In addition, since A4 cells have the potential for multilineage differentiation, it was of interest to determine whether thymic-derived stromal cells could trigger A4 cells to differentiate along the T-cell lineage.

The double negative (CD4-CD8-) subset has been shown to contain T-cell precursor activity with the capacity to undergo differentiation into their various progeny (double positive and single positive) both in vitro and in vivo (Ceredig et al., 1983a; Kisielow et al., 1984). In order to determine whether the stromal clones could contribute to these processes at any stage, they were tested for their ability to support growth and/or differentiation of thymic-derived precursor cells.

Many studies have shown that the thymic microenvironment, together with a complex network of cytokines, promote T-cell differentiation. One factor, interleukin-7 (produced by stromal elements) has been shown to influence particularly immature thymocyte subsets (Murray et al., 1989; Watson et al., 1989). Therefore, the response of co-cultures of stromal cell lines and T-cell precursors, to this factor was analysed.

In addition, as an extension of this study, the possibility of creating an enclosed stromal environment for the developing thymocytes by using established cell lines was explored.
7.2 RESULTS

7.2.1 The ability of isolated clones to support the growth of the murine stem cell clone A4

The A4 clone is completely dependent on IL-3 for its capacity to self-renew. However, during altered culture conditions such as after addition of growth factors, co-culture with stromal cells or changes in cell density and serum-type in the culture medium these cells have been shown to have the capacity to differentiate (Spooncer et al., 1986). In order to analyse the ability of stromal clones to support the growth of this cell line two functional assays were set up. First, the stromal cells-conditioned medium was checked for the presence of IL-3 or other soluble factors capable of supporting A4 growth. Secondly, all cell lines were tested for their capacity to maintain A4 cells through a semi-solid layer of soft agar where factors may be able to diffuse and promote cell growth. None of the clone-conditioned media tested could assist A4 growth (supplied as 2% or 20%) (data not shown). However, the ability to support A4 was found in some cell lines using soft agar (figure 7.1). The results shown are from clones which were selected for further study in chapters 5 and 6. The data shown in figure 7.1 indicate that some clones can promote survival of A4 cells (e.g. from clone 6.10, 6x10^4 cells were recovered of 1x10^5 plated) although it is not possible to establish whether actual proliferation had occurred. Although the growth promoting effect of the cell lines is minimal compared to the IL-3-secreting control culture (Wehi-3B), it is significant in comparison to cultures without stromal cells, where no cells survived (data not shown). These findings may be a result of a factor which is too short-lived to be detected in the conditioned medium and the soft agar culture perhaps allows continuous exposure to this molecule.
7.2.1. Thymic stromal cells can promote A4 growth and differentiation through cell-cell contact

Although various cytokines modulate cell survival and differentiation by the release of diffusible growth factors, there is evidence that cell-cell contact is important. To study
the influence of various cytokines on A4 development, these cells were cultured on

- Figure 7.1 Soft-agar assay testing the ability of thymic stromal cell lines to support

the growth of A4 cells. Stromal cell lines (5x10^5) were overlaid with 1ml of semi-solid

agar (0.3%) and 1x10^5 A4 cells in 2 mls of medium were added. Seven days after
culture the number of viable cells was determined by trypan blue exclusion. The results
shown are the average +/- variance of 2 experiments in which the IL-3 secreting cell line Wehi-3B
was used as a positive control.

![Graph](chart.png)

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was used as a positive control.
7.2.2 Thymic stromal cells can promote A4 growth and differentiation through cell-cell contact

Although stromal cells may modulate self-renewal and differentiation by the release of diffusible growth factors, there is evidence that cell-cell contact is important. To study the influence of thymic-derived stromal clones on A4 development, these cells were cultured on monolayers of irradiated stromal cells. This allowed cell-cell interactions to occur and the A4 response to local conditions could be monitored.

Post-irradiation of the stromal cells, A4 cells were added and left for 19 days after which the cells were analysed by a number of assays. Firstly, samples of A4 cells were collected from each stromal culture and stained with May-Grunwald Giemsa in order to study the cell morphology (materials and methods, section 2.8.1). Secondly, the cells were stained for non-specific esterase activity (materials and methods, section 2.8.2), typical for cells of the monocyte/macrophage lineage. Thirdly, each sample was used in a yeast-uptake assay (materials and methods, section 2.14). This assay provides an easy read out system for identifying MΦ. Hence, differentiated MΦ engulf dyed yeast particles and the number of MΦ can be estimated from prepared cytospins. Finally, A4 cells were stained in cell suspension with mAbs specific for haemopoietic cells (CD45), MΦ (Mac-1) and T-cells (CD3, CD4 and CD8) (all antibodies are listed in table 1.1). The results obtained from these assays are summarised in table 7.1.

All A4 cells recovered from stromal cell co-cultures and control cultures with Wehi-3B expressed CD45 (data not shown). None of the clones were able to induce T-cell differentiation documented by the lack of reactivity with T-cell antigens such as CD3, CD4 and CD8 (data not shown). In contrast, all clones tested were able to induce myeloid differentiation. As shown in table 7.1 each stromal cell line appeared to preferentially induce different myeloid differentiation pathways. The SV40tsA-derived clone 6.10 could maintain ~5% of the cells at an undifferentiated stage. Most of the
remaining cells (95%) matured into macrophages. These results are demonstrated graphically in figure 7.2. In the co-culture with clone 7.5, the majority of A4 cells were stimulated to mature to macrophages, indicated by a >99% reactivity with Mac-1 (data not shown), non-specific esterase activity and morphology (figure 7.2). In contrast, 60% and 40% of the cells recovered from the 15.5 and 15.18 cultures, respectively, had a typical neutrophil morphology (table 7.1 and figure 7.2). These results suggest that one or more of the colony stimulating factors are being produced by the thymic-derived clones listed in table 7.1. Because the majority of the cells matured into macrophages and/or neutrophils it is tempting to propose M-CSF and GM-CSF and perhaps G-CSF, to be responsible for the development of A4 cells in our co-cultures. Another possibility, is that IL-6 is responsible for some of these activities since IL-6 has been shown to be produced by several thymic stromal elements and is known to contain CSF-activity (Murray et al., 1989; Ogata et al., 1989; Wong et al., 1988).

Despite the failure of established clones to stimulate A4 differentiation towards the T-cell lineage, the possibility of such an event could not be ruled out. To test this feasibility A4 cells were seeded into alymphoid foetal thymic lobes, prepared by dGuo-treatment. Although several attempts were conducted both with and without IL-3, no recolonisation was observed (data not shown). This indicates that in these circumstances A4 cells do not contain the capacity to repopulate the thymus. This finding is in agreement with in vivo experiments performed by E. Timms and colleagues, where A4 cells did not restore thymopoiesis in irradiated mice (personal communication E. Timms).

7.2.3 Thymic-derived stromal cells can promote growth of CD4−CD8−adult thymocytes

Adult thymocytes were depleted of CD4, CD8, Mac-1 and Ia expressing cells (materials and methods, section 2.3.5 and 2.3.6) in order to enrich for CD4−CD8− thymocytes. Although the separation was repeated twice, a contamination of ~12% CD4 expressing
Table 7.1 Ability of stromal cell lines to induce differentiation of A4 cells.

a) A4 cells were cultured on irradiated monolayers of stromal cells. The percentage of undifferentiated A4 cell, macrophages and neutrophils recovered from each stromal cell co-culture after a 19 day culture period is shown. The percentages are mean values from four different assays; May-Grunwald and Giemsa, yeast-uptake assay, non-specific esterase activity staining and Mac-1 staining.
Figure 7.2 A4 cells cultured on irradiated 6.10, 7.5 and 15.5 cells. Cytospin preparations of A4 cells stained with May-Grunwald and Giemsa, A4 cells from yeast-uptake assay and non-specific esterase activity staining.
Figure 7.3 Fluorescence profile of adult thymocytes at day 0. Adult thymocytes were depleted of CD4 and CD8 expressing cells by antibody and complement followed by panning (materials and methods section 2.3.5 and 2.3.6). Separated cells were stained with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 mAbs and analysed on the FACScan.
cells was present in the final population (figure 7.3). The cells were co-cultured with stromal cells with and without IL-7. At various time intervals thymocytes were recovered and the number of viable cells estimated.

Figure 7.4 demonstrates the cell recovery from one tsA-derived clone (6.10), three E1a-derived clones (8.40, 15.5 and 15.18), a non-thymic derived fibroblast line and from medium control cultures. In the medium control cultures (with and without IL-7) no viable cells were detected after 4 days. Thymocytes co-cultured with the skin fibroblast line tsA-8 did not survive beyond one week. In contrast, thymic-derived stromal cell lines were able to support proliferation at different levels. In all cases the cell-recovery at each time-point was greater in the presence of IL-7. During the first week in culture the number of viable cells decreased significantly when no IL-7 was added. This decrease was also observed in cultures supplemented with IL-7, but to a lesser degree. One explanation for these observations could be that certain cells remained non-dividing for a week while a selective cell-death of other subsets occurred. Alternatively, certain subsets may have divided but not sufficiently to 'replace' those which died, resulting in a lower cell-recovery. In contrast to clone 6.10 and 8.40, clone 15.5 (with IL-7) and 15.18 demonstrated almost no change in the number of cells recovered (compared to the start-population) during the first 7 days. However, after one week in culture an increase in cell-proliferation could be observed in all thymic-derived co-cultures. The enhanced growth between day 7 and 11 was most dramatic in co-cultures with 8.40 and 15.5 (-IL-7). It appeared that once the cells began active proliferation, subsequent growth was characterised by continuous doubling of the population. The most extensive proliferation was seen in 15.5 and 15.18 co-cultures supplemented with IL-7, where the cell recovery increased 6 and 5 fold, respectively, over the eleven day culture period.
Figure 7.4 Growth of adult CD4<sup>-</sup>CD8<sup>-</sup> thymocytes in co-culture with thymic stromal cell lines with and without IL-7. Thymocytes from BALB/c mice (2x10<sup>4</sup>/well) were cultured in 48-well plates with stromal cells (5x10<sup>3</sup>/well) and medium only. Culture supernantant from IL-7-secreting COS-7 cells were used as a source of IL-7, diluted 1:100 (15.5U/ml). At each time point thymocytes were recovered by vigorous pipetting and the number of viable cells determined by trypan blue exclusion. Cell recovery in the presence of IL-7 (■-■) and in the absence of IL-7 (□-□). Each value represents a mean value from triplicate cultures, from one experiment.
7.2.4 Changes in cell surface markers during co-culture

Thymocytes co-cultured with thymic stromal cells were tested for changes in the expression of several antigens which are known to be involved in T-cell differentiation. Figure 7.5 demonstrates changes in CD4^-CD8^- adult thymocytes of the expression of CD4, CD8, CD3, HSA and Pgp-1, after co-cultivation with clone 15.18 in the presence of IL-7. The thymocyte population recovered from clone 15.18 showed an enrichment of DN cells, from 88% (top left panel, day 0) to 97% (top right panel, day 11). Sampling of cells grown on 15.18 at day 4 and day 7 revealed that the cells did not express CD4 or CD8 at any time tested. Similar results were obtained with clone 15.18 without IL-7 and with clones 6.10, 8.40 and 15.5 in the presence of IL-7. Furthermore, an increase of CD3 expressing cells was observed during co-culture with 15.18 (from 49% to 75%). From these data it cannot be determined whether the increasing number of CD3^+ cells is simply an outgrowth of that population and/or perhaps selective death of the CD3^- cells or alternatively, whether the CD3^- population present at day 0 acquired CD3 during culture. Preliminary experiments indicated that cells recovered at day 11 were not able to recolonise dGuo-treated lobes in vitro. The majority of the cells at this time point are DNCD3^+, a thymocyte subset lacking precursor activity in in vivo transfer experiments (Crispe et al., 1987). However, it appears that some cells recovered at day 11 display a more immature (DNCD3^-) phenotype which conceivably could contain cells with thymus colonisation capacity.

Heat stable antigen positive cells increased in these cultures. Previous studies have shown that HSA^+ cells within the DN subset (not yet expressing CD3) contain precursor activity while the HSA^- population lack this activity and usually express CD3 on the surface (Crispe et al., 1987; Ewing et al., 1988; Pearse et al., 1989). These findings do not seem to correlate with the most common cell type found in our cultures which seems to be DNCD3^+HSA^+. It is interesting to note that co-culture with thymus- derived stromal cells (with or without IL-7) appear to promote cells expressing
HSA at a moderate level rather than with high intensity as observed at day 0. The existence of such a DN population with low levels of HSA has been reported by Crispe et al., (1987), Ewing et al., (1988) and Pearse et al., (1989). In this study the HSA\textsuperscript{low} subset contained 60% CD3 expressing cells compared with 6% within the HSA\textsuperscript{high} fraction and precursor activity was only found in the CD3\textsuperscript{+} fraction. This could explain why no recolonisation was achieved with cells recovered at day 11 (data not shown) since the CD3\textsuperscript{-}HSA\textsuperscript{+} subset presumably was present at very low levels in our cultures.
Figure 7.5

DAY 0 | DAY 11

+IL-7

**CD4-PE**

**CD8-FITC**

**anti-rat FITC**

**CD3 (KT3 1.1)**

88% 49% 75%

53% 95%

**HSA (YMB10)**

54% 84%

**Pgp-1 (IM7.8.1)**
Figure 7.5 Phenotypic analysis of thymocytes before and after co-culture with clone 15.18 in the presence of IL-7. Freshly isolated thymocytes or thymocytes co-cultured with clone 15.18 for 11 days with IL-7 were analysed by flow cytometry (materials and methods section 2.6).
In addition to CD3 and HSA, the percentage of Pgp-1+ cells also increased during co-culture, from 54% to 84%. HSA and Pgp-1 have generally been described to have an inverse relationship in adult DN thymocytes where the majority of HSA+ cells do not express Pgp-1 and cells lacking HSA express Pgp-1 (Wilson et al., 1988a). This pattern does not seem to apply to the cells recovered from described co-cultures where the majority of the cells express both HSA and Pgp-1. However, the outgrowth of this population may be an effect of IL-7 since Pgp-1+HSA+ thymocytes in foetal thymic lobe submersion cultures have been shown to grow in response to IL-7 (Watson et al., 1989).

Although the cell recovery from co-cultures without IL-7 was less than from those supplemented with IL-7, no significant differences in cell surface expression could be observed (data not shown). If this result is an effect of IL-7, one can infer that the thymic-derived stromal cells may secrete IL-7, but at lower levels than was supplied exogenously. However, no thymocytes survived for more than three days in IL-7 supplemented cultures without stromal monolayers which suggests that IL-7 alone is not responsible for thymocyte proliferation but rather that the stromal cells themselves play an important role in inducing the effect. First, perhaps IL-7 binds to the stromal cells and/or extracellular matrices produced by them and is hence more readily available for the thymocytes. Second, IL-7 may induce secretion of other factors by the stromal cells which can influence the thymocytes.

### 7.2.5 Growth effect of IL-7 on CD4-CD8-CD3+ foetal thymocytes in co-culture with clone 15.5

A similar experiment to those described in section 7.2.4 and 7.2.5 was performed with day 15-16 foetal thymocytes. A single cell suspension of foetal lobes was prepared by homogenising intact lobes (materials and methods section 2.13.3). As shown in figure 7.6, 97% of the cells did not express either CD4 or CD8 and <1% expressed CD3. In
this experiment we decided to focus on cell line 15.5, which had been shown to be efficient in promoting proliferation of adult DN thymocytes.

Thymocyte proliferation was monitored at different time points during a 20 day culture period. As shown in figure 7.7 (a) the growth profile resembled that of adult DN thymocytes. Seven days after seeding the number of viable cells recovered had not changed significantly where IL-7 was added while a ~4 fold decrease could be observed in the absence of IL-7. However, after one week in culture, a dramatic proliferation occurred and between day 7 and day 20 the number of cells had increased ~5 fold in the presence of IL-7 and ~3 fold in the absence of IL-7. Figure 7.7 (b), (c) and (d) show the 15.5+IL-7, the 15.5-IL-7 and the IL-7-medium control culture, respectively, at day 13.

7.2.6 Phenotypic analysis of foetal thymocytes in co-culture with 15.5

Co-culture of clone 15.5 and the most immature foetal thymocyte subset, CD4-CD8-CD3+, promoted thymocyte growth and maintenance with and without IL-7. In order to analyse whether the phenotype of these cells changed during culture, the cells were initially examined for CD4 and CD8 expression after 4, 7, 13 and 20 days of culture, using two colour flow cytometry. Freshly prepared day 15-16 foetal thymocytes, used to initiate these cultures, consisted of 97% DN cells, shown in figure 7.8 (top left panel). The CD4-CD8- thymocytes remained CD4-CD8- during 20 days in culture (with or without IL-7) at all time points tested. Figure 7.8 top four FACS-profiles showing the staining result at day 13 (with IL-7) and day 20 (with and without IL-7) clearly demonstrate this finding.

Further analysis of other cell surface antigens was also performed and the data at day 0, 13 and 20 of culture are summarised in the rest of figure 7.8. Cells expressing CD3, which is almost completely absent at day 0 (~1%), appeared to increase during co-
culture. The presence of IL-7 resulted in a recovery of ~16-17% CD3 positive cells at day 13 and day 20. This increased CD3 expression was slightly greater in the absence of IL-7 (day 20) where ~26% CD3+ cells were recovered. Since the CD3+ subset was very small in the initial population it is tempting to suggest that some of the cells have converted to express CD3 during culture and that this can occur without added IL-7. Preliminary staining experiments have indicated that both αβ and γδ-TCR expressing cells are present in the CD3+ population (data not shown).

The majority (97%) of day 15-16 foetal thymocytes expressed HSA at high levels (figure 7.8). However at day 13 this expression had ceased almost entirely and only 8% were found to be HSA+. At day 20, both with and without IL-7, a slight increase of HSA expression was obtained. A reverse pattern was observed for the expression of Pgp-1. At day 0, 25% of the thymocytes expressed Pgp-1 and during culture the majority (96-99%) of the cells converted to a Pgp-1+ phenotype both with and without IL-7.

Since the thymocytes cultured in the presence of IL-7 have been shown to contain subsets which can respond to IL-2 and become cytolytic (Watson et al., 1989; Widmer et al., 1990) it was of interest to determine the levels of IL-2R expression in these cultures. Day 15-16 foetal thymocytes displayed an IL-2R staining profile consisting of two peaks, one negative (66%) and one positive (54%). This pattern of expression changed during culture with 15.5 and IL-7 and at day 13 and 20 it appeared that the two distinct populations disappeared and it was difficult to separate the cells into two distinct subsets at these time points. From this data it is not possible to determine whether the IL-2R- subset has shifted to an intermediate level of IL-2R expression and/or whether the IL-2R+ cells are maintained in culture and represent both intermediate and high level expressing subsets. However, the percentage of IL-2R+ cells increased to ~70% when the cells were maintained in IL-7. This pattern was not
Figure 7.6 Fluorescence profiles of day 15-16 foetal thymocytes. Freshly isolated thymocytes from cell suspension and analysed on the FACScan.
Figure 7.7 Growth effect of thymocytes co-cultured with clone 15.5 with and without IL-7. (a) Thymocytes from day 15-16 of gestation BALB/c embryos were co-cultured with clone 15.5 (1x10^4 cells/well) at 2x10^4 cells/well in 24-well plates. Cultures with IL-7 were supplemented with supernatant from IL-7 secreting COS-7 cells diluted 1:100 (15.5U/ml). At each time point thymocytes were recovered by vigorous pipetting and the number of viable cells determined by trypan blue exclusion. Cell recovery in the presence of IL-7 (■-■) and absence of IL-7 (□-□). Each value represents a mean value of triplicate cultures. (b) Co-culture of 15.5 and day 15-16 foetal thymocytes with IL-7 and (c) without IL-7 at day 13. (d) day 15-16 foetal thymocytes culture in medium supplemented with IL-7 at day 4.
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<td>17%</td>
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<td>72%</td>
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<tr>
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Figure 7.8 Phenotypic analysis of thymocytes before and after co-culture with clone 15.5, with and without IL-7. Freshly isolated thymocytes or thymocytes co-cultured with clone 15.5 for 13 and 20 days with and without IL-7 were analysed by flow cytometry (materials and methods section 2.6).
obvious in the cultures without IL-7, where the IL-2R+ did not increase and the FACS profiles appeared similar to day 0. Thy-1 was expressed by >92% of the cells at day 0. After co-culture a reduction in Thy-1 expression was observed. Similar profiles are demonstrated at day 13 and 20 (with IL-7) although the expression was slightly lower in culture without IL-7 at day 20.

### 7.2.7 Formation of stromal cell aggregates which could provide an environment for developing thymocytes

It has been shown by Owen and colleagues that foetal thymus lobes disrupted through enzyme digestions can reaggregate and form 'new lobes' (unpublished data). To explore the possibility of using characterised cell lines for the purpose of reconstructing enclosed microenvironments, two different methods were employed. First, single cell suspensions of individual clones or a mixture of different cell lines (~1500 cells) were incubated overnight in a hanging drop culture to allow contact and thereafter cultured on Nuclepore filters floating in medium. Second, single cell suspensions of individual clones or a mixture of cell lines were plated as 10μl drops (~1500 cells/drop) onto Nuclepore filters floating in medium (drop cultures).

In initial experiments the capacity of individual cell types to form aggregates was investigated. Table 7.2 summarises the results using both methods of aggregation, hanging drop cultures and drop cultures. All clones except the E1a-derived clones 8.40 and 14.5 formed aggregates resembling thymic lobes. When the cells were incubated as a hanging drop culture only one lobe-like structure was generated while cells layered as a drop on filters in several cases formed multiple aggregates. In most cases as the lobe-like structures grew, daughter lobes were produced and hence could be expanded in numbers. Aggregates formed in this way are shown in figure 7.9. When multiple cell types were mixed, similar structures were generated.
This way of mixing different thymic stromal cells provides an opportunity to examine whether cells of different origins will organise themselves in a particular fashion in the lobe-like aggregates. In other words, can cells representing fibroblasts, epithelial cells and, maybe macrophages, form aggregates and have the same or similar cellular distribution to normal thymus. In an attempt to investigate this possibility aggregates composed of different stromal clones were snap-frozen, sectioned and stained with haematoxylin-eosin to study the morphology. Aggregates at day 4 and day 9 and sections of a 'lobe' composed of clones 5.10, 15.5 and 15.18 are shown in figure 7.10 (a-d). Cells located around the edges of the structure seem to run alongside, forming a type of capsule. Inside the structure, the cells appear healthy and cell-cell contact can be observed. These preliminary results suggest that formation of lobe-like structures in this way could perhaps provide a system where the contribution of individual cell-types could be analysed. However, this system requires that each cell-type can be easily identified in the aggregates by using specific cell surface markers or other methods of detecting and distinguishing between cell-types.
Table 7.2 Abillity of SV40tsA and Ela-derived thymic stromal cell lines to form lobe-like aggregates

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<td>clone</td>
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<td>15.5^{20}</td>
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Figure 7.9 day 4

5.10

6.10

8.40

15.5
Figure 7.9 Light microscope photographs of aggregates formed by thymic stromal cell lines. Cultures at day 4 and day 9 after plating from clones 5.10, 6.10, 8.40 and 15.5.
Figure 7.10 Aggregate composed of clones 5.10, 15.5 and 15.18. Light microscope photograph of aggregates at day 4 (a) and day 9 (b) after plating. A section of a aggregate stained with haematoxylin-eosin (c and d).
7.3 DISCUSSION

A panel of SV40tsA- and E1a-derived thymic stromal cell lines have been analysed with respect to their capacity to support growth and/or differentiation of different precursor cell populations. Experiments using A4 cells yielded some information regarding what haemopoietic growth factors may be produced by the immortalised stromal cells. Self renewal of A4 cells, which can be maintained by culturing the cells in IL-3, was not achieved when supplied with conditioned medium from the stromal cell lines. However, when A4 cells were co-cultured with the stromal clones for 7 days using soft agar as a barrier for cell-cell contact, a limited capacity for maintenance was observed. This would suggest that the stromal cells probably do not secrete IL-3 but perhaps an unidentified factor which is responsible for A4 growth. However, since no phenotypic analysis was performed of the recovered A4 cells after this culture period, it is not possible to conclude whether the cells self-renewed or perhaps differentiated during these conditions. Nonetheless, during co-culture with stromal clones when cell-cell contact was permitted, differentiation of A4 cells occurred. This is presumably a result of haemopoietic growth factors produced by individual cell-types. Although the exact nature of these factors cannot be determined, it is interesting to note the following (i) cell lines 5.10 and 7.5 which were considered to be of fibroblastoid origin in chapter 5 and 6 appear to support differentiation of mainly macrophages, (ii) 6.10 and 8.40 which are both of embryonic origin and may be epithelial-like cells show similar capacity for promoting A4 differentiation and (iii) clones 15.5 and 15.18 isolated from adult T-ROS/TNC cultures preferentially support macrophage and neutrophil differentiation. Taken together, stromal elements of fibroblastoid nature may be limited to produce only M-CSF while other cell types like epithelial cells can secrete a wider range of growth factors like M-CSF, GM-CSF and perhaps G-CSF and IL-6. Furthermore, studies by (Metcalf 1987) showed that not only the type of CFS but also the concentration influence the fate of progenitors. Therefore, the data obtained may be
a result of A4 cells exposed to different concentrations of the CSF secreted by a stromal cell line rather than different CSFs.

The failure to stimulate A4 cells to grow and undergo T-cell differentiation in thymic organ cultures suggests that A4 cells are not multipotential but rather are limited to the myeloid and erythroid lineages. Alternatively, perhaps A4 cells represent early BM-derived cells which require some sort of stimulation (still unidentified) prior to those provided by the thymus in order to undergo T-cell differentiation.

Co-culture of thymic stromal clones with adult (CD4^-CD8^-) and day 15-16 foetal (CD4^-CD8^-CD3^-) thymocytes resulted in thymocyte growth and addition of IL-7 to such cultures enhanced the proliferation. This effect of IL-7 on the viability has been demonstrated previously (Watson et al., 1989). However, cells in medium control cultures responded poorly to IL-7. This finding indicates that the presence of stromal cell monolayers created culture conditions which increased the IL-7 induced response. From these experiments it is not possible to determine whether the increased thymocyte growth is a direct consequence of IL-7 or whether IL-7-induced secondary events are responsible for the increased proliferation. That thymocyte growth was promoted in the absence of IL-7, implies that stromal cells on their own have the capacity to support thymocyte proliferation either by secreting IL-7 or other factors with similar activity. In this context it is of interest to determine whether cell-cell contact is crucial or if soluble factors alone can mediate the effect. This could be tested by using a transwell system where no cell-cell contact is permitted. Furthermore, to test the effect of IL-7 on the stromal clones in terms of induction of other factors, conditioned medium could be collected from cells cultured with and without IL-7 and tested for the presence of various cytokines.

Clones 15.5 and 15.18 showed similar abilities to support growth, suggesting that since they originate from the same type of culture (T-ROS/TNC) they may have similar
characteristics. The tsA-derived clone 6.10 and clone 8.40 showed a more limited capacity in co-culture compared to 15.5 and 15.18. Since 6.10 is thermolabile for growth, these cells may have been affected by being cultured at 37°C which is in between the permissive (33°C) and non-permissive (39°C) temperature. Perhaps 6.10 cells are not capable of staying metabolically active during longer culture periods at 37°C and therefore display a more restricted potential for inducing thymocyte growth. Preliminary findings indicate that a possible temperature dependent effect can be avoided by serial transfer of the thymocytes to new monolayers of stromal cells.

A number of phenotypic changes occurred during co-culture with adult and foetal thymocytes. The cell populations recovered from cultures with and without IL-7 showed nearly identical expression of a variety of surface antigens. The most prevalent adult thymocyte populations expanded in culture lacked CD4 and CD8 and expressed CD3, HSA and Pgp-1. According to the expression of surface CD3 this population can be classified as mature, although HSA and Pgp-1 have been shown usually to be present on immature DN thymocytes not yet expressing CD3. However, this population has been observed previously in normal adult thymus but at low levels (Papiernik and Pontoux 1990). Furthermore, it is not clear whether this population (DNCD3+HSA+Pgp-1+) is part of a mainstream differentiation pathway or whether it is a cell type promoted to expand during these particular culture conditions. Perhaps this population represents a subset which is usually eliminated because of failure to mature further. This hypothesis could be tested by adding anti-CD3 antibodies to see whether CD3 engagement would induce apoptosis.

In an attempt to determine whether the outgrowth of CD3+ cells observed with adult thymocytes was due to proliferation of CD3+ cells or differentiation of CD3- cells, foetal thymocytes lacking CD3, CD4 and CD8 were used in subsequent experiments. The most frequent foetal thymocyte subset expanded during co-culture displayed a phenotype different from adult thymocytes proliferating in these type of cultures. It
appeared that certain CD3- cells had differentiated to express CD3 (16-26%) both with and without IL-7. However, the majority of the cells remained CD3-, lost the HSA antigen and became Pgp-1+. Studies with adult thymocytes have suggested that as cells mature within the DN subset, Pgp-1 is lost while HSA is still present on the majority of CD4-CD8- cells (Egerton et al., 1990). Day 15-16 foetal thymocytes cultured with clone 15.5 do not seem to have followed this general differentiation pathway. In contrast, they seem to represent a population that has not been described in any great detail previously. However, since most studies concerning differentiation pathways of the DN subset have been performed on adult thymocytes, it is conceivable that they are products of embryonic precursor cells which differ phenotypically from those of adult origin.

Another phenotypic effect observed during culture is the reduction in Thy-1 expression from 97% high expressing cells at day 0 to a mixture of Thy-1-, Thy-1low and Thy-1high populations at day 11 and 20. Decreased Thy-1 expression within the thymus has been taken as a manifestation of maturation from the stage of immature thymocytes to the more mature cortical and medullary thymocytes (Scollay and Shortman 1983). Therefore, the change in level of Thy-1 expression during co-culture may indicate that maturation took place.

Interleukin-2 has been shown to stimulate growth and functional activity in thymocyte cultures previously exposed to IL-7 (Widmer et al., 1990). Furthermore, transient expression of IL-2R within the DN subset has been shown to mark a developmental stage at which the TCRβ-chain gene rearrangement and the initiation of TCRα and β-chain gene expression occurs (Pearse et al., 1989). Since the majority of cells after co-culture express IL-2R, the effect of adding IL-2 to these culture should be examined and furthermore, to determine whether the cells expanded in these cultures have any of their TCR genes rearranged, mRNA analysis and TCR gene probing of the cells at day 0 and during culture would be necessary. In addition, by double and triple staining
the relationship between the CD3, HSA, Pgp-1 and IL-2R antigens on the expanded thymocyte populations could be established.

Taken together, perhaps the co-culture of clone 15.5 with and without IL-7 mediated an effect on the thymocytes at a certain stage during differentiation, and in order for the thymocytes to mature further in these cultures (e.g. to express CD4 and/or CD8), other stromal cell types may be required. It would, therefore, be of interest to use stromal clones with characteristics different from 15.5 and to analyse the outcome.

In relation to both A4 and thymocyte co-cultures it would be of interest to add other cytokines important in lymphopoiesis, such as IL-1 and IL-4. Furthermore, since IFNγ has been shown to induce MHC antigens on stromal cells the influence of IFNγ treatment prior to co-culture should be tested.

The advantage of studying the effect of one stromal cell type or one factor on thymocyte development is that different stages during intrathymic differentiation can perhaps be delineated. However, to expose cells to only one type of stimulant may result in a different response compared to normal circumstances, when the developing thymocytes are in a complex environment. Therefore, a model in which the effects of several stromal cell lines on developing thymocytes could be tested, was explored. Preliminary results showed that stromal cell lines can aggregate and form lobe-like structures. Since very few stromal cell-related antigens are expressed by our cell lines it would be of interest to examine whether the formation of lobe-like structures induces any changes in antigen and intermediate filament expression. The next step would be to mix different subpopulations of thymocytes (or other sources of precursor cells) with one or several cell lines and form aggregates to see whether the contribution of selected stromal cells in T-cell differentiation can be evaluated.
CONCLUSIONS AND FUTURE PERSPECTIVES

The first part of this thesis relied on the already established mouse foetal thymic organ culture system as a model for studying T-cell development (chapters 3 and 4). The second part described the establishment of thymic stromal cell lines by the use of SV40 and E1a as immortalising agents, characterisation of these cell lines and investigation of the possibility of using them as accessory cells for studying T-cell lymphopoiesis in vitro (chapters 5-7).

Haemopoietic stem cells are found at different sites during development; the embryonic yolk sac, foetal liver and adult bone marrow. The lineages of stem cells or prothymocytes in foetal and adult life which are destined to home to the thymus in order to generate and maintain T-cell development, have not been well characterised. Furthermore, it is not clear whether pluripotent stem cells, with the capacity for self renewal seed the thymus directly or whether T-cell committed stem cells, with a limited capacity for self renewal, are generated in the foetal liver/adult bone marow before thymus homing. A number of approaches such as retroviral marking and cell separation techniques have been used to document cells with multipotential capacity for differentiation in vivo. Equivalent studies in vitro have been limited although defined culture conditions, supplemented with purified growth factors or stromal cells, have allowed investigation of erythroid, myeloid and B lymphoid differentiation. The T-cell differentiation process remained difficult to study until the mouse foetal thymic organ culture system was developed. In this system maturation of different T-cell lineages could be followed by characterisation of cell-surface antigens as markers for different stages in development.

Although the thymic organ culture system has previously been used for documenting precursor activity among thymocyte populations, no analysis has been carried out in order to determine at what frequency cells of different origins can colonise dGuo-treated thymic rudiments. Limiting dilution analysis (chapter 3), proved useful in
determining repopulation activity in adult and foetal populations. By titrating different unfractionated and fractionated cell populations it was possible to estimate the relative abundance of precursors within a given population and also to quantitate small differences between populations in the ability to colonise the thymus.

Do stem cells with self renewal capacity exist in the thymus? and if so can they be expanded in vitro by special culture conditions? Endpoint titrations pursued in chapter 3 would not be an adequate way to distinguish between colonisation by one cell with high proliferative capacity and colonisation by more than one cell with less proliferative potential. However, by combining limiting dilution with continuous repopulations into alymphoid lobes different precursor populations could be screened for cells with high replicative capacity (chapter 4).

The results obtained from serial passaging suggest that mature (CD4⁺CD8⁻CD3⁺) and immature (CD4⁺CD8⁻CD3⁻) subpopulations and in addition a population of CD4⁺CD8⁺ cells (which could be CD3⁻ and /or CD3⁺) of foetal origin (liver and thymus) can be expanded in these cultures. From these studies it is not possible to determine whether they are related through precursor-progeny relationships or whether they have developed as separate lineages from several precursor cells. Since genes such as the TCR genes in the haemopoietic system are developmentally regulated, it would be useful to isolate and characterise the status of TCR β and γ-genes which are the first to be rearranged during T-cell maturation and neccessary for proper lineage expression. This type of analysis could perhaps elucidate a precursor-progeny relationship between certain expanded subsets or show whether they are dead-end products of the developmental sequence, which have failed to mature to single CD4 and in some cases to single CD8 expressing cells. A second approach which may yield insight into the relationships and differentiative potential of these populations could be to isolate each particular population through cell sorting and to subsequently attempt their expansion and/or demonstrate their in vitro multipotentiality by exposing them to other special
culture conditions. These could involve supplement of growth factors or co-culture with stromal elements. A third approach could involve retroviral marking of precursor cells introduced into alymphoid lobes. This labelling technique would allow different thymocyte 'clones' to be identified by their unique viral integration site and hence, their fate to be followed during multiple colonisations. A fourth approach could be to seed these cells singly into dGuo-treated lobes and characterise the progeny and in addition, investigate whether cells enriched by serial passage *in vitro* contain the potential for *in vivo* reconstitution of irradiated recipients.

Furthermore, it would be of interest to see whether the continuous recolonisation observed for up to 6 passages with unfractionated foetal liver and thymocytes could be achieved with other selected populations. Thus, the CD4^{low}CD8^{-} thymocyte population isolated from adult thymus (0.05%) (Wu et al., 1991), the foetal liver population defined by the mAb AA4.1 (0.5-1%) (Jordan et al., 1990) and the adult bone marrow population isolated by (Spangrude et al., 1988), utilising a series of cell surface markers, which have been shown to be enriched for T-cell precursor activity (Wu et al., 1991) and multipotential capacity (Jordan et al., 1990; Spangrude et al., 1988) would be interesting targets for serial colonisations into alymphoid lobes *in vitro*.

The mouse foetal organ culture and *in vivo* transfer experiments allow differentiation of mature T-cells from early progenitors, and by studying the differentiation process at various stages, cell lineages can be implied. However, in order to study directly the development of distinct T-cell lineages in which the precise influence of different stromal cells in the microenvironment can be evaluated a simpler assay system is required. Therefore, one aim of this thesis was to establish an *in vitro* system in which the contribution of distinct stromal components could be studied. To achieve this goal, retroviral constructs were utilised to generate immortalised cell lines from foetal and adult thymus.
By studying the characteristics of established cell lines (chapter 5 and 6) the majority generated by SV40tsA were assigned to a fibroblastic lineage. However, E1a appeared to have the capacity for immortalising a larger variety of cell types both from primary and enriched TNC/T-ROS cultures. The results obtained from this study, in conjunction with other reports where stromal clones have been isolated, (table 5.2 and (Cattermole et al., 1989)) suggest that in order to establish cell lines representing all stromal cell lineages a number of approaches have to be combined, (i) continuous culture of primary as well as of selected, separated populations (such as antibody binding and panning and TNC/T-ROS isolation), (ii) immortalisation of different types of cultures with different virus constructs, since each virus appears to infect preferentially certain cell types, (iii) combination of cell separations with addition of growth factors which have been shown to enhance growth of a particular stromal cell type (e.g IL-1 and GM-CSF enhance growth and maturation of dendritic cell precursors in vitro), (iv) continuous culture and/or retroviral infection of stromal cells from adult as well as from different gestational days of embryonic thymus since the make-up of the microenvironment may change during development and by growing cells from only one time-point the selection may be limited and (v) isolation of stromal cells from transgenic animals such as the tsA mouse (Jat et al., 1991). With the use of antibodies this would obviously be an efficient way to isolate different populations from the thymus already immortalised and ready for use.

The binding of mainly DP thymocytes to clones 15.5 and 15.18 was described in chapter 6. However, during special conditions, such as after IFNγ treatment of stromal cells and/or after culturing of SV40tsA-derived clones at the non-permissive temperature, adhesion with other isolated clones was induced and/or enhanced. These findings suggest that adhesion can be facilitated by a number of mechanisms.

To study the nature of cell-cell interactions observed under different circumstances ('normal', after IFNγ induction and after temperature switching) a number of methods
could be employed. Firstly, determine the molecules involved in adhesion by antibody blocking. Secondly, phenotype the cells bound, to see whether different conditions attract certain thymocyte subsets (DP, DN and SP), which could perhaps tell us something about the stage at which a particular stromal cell type is important for T-cell differentiation. And finally, investigate whether adhesion involving different molecules and cell populations (stroma and thymocytes) mediate specific functional effects. This could include a number of studies such as (i) to determine whether binding can induce thymocytes to differentiate further or to be eliminated, (ii) to investigate if certain cytokines are produced as a result of an interaction, and (iii) to see whether rosettes formed at 4°C after being cultured at 37°C will form multicellular complexes in the form of TNC. This phenomenon has been described in a thymic epithelial cell line which was shown to enclose thymocytes in vitro and induce apoptosis of the engulfed cells (Hiramine et al., 1990).

Although the antibody blocking studies in section 6.2.2 are preliminary, there is an indication that CD8 and, therefore, perhaps class I are involved in the adhesion process between adult thymocytes and clones 15.5 and 15.18. However, adhesion between CD8 and class I is not considered to be of high affinity and therefore, this finding raises a number of questions; Why can binding between these two molecules be detected if it is of low affinity? Does antibody bound to CD8 downregulate other adhesion molecules neccessary for binding? Does CD8 associate with other molecules on thymocytes (e.g CD8 and CD1 in man) (Ledbetter et al., 1985) which can have an effect on adhesion?

Experiments in chapter 7 made use of established stromal cell lines as accessory cells for studying haemolymphopoiesis as a step towards the goal of establishing in vitro culture systems for T-cell differentiation. In these experiments a bone marrow-derived stem cell clone (A4) and adult /foetal DN thymocytes representing early precursor cells were co-cultured with stromal clones. These studies suggest that some of the cell lines
may have the potential to induce the expansion of immature thymocytes *in vitro* and perhaps support differentiation of T-cells at an early stage in development by inducing their expression of CD3. However, to explore the full potential of this *in vitro* culture approach, other cell populations representing different stages in the developmental sequence have to be utilised, such as other thymocyte subsets, foetal liver cells and bone marrow cells. Furthermore, in order to understand the relationships and the differentiative potential of cells at the various branch points in T-cell lineage development, it would probably be an advantage to use well defined populations (by cell surface marker expression) in co-culture with stromal cells rather than unfractionated populations.

Developmentally regulated events, such as TCR rearrangements and changes in cell surface antigen expression are used as markers to document differentiation of lymphoid cells in the thymus. These changes can be identified readily by a range of molecular probes and mAbs. However, the proliferative and differentiative signals provided by the microenvironment have not been well documented. Therefore it will be important to define what cytokines are produced by different stromal cell types and what effect one factor or a combination of factors may have on T-cell development. These studies could involve; (i) probing for cytokine mRNA in normal thymus and among transformed and non-transformed stromal cell lines, to analyse and compare the distribution of different factors, (ii) blocking of cytokine production in co-culture with lymphoid elements by anti-cytokine mAbs, (iii) transfecting an already established stromal cell line with the cytokine of interest (e.g IL-1, IL-4, IL-7 and CSFs), and studying its influence on T-cell differentiation, (iv) studying T-cell differentiation in transgenic mice with a cytokine-encoding transgene and furthermore, establishing stromal cell lines of different lineages from these mice to evaluate transgene expression in different cell types, (v) studying thymus development *in vivo* and isolated stromal cells *in vitro*, from mice with an ablated cytokine gene (e.g IL-2).
The possibility of using immortalised cell lines to restore an enclosed thymic microenvironment (chapter 7) could be explored further. In addition to mixing different cell lines, one possibility of characterising a stromal cell line's behaviour could be to mix an immortalised clone with stromal cells from normal thymus and to identify where in the lobe the clone is placed after aggregation. In the case of SV40-transformed cells, anti-large T mAbs could be used to identify readily these cells among normal untransformed cells.
REFERENCES


