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6. THE ONTOGENY OF THE IMMUNE SYSTEM

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ESTABLISHMENT AND MANAGEMENT OF B LYMPHOCYTE REPERTOIRES

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SUMMARY

This paper focuses on some aspects of the establishment and maintenance of antibody repertoires. We will discuss a developmental program where the perinatal B cell repertoire is established through i) genetic mechanisms imposing the expression of a highly connected, germline encoded idiotypic network ii) a limited somatic diversification process at this stage of ontogeny which guarantee the expression of these germline encoded properties iii) cellular selection favouring clones displaying these properties. In the adult similar genetic constraints appear to apply to the emergent repertoire of pre-B cells of the bone marrow. In contrast to the perinatal period, however, somatic diversification (e.g. N-sequence additions) at this stage is abundant and clones displaying high connectivity appears to be selected against.

INTRODUCTION

A central problem of immunology today concerns the development and control of lymphocyte repertoires. The lymphocyte repertoire consists of individual B lymphocytes expressing antigen receptors (surface or secreted 19), with V-regions generated through recombination of V, (D), and J gene segments during early stages of lymphocyte differentiation. As a consequence of understanding the genetic mechanisms responsible for the generation of 19 diversity, increasing interest has been directed to defining the mechanisms controlling the development and the maintenance of this diversity. By estimating the number of different V, D, J (for 19 heavy chain) and V and J (for light chain) gene segments present in the germline (*Tonegawa*, 1983), it is obvious that the number of potential combinations by far exceed the number of B lymphocytes that are present in a mouse at any time (*Jerne*, 1971). This observation rises the question of on which bases the V-regions represented in the periphery are selected. The rules and mechanisms mediating these processes concerning are in the focus of this review.

GERMLINE ENCODED CONNECTIVITY

It is well established that the B lymphocytes in perinatal and in adult individuals differ considerably with respect to specificity repertoires (*Silverstein* et al., 1963, *Klinman* and *Press*, 1975) and to V gene utilisation (*Yancopoulos* et al., 1984, *Perlmutter* et al., 1985, *Dildrop* et al., 1985). One important

difference relates to the degree of connectivity displayed by the immune system (IS) at different points of ontogeny. Connectivity, measured as mutual recognition between monoclonal IgM antibodies derived from B cells at different stages in ontogeny, has been demonstrated to be considerably higher in foetal liver (FL) and newborn (Nb) spleen as compared with adult (Ad) spleen (*Holmberg* et al., 1986, *Vakil* and *Kearney*, 1986). This V-region connectivity among B cells appears to result in part from the programmed expression of germline encoded V gene specificities and in part from a learning process based upon interactions between the developing B cell clones and different "self"-ligands.

Molecular analyses of B cell hybridomas displaying mutual reactivity have demonstrated the germline origin of the 19 receptors included in such a network of V-region interactions (*Carlsson* and *Holmberg*, 1990, *Carlsson* et al., 1991). Moreover, mechanisms mediating somatic diversification of the junctional region between the V_H, D, and J_H gene segments are minimised early in ontogeny. This is evident particularly for the addition of N-region nucleotides (*Alt* and *Baltimore*, 1982) which are rare in sequences of foetal and neonatal origin but abundant in sequences of adult origin (*Carlsson* and *Holmberg*, 1990, *Holmberg* et al., 1989, *Gu* et al., 1990, *Feeney*, 1990). As argued before, the limited somatic diversification of perinatal V-regions may guarantee the expression of germline encoded specificities in the early IS (*Holmberg* et al., 1989).

In parallel with the functional characteristics of high connectivity, the perinatal B cell repertoire expresses V_H genes in a non-random fashion. Thus, B cells of the FL and the neonatal spleen preferentially utilise V_H genes of the D-proximal V_H gene families (V_H7183, V_HQ52), whereas the repertoire expressed in the adult spleen shows no obvious bias in this respect (*Yancopoulos* et al., 1984, *Dildrop* et al., 1985, *Jeong* and *Teale*, 1988, *Freitas* et al., 1989). A non-random V gene utilisation may result from mechanisms favouring (or disfavouring) certain V gene segments during the process of V(D)J assembly at the early pre-B cell stage. Alternatively, certain V(D)J rearrangements may be intra- or inter-cellularly selected on the basis of their specificity. Evidence has accumulated during the last few years suggesting that mechanisms of both types contribute to the establishment of the mature B cell repertoire.

INTRINSIC RATES OF V(D)J REARRANGEMENTS

The first observations demonstrating a non-random utilisation of V_H genes in early B cells were derived from analyses of Abelson murine leukaemia virus (A-MuLV) transformed pre-B cell lines, which continuously undergo V_H to DJ_H rearrangements in culture (*Yancopoulos* et al., 1984). Together with similar analyses of FL hybridomas, these studies suggested that the observed bias in the foetal and perinatal B cell repertoires was a result of mechanistic constraints on the 19 gene assembly process, i.e. chromosomal positioning and accessibility to the recombination machinery (*Alt* et al., 1986, *Blackwell* et al., 1986). Clearly, V_H gene families positioned in the proximity of the D region of the IgH locus are preferentially utilised in the process of V_H to D-J_H rearrangements. This preference is most marked for one particular V_H gene of the

V_H7183 family, the V_H7183.1 gene segment (previously denoted 81 X), which in A-MuLV B cell lines is utilised in almost 30% of all rearrangements (*Yancopoulos* et al., 1984, *Reth* et al., 1986, *Lawler* et al., 1987). More recent analyses of V_H gene utilisation using PCR technology to amplify V_H7183 rearrangements from genomic DNA have confirmed these findings (*Carlsson* et al., 1992). Further support for a biased rearrangement machinery was evident by analysing the frequency with which the V_H7183.1 gene occurred in non-productive rearrangements. Non- productive rearrangements are presumable non-selectable and would therefore to a large extent reflect the rearrangement machinery. The analyses of non-productive V_H7183 rearrangements revealed that the relative frequency of V_H7183.1 rearrangements remains constant at about 70% of the total V_H7183 rearrangements, irrespective of organ localisation and the developmental stage (*Huetz* et al., 1992).

In conclusion, these data strongly suggest that mechanistic constraints exist favouring the rearrangement of this gene segment over the other members of the V_H7183 gene family. However, chromosomal positioning cannot be the only factor determining the frequency of individual V_H gene segment rearrangements. The V_H7183.8 gene segment (previously denoted V_HE4.Psi), which is more proximal to the D region in the BALB/c genome, rearranges with a lower frequency than the V_H7183.1 gene segment in AMuLV-transformed pre-B cell lines (*Yancopoulos* et al., 1984) and are rarely found in the V_H7183-DJ_H PCR libraries (*Carlsson* et al., 1992, *Huetz* et al., 1992).

POSITIVE SELECTION OF EARLY B CELL REPERTOIRES

Although mechanistic constraints on the V_H-D-J_H recombination machinery may favour the rearrangement of certain D-proximal V_H genes, cellular selection also appears to contribute to the establishment of the perinatal B cell repertoire. Evidence for positive selection has been obtained from sequencing V_HDJ_H joinings of genomic DNA from B cell populations in perinatal and adult life. Thus V_H7183 rearrangements of adult origin show expected frequencies of out-of-frame rearrangements, whereas essentially all V_H7183 rearrangements of perinatal origin are in-frame (*Carlsson* et al., 1992). Further support for positive selection of B cells in neonatal individuals is indicated by that approximately 30% of the V_H7183.1-DJ_H rearrangements derived from neonatal pre-B cells are productive, while >80% of the V_H7183.1-DJ_H rearrangements are productive in the neonatal B cells (*Huetz* et al., 1992). These observations constitute the first formal evidence for positive selection of precursor B cells during the perinatal period of an individual, and argues against that V_H7183.1 utilising pre-B cells are not able to clonally expand as suggested by *Decker* et al. (1991).

The expansion of B cells during the perinatal period is not a result of intrinsic properties of the cells produced at this point in life since adult B lymphocytes transferred to neonatal recipients behave like the endogenously produced B cells with respect to growth and persistence (*Thomas-Vaslin* et al., 1991). Furthermore, recent analysis of adult mice with severe combined immunodeficiency (SCID) suggest that the ontogenic program of V_H gene repertoires can be "replayed" if these mice are reconstituted with adult BM cells from normal donors. Thus, up to 2 weeks

after reconstitution, SCID mice display a V_H repertoire resembling that of normal, neonatal mice with a considerable part of the 7183.1- DJ_H rearrangements being productive. However, 8 weeks after reconstitution the recipient mice have established a V_H repertoire resembling the normal adult mice.

NEGATIVE SELECTION OF ADULT B CELL REPERTOIRES

In adult life, most mature B cells are believed to be produced from the differentiation of precursor cells in the bone marrow, rather than by division and clonal amplification of pre-existing peripheral B cells. Although the V_H utilisation in adult, peripheral B cell repertoires appears to roughly represent the germline gene complexity of each of the V_H gene families, in situ hybridisation studies show that the intrinsic biases of V_H gene rearrangements in the adult bone marrow are the same or similar to those observed during the perinatal period (*Freitas* et al., 1990). This is further supported by the fact that the ratio of V_H7183.1 to other V_H7183 genes observed among non-functional rearrangements is constant all through ontogeny as previously discussed (*Huetz* et al., 1992).

Similar to the perinatal situation, cellular selection appears to contribute to the modulation of the emerging B cell repertoire. While in the perinatal period, positive selection of B cells predominates the adult repertoire is, in part, formed through negative selection. Examples of the action of such negative selection has been demonstrated in transgenic systems (*Russel* et al., 1991, *Hartley* et al., 1991, *Brombacher* et al., 1991). More recently, a striking example of negative selection of B cells utilising the V_H7183.1 gene segment in adult mice has been reported. This gene segment is utilised in >70 of the functional V_H7183 rearrangements during the perinatal period, while functional rearrangements of this gene is almost absent in adult peripheral organs (i.e. spleen, mesenteric lymphnodes, and Peyer's patches) (*Huetz* et al., 1992, *Decker* et al., 1991). Thus, during ontogeny a negative selection of B cells utilising the V_H7183.1 gene segments occurs. This negative selection of B cells appears to occur during the transition of B cells from the BM to the periphery since approximately 30% of the V_H7183.1-DJ_H rearrangements are productive in the pre-B and B cell compartment of the adult BM (*Huetz* et al., 1992).

IMMUNOPHYSIOLOGICAL REMARKS

Based on the data discussed above it could be hypothesised that the observed positive selection of perinatal B cells occurs on the basis of the properties of connectivity ascribed to these clones. Antigen receptor receptors with properties of "high connectivity" should be more likely to find complementary ligands in an immune system, which at this time is relatively "empty" in terms of antigen receptor specificities. In this context it is interesting to note that Kearney and co-workers who have found that hybridomas expressing the V_H7183.1 gene are among the most highly connected. These Ig molecules binds in ELISA assays other syngenic antibodies and antigens at a high frequency (*John Kearney*, personal communication). The binding to other sur-

face receptor Ig molecules at a high frequency may be a way to trigger other B cells to expand.

Since lymphocyte responses are a function of receptor occupancy the dose response curve is bell shaped, i.e. to few and to many receptors occupied by ligand leads to retention of the cell in the inactivated state (*Varela* and *Coutinho*, 1991), it might be argued that a highly connected antibody (e.g. V_H7183.1 encoded) would be positively selected as long as the B cell repertoire is expanding. Such clones would instead be negatively selected as the system "fills up", due to increasing receptor occupancy. The intrinsic bias of the rearranging machinery together with the minimisation of somatic diversification would guarantee the B cell repertoire to start as a highly connected idiotypic network, and thus give the system the means to establish, in a ordered and controlled fashion, the B cell repertoire.

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B CELL POPULATIONS IN ANTIGEN-FREE MICE

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SUMMARY

B cell populations in germfree mice fed a chemically defined ultrafiltered "antigen-free" diet (GF-CD) and conventional mice fed a diet of natural ingredients (CV-NI) are being investigated by immunophenotyping of the B cells using FACS analysis, and by immunohistological examination. The results show that small, follicular B cells and large, marginal zone B cells can be found in the spleen of GF-CD mice in numbers comparable to those in CV-NI mice. The numbers and sizes of mesenteric lymph nodes and Peyer's patches in GF-CD mice were smaller than in CV-NI mice and no germinal centres were detected. In the peritoneal cavity of GF-CD mice the number of B1 (Ly-1 B) cells is the same as in CV-NI mice and the B1 cells have the same level of IL5-receptor expression in both groups of mice. The most striking difference between GF-CD and CV-NI mice was the very severe reduction of IgA plasma cells in the lamina propria of GF-CD mice compared to that of CV-NI mice.

INTRODUCTION

The B cell repertoire can be subdivided into the potential, the available and the actual repertoire (*Coutinho* et al., 1984). The potential B cell repertoire is being formed by DNA rearrangements of germline genes encoding the heavy (V-D-J) and light (V-J) variable regions of immunoglobulins (*Early* et al, 1980). Only part of this potential Ig repertoire is represented among the available, immunocompetent B cells in the peripheral lymphoid organs. During an immune response some of the available B cells are being selected by antigens to either differentiate into Ig-secreting cells (actual repertoire) or to become memory B cells. By comparing the B cell repertoires in germfree mice fed a chemically defined synthetic diet (GF-CD) with conventional mice given a diet of natural ingredients (CV-NI), we can discriminate the endogenous and exogenous selective forces in the shaping of the B cell repertoire.

In the available repertoire, an influence of exogenous antigenic stimulation can be seen. In conventional mice the available splenic B cells have a seemingly "random" usage of V_H gene families in accordance with the size of the different V_H gene families (*Dildrop* et al., 1985; *Holmberg* et al., 1986; *Schulze* and *Kelsoe*, 1987). This "random" usage is probably antigen-se-

lected, because in adult conventional mice differences in V_H gene usage within a V_H gene family are observed between pre B cells in bone marrow and splenic B cells (*Gu* et al., 1991). In neonatal mice there is a biased usage of the V_H gene families that are most proximal to the constant Ig-region genes (*Holmberg*, 1987; *Bos* and *Meeuwsen*, 1989). A comparable biased usage was seen in adult GF-CD mice (*Bos* and *Meeuwsen*, 1989) and in GF mice (*Freitas* et al., 1991). The "normalisation" of V_H gene family usage in splenic B cells of adult conventional mice is thus apparently influenced by exogenous antigenic stimulation. The available repertoire in conventional neonatal mice and adult GF-CD mice is comprised of many B cells producing multireactive antibodies (*Bos* et al., 1989a). Such antibodies have also been reported to be produced by B1 cells (formerly called Ly1 B cells; *Kantor*, 1991) (*Hayakawa* et al., 1986).

GF-CD mice have a seemingly normal IgM production and a severely reduced production of IgG and IgA, as reflected by total numbers of Ig-secreting cells and serum Ig levels (*Bos* et al., 1988, 1989b). The frequency of antigen-specific IgG- and IgA-secreting cells greatly differs from that of the same isotypes in conventional mice, and is similar to that of the IgM-secreting cells (*Bos* et al., 1988, 1989b). Obviously, exogenous antigenic stimulation plays an important role in the development of the actual B cell repertoire.

Whether the described differences in the available and the actual B cell repertoire are reflected in differences in B cell populations between GF-CD and CV-NI mice is now being investigated by immunophenotyping of the B cells using FACS analysis, and by immunohistological examination.

Immunohistological staining of tissue sections and multiparameter flow-cytometric analysis of single cell suspensions of conventional murine tissues have demonstrated heterogeneity in the phenotype of B cells. For example, in immunohistological sections of the spleen of adult conventional mice at least two subpopulations of B cells are found: $IgM^{dull}IgD^{bright}$, which can be found in lymphoid follicles (follicular B cells or population I) and a much smaller $IgM^{bright}IgD^{dull}$ population, which predominantly can be found in the marginal zone (MZ) surrounding the follicles (MZ B cells or population III) (*Hardy* et al., 1984; *Kroese* et al, 1991). Upon antigenic stimulation so called germinal centres (GC) are formed in the follicles, containing large, rapidly dividing B cells. In addition to these B cell subsets a distinct lineage of B cells is formed by B1 cells (previously called Ly1 B cells). B1 cells differ developmentally from B cells which arise in the bone marrow (conventional B cells), as they are derived from foetal omentum and foetal liver (*Solvason* et al., 1991) and in the adult mice have a self-renewing capacity. They are predominantly found in the peritoneal cavity and exhibit unique phenotypic and functional properties (*Herzenberg* et al., 1986). Interestingly, MZ B cells and B1 cells in conventional mice share the same $IgM^{bright}IgD^{dull}$ phenotype and are both described as large cells, being in some state of "activation" (*Herzenberg* et al., 1986).

In this study we show that small, follicular B cells and large, MZ B cells can be found in the spleen of GF-CD mice in cell numbers comparable to those in CV-NI mice. The numbers of microscopically detectable MLN and PP in GF-CD mice were smaller than in CV-NI mice and no GC were detected.

In the peritoneal cavity of GF-CD mice and CV-NI mice both conventional B cells and B1 cells were found and the B1 cells have identical levels of IL5-re-

ceptor expression in both groups of mice. The most clear difference between GF-CD and CV-NI mice was the very severe reduction of IgA plasma cells in the lamina propria of GF-CD mice compared to that of CV-NI mice.

MATERIALS AND METHODS

Animals

BALB/cAnN mice were reared and maintained by American Biogenetic Sciences (Notre Dame, IN), either germ-free and fed a chemically defined ultrafiltered "antigen-free" diet L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (*Pleasants* et al., 1986), or were maintained conventionally and fed natural ingredient diet L-485. CV-NI mice and GF-CD mice (in mini-isolators) were sent to the University of Groningen, The Netherlands, and kept under comparable conditions as in Notre Dame for four weeks before usage. Mice were fed their daily supply of fatty acids one hour before sacrifice.

Cell suspensions

Single cell suspensions from spleen were prepared in PBS, containing 10% NCS (PBS-NCS) by mincing tissue fragments over a stainless steel grid and filtering through a nylon mesh. Peritoneal cells were collected by rinsing the peritoneal cavity of adult mice with ca. 15 ml of PBS-NCS.

Antibodies

The following rat monoclonal antibodies were used: anti-IgM (331.12) (*Kincade* et al., 1981), anti-Ly1 (53-7.8) (*Ledbetter* et al., 1979), anti B220 (RA3-6B2) (*Coffman* and *Weissman*, 1981) and anti-IgA (71.14) (*Butcher* et al, 1982) and anti-IL5 receptor (*Rolink* et al., 1989) (a generous gift of Dr. A Rolink, Basel Institute for Immunology, Basel, Switzerland). For IgD staining a mouse anti-mouse anti-IgD^a was used (9.1) (*Stall* and *Loken*, 1984). Conjugation of antibodies to biotin and fluorescein has been described previously (*Hardy* et al., 1986).

Immunofluorescence staining and FACS analysis

Two-colour immunofluorescence staining of cells using FITC- and biotin-conjugated mAb was carried out in PBS-NCS as described in detail elsewhere (*Hardy* et al., 1984). Biotinylated antibodies were revealed by Streptavidine-Phycoerothrine (SA-PE; Southern Biotechnology Associates, Birmingham, AL) as second step reagent. Cells were analysed on a fluorescence activated cell sorter (FACStar; Becton Dickinson, Mountain View, CA). Non-lymphoid cells were gated out on the basis of forward and perpendicular site scatter pattern. For each analysis data from 10,000-20,000 cells were collected.

Immunohistology

Cryostat sections were prepared from spleen, MLN, PP and lamina propria. MLN and PP were only detectable in GF-CD mice by examination under a dissecting microscope, because of the white appearance of the lymph veins due to ingestion of fat one hour before. Staining of the cryostat sections with mAb using an indirect immunoperoxidase technique was performed as described elsewhere (*Kroese* et al., 1987). As second-stage antibody, peroxidase-conjugated polyclonal rabbit anti-rat Ig (Dakopatts, Copenhagen, Denmark) was used. For anti-IgD^a an Avidine-peroxidase conjugate (Southern Biotechnology Associates, Birmingham, AL) was used.

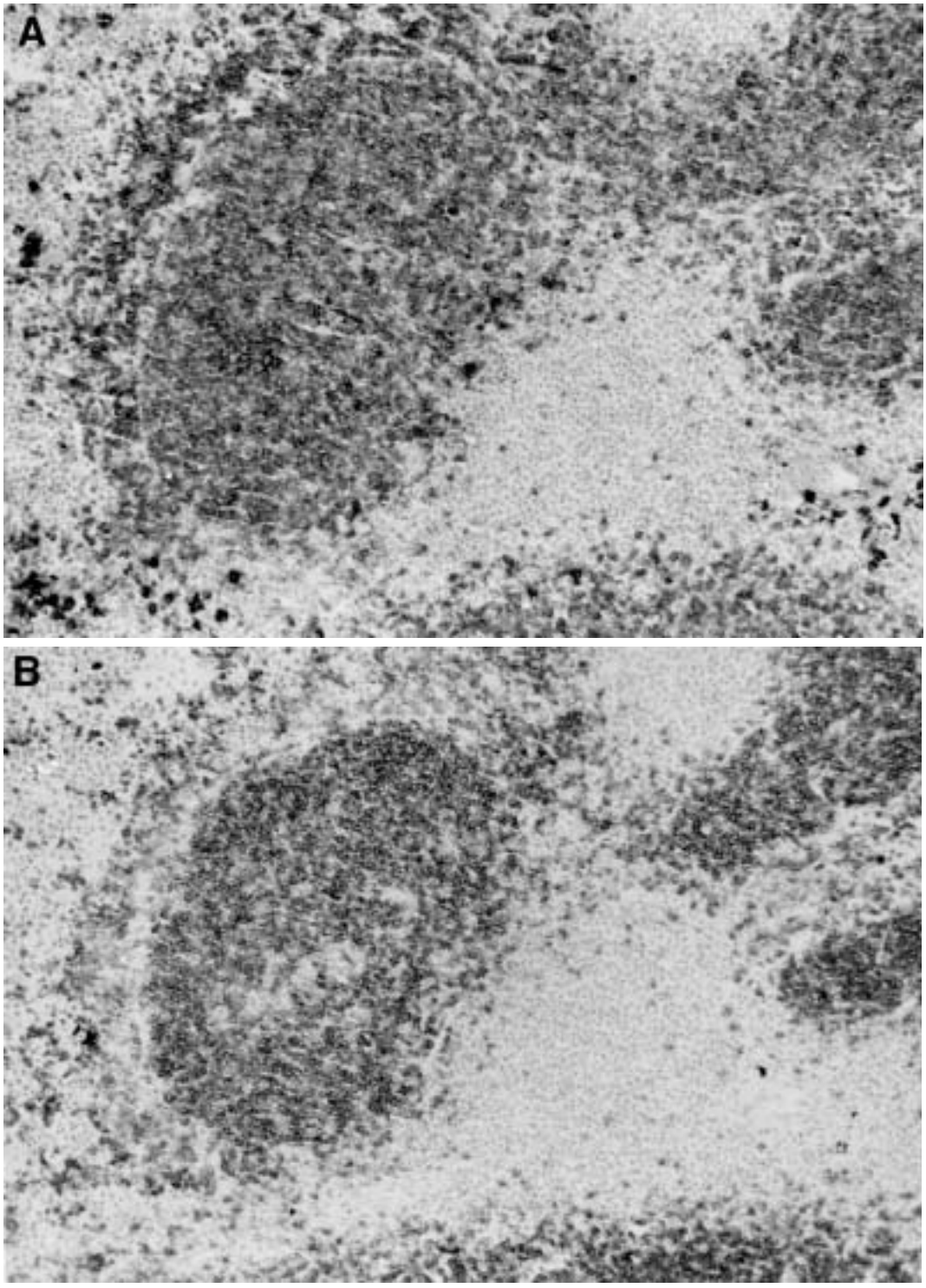

Figure 1: Serial cryostat sections of the spleen of a GF-CD mouse stained with (A) anti-IgM and (B) anti-IgD (x60).

Table 1: Flow cytometric analysis of B cell subpopulations in spleen and peritoneal cavity of GF-CD and CV-NI BALB/c mice.

Organ	phenotype	GF-CD	CV-NI
Spleen	IgD^{high}/IgM^{low} (follicular B cells)	47%[1]	52%
	IgD^{low}/IgM^{high} (MZ B cells)	8%	8%
Peritoneal cavity	IgD^{high}/IgM^{low} (conventional B cells)	14%	27%
	IgD^{low}/IgM^{high} (B1 cells)	68%	52%
	$Ly\text{-}1^{+}/IgM^{high}$ (B1a cells)	40%	29%
	$Ly\text{-}1^{-}/IgM^{+}$ (B1b+conventional B cells)	53%	62%
	$IgM^{+}/IL5R^{+}$	74%	71%

[1]Percentage represents the mean percentage of all lymphoid cells in the different organs of individually analysed 8-12 wk old GF-CD BALB/c mice (n=3) and CV-NI BALB/c mice (n=3).

RESULTS

B cell populations were examined by flow cytometry and/or immunohistology in spleen, MLN, PP, lamina propia and peritoneal cavity.

Spleen

In the spleen of conventional mice the majority of small resting, $IgM^{low}IgD^{high}$ B cells can be found in the follicles. Furthermore, large B cells, which are $IgM^{high}IgD^{low}$, can be seen in the marginal zone surrounding the follicles. Upon antigenic stimulation so called germinal centres (GC) are formed in the follicles, containing large, rapidly dividing B cells.

In the spleen of GF-CD mice essentially the same B cell subpopulations were observed by immunohistology, except for the total lack of GC. Figure 1 shows serial frozen sections of the spleen of GF-CD mice stained with anti-IgM (Figure 1a) and anti-IgD (Figure 1b). Follicles were present, surrounded by well-developed marginal zones. Furthermore, IgM plasma cells were found in the red pulp of the spleen of GF-CD and CV-NI mice (Figure 1a).

Also quantitatively the same numbers of $IgM^{low}IgD^{high}$ and $IgM^{high}IgD^{low}$ B cells were found by flow cytometry. In GF-CD and CV-NI mice there were 47% and 52% $IgM^{low}IgD^{high}$ B cells and 8% and 8% $IgM^{high}IgD^{low}$ B cells, respectively (Figure 2, Table 1).

Mesenteric lymph nodes

MLN are very hard to find in GF-CD mice, showing that their size is clearly dependent on exogenous antigenic stimulation. However, because of the rapid uptake of the fatty acids fed one hour before sacrifice, the lymph veins can easily be traced with use of a dissecting microscope. In this way we were able to find MLN in GF-CD mice. Immunohistological examination showed a normal architecture of these lymph nodes. A cryosection stained with anti-B220, a common B cell marker, showed normal follicular B cell areas (Figure 3). In MLN of CV-NI mice several GC were observed, while in GF-CD mice only one small, beginning GC was detected (data not shown).

Peyer's patches

Gut associated clusters of lymphoid follicles, known as Peyer's patches (PP) can be found along the intestinal tract of conventional mice. PP could only be detected in low numbers in GF-

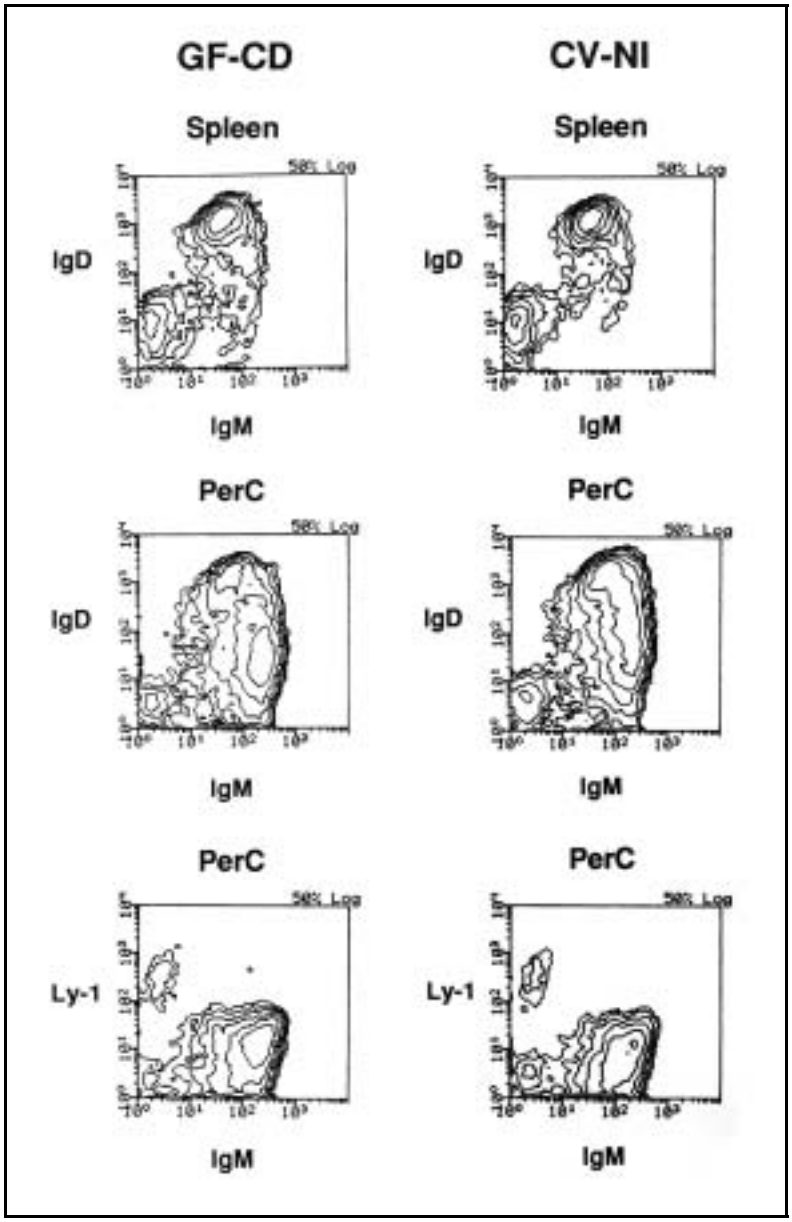

Figure 2: Two colour FACS analysis of splenic and peritoneal cavity (PerC) cell suspensions of 8-12 wk-old GF-CD and CV-NI mice, stained with combinations of anti-IgM/anti-IgD and anti-IgM/Ly-1. Figures show representative samples; calculations are shown in Table 1.

CD mice after careful examination of the gut with a dissecting microscope. The follicles of PP of CV-NI mice contained many GC with many sIgA-positive B cells (data not shown). By contrast, follicles of PP in GF-CD mice contained follicular B cells as shown by B220 staining (Figure 4) and IgM and IgD, but they did not contain GC and sIgA positive cells were absent.

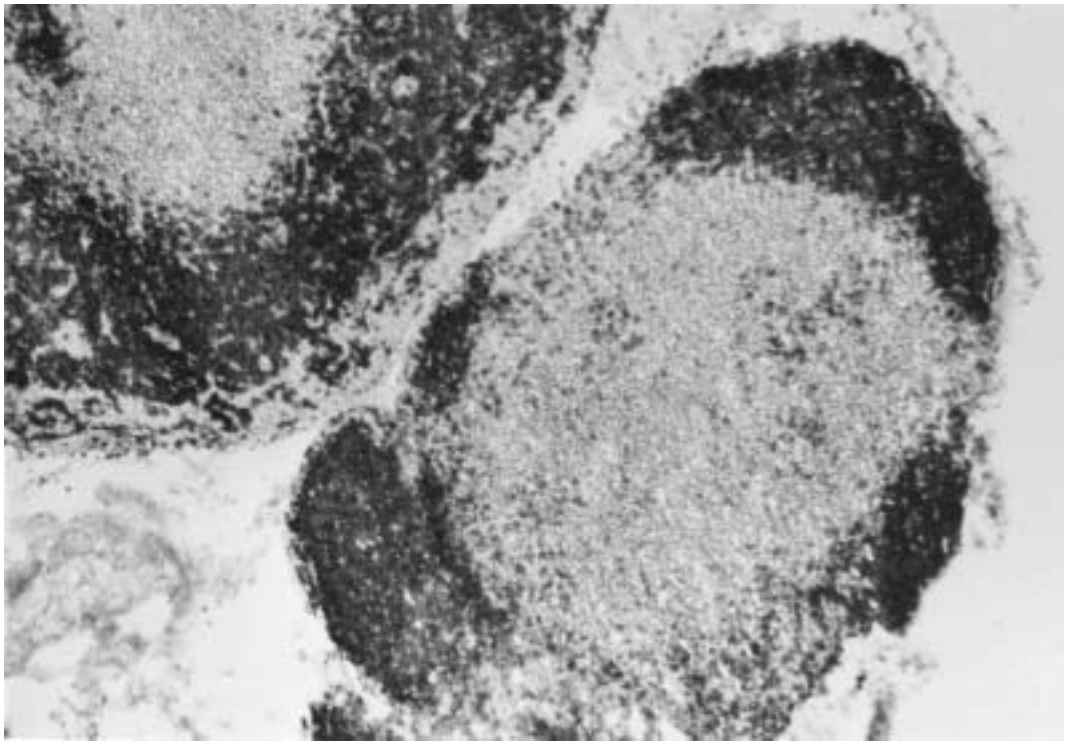

Figure 3: Cryostat section of a mesenteric lymph node of a GF-CD mouse, stained with anti-B220 (x60).

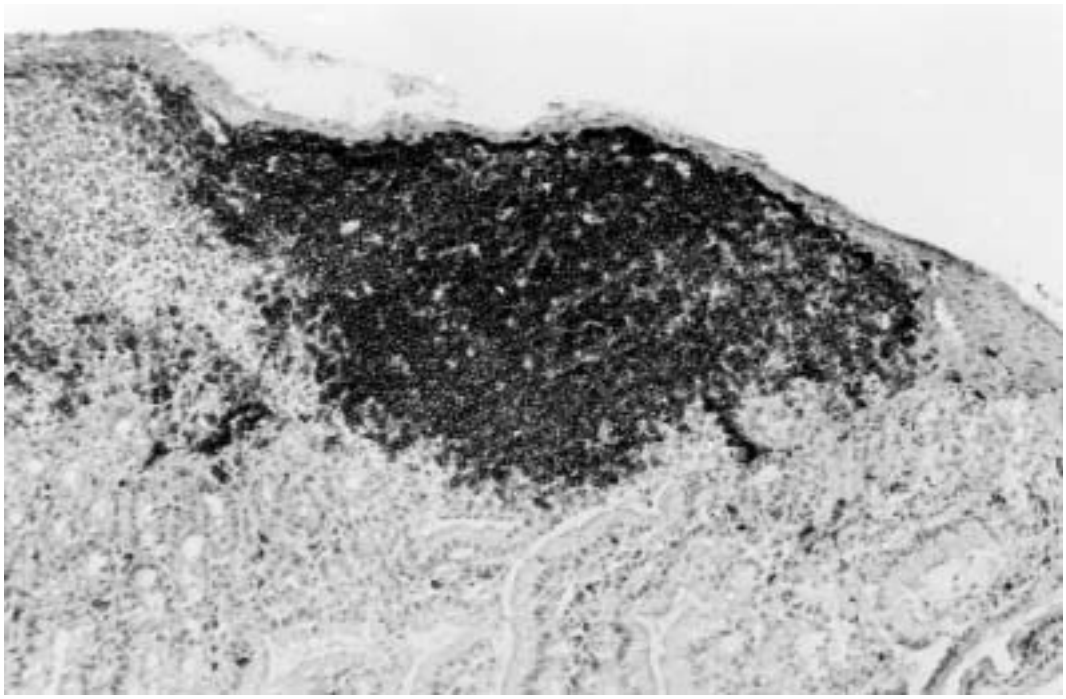

Figure 4: Cryostat section of a Peyer's patch of a GF-CD mouse, stained with anti-B220 (x60).

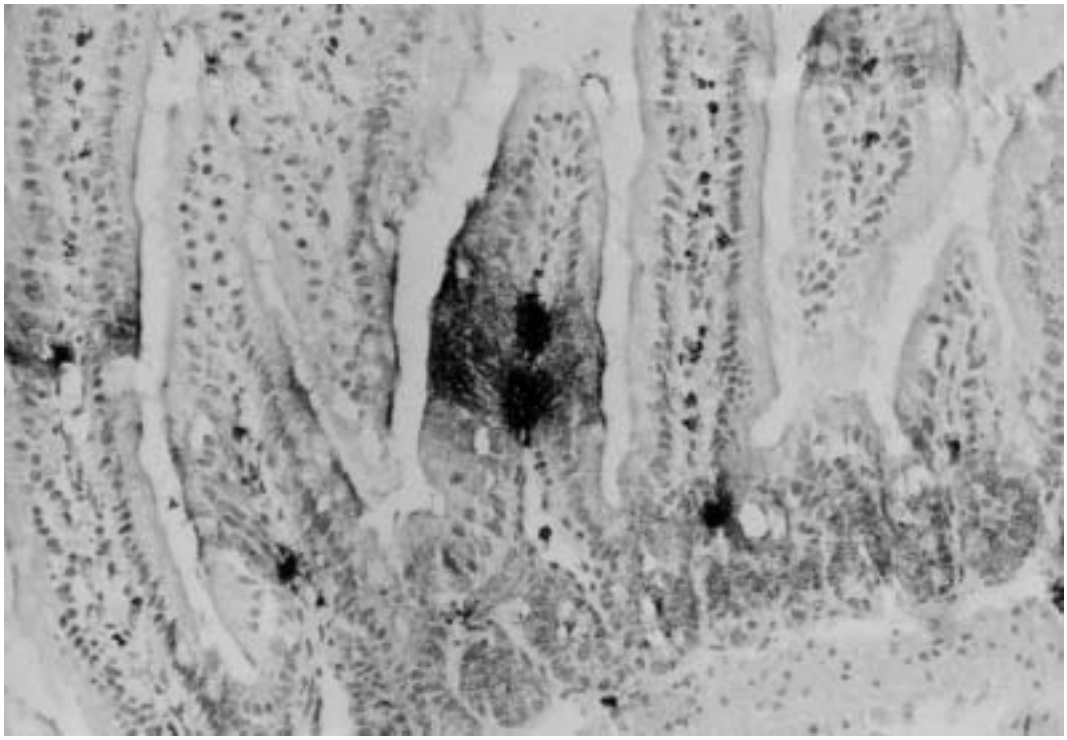

Figure 5: Cryostat section of the lamina propria of the gut of a GF-CD mouse, stained with anti-IgA (x100).

Lamina Propria

In the lamina propria of CV-NI mice very large numbers of IgA plasma cells were located (data not shown). Previous studies have shown that more than 80% of all Ig-secreting cells in conventional animals can be found in the lamina propria and these produce mainly IgA (*van der Heiden* et al., 1987). By contrast, the lamina propria of GF-CD mice contained extremely few IgA plasma cells (Figure 5) and no IgM plasma cells. In cryosections of the gut of GF-CD mice IgA can be detected surrounding the rare IgA plasma cells, suggesting the secretion of IgA into the intestinal tract (Figure 5).

Peritoneal cavity

In CV-NI mice high numbers of B cells are found in the peritoneal cavity. Most of the peritoneal B cells belong to a distinct lineage of B cells, called the B1 cells (formerly called Ly1 B cells), of which the majority express low amounts of CD5. The B1 cells in the peritoneal cavity have a characteristic $IgM^{high}IgD^{low}$ phenotype in contrast to conventional B cells that are $IgM^{low}IgD^{high}$. In GF-CD and CV-NI mice both conventional B cells and B1 cells were found. GF-CD and CV-NI mice contained 14% and 27% of $IgM^{low}IgD^{high}$ conventional B cells and 68% and 52% $IgM^{low}IgD^{high}$ B1 cells respectively (Figure 2 and Table 1). The Ly-1 (CD5) positive B1 cells (B1a cells) were 40% and 29% in GF-CD and CV-NI mice, respectively (Figure 2 and Table 1). IL5 receptor expression on peritoneal B cells was examined by double staining with anti-IgM and anti-IL5 receptor mAbs. Table 1 shows that both in GF-CD and in CV-NI mice a similar high percentage

of the B cells express the IL5 receptor (74% and 71%, respectively). There was a positive correlation between the level of IgM expression and IL5R expression, showing that IgM^{high} (B1 cells) had the highest expression of IL5 receptor (data not shown).

DISCUSSION

B cell populations in GF-CD mice

Frequencies of B cells and B cell subsets in various lymphoid organs such as spleen, lymph nodes and PP are independent of exogenous antigenic stimulation, since B cell follicles and the marginal zone surrounding the follicles in the spleen were the same in GF-CD and CV-NI mice. Also, the number of peritoneal B1 cells was comparable in both groups of mice. However, there were some differences observed between GF-CD and CV-NI mice.

Firstly, the number and size of gut-associated lymphoid tissues such as MLN and PP were severely reduced, but still detectable, in GF-CD mice compared to CV-NI mice. This finding is in contrast to that of others who failed to detect such organs in GF-CD mice (*Hooijkaas* et al., 1984; *Pereira* et al., 1986). This discrepancy is probably due to technical differences in the dissection procedure, since we only detected MLN in GF-CD mice if the animals had ingested fatty acids one hour earlier. Both these and our studies, however, clearly show that full development of gut- associated lymphoid tissue is dependent on exogenous antigenic stimulation.

Secondly, virtually no GC were found in the lymphoid organs of GF-CD mice, not even in the PP, where in normal animals many GC can be found. GC are thought to be the site where memory B cells are formed and affinity maturation and isotype switching of antigen-specific B cells takes place after immunisation (*Kroese* et al., 1990). Earlier studies with GF mice showed some GC formation in the MLN (*Pollard* et al., 1967). Probably this is due to the antigenic stimulation from antigens of the sterilised conventional food of such animals.

Thirdly, there were almost no sIgA-positive cells in the PP and only few IgA plasma cells were found in the lamina propria of GF-CD mice. This is in agreement with earlier findings in GF mice, which show also a clear reduced number of sIgA-positive cells in the PP (*Weinstein* and *Cebra*, 1991) and in the number of intestinal IgA-secreting cells (*van der Heiden* et al., 1989). The sIgA-positive cells in the PP are considered as precursors for both IgA plasma cells in the lamina propria and IgA memory cells (*Gearhart* and *Cebra*, 1979). Interestingly, there were still some IgA plasma cells found in the lamina propria of GF-CD mice. Recently, it has been found that not only PP cells can give rise to IgA plasma cells, but that peritoneal B1 cells are also a source for intestinal IgA-plasma cells (*Kroese* et al., 1989). Since GC are absent in the PP of GF-CD mice, possibly the IgA plasmacells in the gut of GF-CD mice belong to this lineage. Where and how the B1 cells get triggered to become IgA plasma cells is still unknown.

Available repertoire

In GF-CD mice and in GF mice a biased V_H gene family usage of the V_H gene family PC7183 that is most proximal to the constant Ig genes is observed (*Bos* and *Meeuwsen*, 1989; *Fre-*

itas et al., 1991). A comparable biased V_H gene family usage has been observed in neonatal B cells and in pre-B cells in the bone marrow of adult conventional mice (*Manlynn* et al, 1990). In adult conventional mice a more stochastic V_H gene family usage was seen according to the size of the V_H gene families (*Dildrop* et al., 1985; *Holmberg* et al., 1986). Also, within the large V_H gene family J558, differences were observed in V_H gene usage between bone marrow pre-B cells and splenic B cells, suggesting that the seemingly random usage in adult conventional mice is caused by antigenic selection (*Gu* et al., 1991). In GF mice, it was shown that injection of IgG derived from conventional mice, causes a normalisation of V_H gene family usage (*Freitas* et al., 1991). This normalisation could either be caused by direct polyclonal antigenic stimulation by the (foreign) injected IgG or IgA antibodies and/or by idiotypic-anti-idiotypic interactions of the injected antibodies with the B cells of the recipient. Until now the available repertoire has been studied in hybridomas after LPS stimulation (*Dildrop* et al., 1985; *Holmberg* et al., 1986; *Bos* and *Meeuwsen*, 1989), by analysis of the total RNA of spleen or bone marrow cells (*Manlynn* et al., 1990) or by *in situ* hybridisation (*Jeong* and *Teale*, 1988). The contribution of different B cell subpopulations in these assays is unknown. What are possible explanations for the differences between the available repertoire in adult GF-CD and CV-NI mice?

Firstly, memory B cells, which are considered to be immunocompetent B cells that can contribute to the available B cell repertoire. The lack of GC in GF-CD mice, suggests that the memory B cell repertoire in GF-CD mice is absent. This absence may explain (partly) the observed "normalisation" of the V_H gene family usage in conventional mice.

Secondly, a source for the differences in the available repertoire between adult GF-CD and CV-NI mice might be the MZ B cells. MZ B cells are relative large cells (*Herzenberg* et al., 1986; *Liu* et al., 1992). The contribution of MZ B cells to the analysed available repertoire is unknown. MZ B cells represent about 13% of the splenic B cells, but if they are preferentially stimulated by LPS or if these relatively large B cells contain more RNA than the small follicular B cells they may contribute significantly to the V_H gene family distribution as found among splenic B cells. This explanation is unlikely, because the number of MZ B cells seems independent of exogenous antigenic stimulation, as no differences were found by FACS analysis and immunohistology of MZ B cells in GF-CD and CV-NI mice.

Finally, the observed differences in the available repertoire between GF-CD and CV-NI mice might be due to a selection process of the follicular B cells. Where and how this selection takes place is still unknown.

Actual repertoire

Since memory formation and isotype switching may take place within the GC, the lack of GC in GF-CD mice is in agreement with our earlier findings that GF-CD mice have a severely reduced production of IgG and IgA, both at the level of Ig-secreting cells and at the level of serum Ig (*Bos* et al., 1988, 1989b). Furthermore, the specificity repertoire of the low numbers of IgG- and IgA-secreting cells greatly differs from that of the same isotypes in conventional mice, and is much like the IgM specificity repertoire (*Bos* et al., 1988, 1989b). Obviously, exogenous antigenic stimulation plays an important role in the development of the actual B cell repertoire. The emerging actual B cell repertoire after immunisation of GF-CD mice has not yet been investigated.

In this regard it is interesting that the production of monoclonal antibodies in GF-CD mice seems to be more efficient then in conventional mice (*Ploplis*, unpublished observations). GF-CD mice contain normal numbers of large, presumably activated, B cells in the spleen as judged by FACS analysis (*Pereira* et al., 1986). Furthermore, the numbers of IgM-secreting cells in spleen, BM and MLN is the same in GF-CD and CV-NI mice (*Pereira* et al., 1986; *Bos* et al., 1988). Also, the specificity repertoire of these IgM-producing cells was comparable between GF-CD and CV-NI mice (*Bos* et al., 1988). A possible source for these so called "natural" IgM antibodies could be the B1 cells. B1 cells are shown to be a separate lineage of B cells which can self renew largely independent of the bone marrow (*Herzenberg* et al., 1986). In transfer experiments with allotypic Ig markers it has been shown that this relatively small compartment of B cells (estimated to represent 1% of all B cells) can produce up to 50% of all serum Ig (*Kroese* et al., 1989). The B1 cells are relatively larger then conventional B cells and a large proportion of them express the IL5 receptor (*Wetzel*, 1989). They seem to be a selected B cell population, because they use a restricted set of Ig genes and limited specificities (*Hayakawa* et al., 1986). How they are selected is unknown. On the other hand, other studies showed no differences in the B1 cell specificity repertoire of GF and conventional mice, suggesting that the B1 cell specificity repertoire is established independent of exogenous antigenic stimulation (*Lalor* et al., 1989). In the GF-CD and CV-NI mice we showed comparable numbers of B1 cells and the same level of IL5 receptor expression. This is in agreement with others who found normal numbers of B1 cells in GF mice (*Forster* et al, 1991). If exogenous antigenic stimulation plays a role at all in the selection of the B1 cell specificity repertoire, it is not reflected in the number or phenotype of the B1 cells.

In conclusion, mice that are kept under conditions that will minimise exogenous antigenic stimulation, have a normal architecture of the B cell areas in the lymphoid organs and numbers of different B cell subpopulations that are similar to those of conventional mice. The available repertoire of adult GF-CD mice is different from that of conventional mice, suggesting a role for exogenous antigenic stimulation in the establishment of the available repertoire. The actual IgM specificity repertoire develops independent of exogenous antigenic stimulation. This is in contrast to the actual IgG and IgA repertoire, which is clearly influenced by exogenous antigenic stimulation. Reconventionalisation and immunisation of GF-CD mice can further clarify the role of exogenous antigenic stimulation in the shaping of the B cell repertoires.

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WHERE DO CD5 B CELLS COME FROM?

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The definition of the "$CD5^+$ B cell population" in mice is loosely based on the presence of any of several attributes including: expression of CD-5 and/or CD11b, the absence of CD23, low CD45R(B220), high CD44, low Igδ, increased size and decreased density, increased plastic adherence, a limited repertoire of immunoglobulins, unusual tissue distribution, long-life and/or self-renewal capacity, and foetal origin (*Hayakawa* and *Hardy*, 1988; *Herzenberg* et al., 1986). For instance, a recent proposal holds that a population of B cells (called B-1 cells) arise early in ontogeny, are self-replenishing, absent in the adult marrow and lymph nodes, predominate in the pleural and peritoneal cavities and make up only a small fraction of splenic B cells (*Allison*, 1991). The majority of these cells (the B-1a population) express CD5 and the minority (B-1b) do not. The proposal goes on to term all other B cells as the B-2 population.

The contention of this review is that this attempt at a consensus definition fails. What if B cells of adult origin become $CD5^+$? Are all CD5 expressing cells B-1a cells? Are all peritoneal $CD5^+$ cells of foetal origin? Are B-1b cells of foetal origin? With these and related questions in mind I will present preliminary results that question the proposition that only B cells of foetal origin can become "CD5 B cells." After briefly reviewing other work addressing the issue of differences between B cells of adult and foetal origin I suggest an alternative view - the specificity hypothesis - of the origin of CD5 B cells is necessary. Finally, I discuss recent published studies from several laboratories with immunoglobulin transgenic mice that provide evidence for this second view.

Previously we asked if adult splenic B cells could be induced to express CD5. We thought that activation induced by the cross-linking of sigM would be likely to accomplish this. Indeed, that is precisely what we found (*Ying-Zi* et al., 1991). We showed that goat anti IgM as well as monoclonal rat anti IgM induced CD5 expression. We showed that this induction began within eighteen ours and involved all B cells by four days. We also showed that even selected $CD5^-$ B cells could be so induced.

This induction results in an increase in processed CD5 mRNA as detected by PCR amplification from cDNA. (Preliminary studies of Ram Bandyopadhyay and Henry H. Wortis.) We have not found sufficient message in freshly isolated $CD5^+$ peritoneal or *in vitro* induced splenic cells for detection by Northern blotting. At the present time we do not know if the accumulation of processed CD5 mRNA results from increased transcription, the onset of processing or message stabilisation. Studies to establish this are currently underway.

In recent preliminary studies (Chris Huang, John Iacomini, Thereza Imanishi-Kari and Henry H. Wortis) we asked whether B cells of adult origin could become $CD5^+$. Other groups have reported that $CD5^+$ cells are derived from adult bone marrow transfers

Table 1: Features of B cells derived from foetal and adult precursors

Feature	Foetal	Adult	Reference
$CD5^+$	many	few	*Riggs* et al., 1990 *Thomas-Vaslin* et al., 1992 *Iacomini* and *Imanishi-Kari*, 1992 *Hardy* et al., 1991, 1992 *Solvason* et al., 1991
$CD11b^+$	some	some	*Kantor* et al., 1992
$CD5^-CD11b^-$	few	many	*Riggs* et al., 1990 *Thomas-Vaslin* et al., 1992 *Iacomini* and *Imanishi-Kari*, 1992 *Hardy* and *Hayakawa*, 1991 *Hardy* et al., 1991, 1992 *Solvason* et al., 1991
Peritoneal	many	few	*Riggs* et al., 1990 *Thomas-Vaslin* et al., 1992 *Iacomini* and *Imanishi-Kari*, 1992 *Hardy* and *Hayakawa*, 1991 *Hardy* et al., 1991, 1992 *Solvason* et al., 1991
Self-renewing	many ?	few ?	not reported
N-less	many	few	*Gu* et al., 1990 *Feeney*, 1990 *Carlsson* and *Holmberg*, 1990 *Bangs* et al., 1991 *Meek*, 1990
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TdT [terminal Deoxynucleotidyl transferase] (pre-B)	few	many	*Desiderio* et al., 1984
mlc-2 [myosin light chain-2] (pre-B)	none	all	*Oltz* et al., 1992

(*Riggs* et al., 1990; *Thomas-Vaslin* et la., 1992; *Iacomini* and *Imanishi-Kari*, 1992), but this has not been a universal experience (*Hardy* and *Hayakawa*, 1991). The sources of these differences have not been studied in detail, but there are three important considerations. If the frequency of potential $CD5^+$ cells is much less in the marrow than in the foetal liver then early examination of the recipients (e.g. after three months) might fail to reveal cells of bone marrow origin. Conversely, if the bone marrow preparation is contaminated by self-renewing cells of foetal origin a false positive result might be obtained.

In our experiments we transferred adult bone marrow from C57BU6 mice into sublethally irradiated C3H.SCID mice. Ten months later we found that a fraction of the splenic B cells were $CD5^+$. We placed B cell enriched splenic cells from the C57BU6 into SCID mice into *in vitro* culture with either LPS or anti-lg. We harvested the cells and double-stained them with FITC anti CD45(B220) and biotinylated anti CD5 plus phycoerythrin streptavidin and analysed them by flow cytometry. All of the anti Ig stimulated cells became $CD5^+$ while few, if any, of the LPS stimulated cells did so.

To determine whether these $CD5^+$ B cells were truly of bone marrow origin (and not derived from a few passenger cells of foetal origin) we took advantage of the observation that B cells of foetal/neonatal origin lack N sequences in their rearranged heavy chains while cells of adult origin have N insertions. We asked whether the junctional sequences of the rearranged heavy chains of these $CD5^+$ B cells reflected an adult or foetal/neonatal origin.

We examined the junctional sequences of rearranged genes containing members of the S107 V_H gene family. We did this because this family is believed to contain only four germ-line V_H genes and the sequences for these genes in the closely related C57BU10 strain (as well as the BALB/c strain) are known. This knowledge makes assignment of N sequences in VD junctions possible. Our strategy was to generate cDNA and use it as template for PCR based amplification using a 5' primer for V_H genes of the S107 family and a 3' CH1 cμ primer. Thus far, all of the heavy chain sequences that we have examined contain N sequences. We conclude that $CD5^+$ B cells can be derived from adult bone marrow.

Table 1 summarise results of studies on the origin of B cells expressing selected phenotypic features. This list is limited to studies in which an attempt was made to exclude the possibility that mature B cells were included in the "progenitor population".

From these published data it is evident that only one of the examined traits, *mlc-2* expression, might be a definitive lineage markers of foetal or adult derived mature B cells. The product of the gene encoding a myosin light chain (*plrlc*), a member of the *mlc-2* family of proteins, is limited in expression to pre-B cells of adult origin (*Oltz* et al., 1992). However, this gene is not expressed in mature B cells. The table also makes it clear that frequencies of expression of several traits do differ markedly between cells of adult and foetal origins. What the data do not tell us is whether cells are predetermined at the progenitor level to express a given mature phenotype.

Pre-B cell surface antigens that are candidate lineage specific molecules are listed in Table 2. The two obvious candidates, CD5 and CD11b do not appear on freshly isolated sig cells nor are they uniformly expressed on Abelson-MuLV transformed pre-B cells derived from either foetal or adult compartments (unpublished results) and therefore are not markers of a lineage *per se*. The absence of CD45RA and Qa-2 was noted in pre-B cells of early embryos. Because foetal B cells appear to develop in a single wave (*Strasser* et al., 1989) it is possible that more primitive B lineage cells were sampled and these cells did not yet express these markers. Several years ago it was reported that the combination of lack of Thy-1 expression and presence of surface asialo-ganglio-N-tetraosylceramide (aGM,) was unique to foetal progenitors of B cells (*Hardy* et al., 1987). However, foetal cells with this phenotype did not yield particularly high numbers of peritoneal $CD5^+$ B cells.

Typical peritoneal $CD5^+$ cells have several characteristic phenotypic features, which suggested to us that they are activated B cells. They are large, low density cells with high levels of CD44 with little or no IgD that express IL-5 receptors (*Hitoshi* et al., 1990). We looked for some of these features in our *in vitro* induced $CD5^+$ cells. We found that become large cells with elevated CD44 without losing IgD (*Ying-Zi* et al., 1991). We speculated that in addition to sig cross-linking another signal, perhaps provided by a cytokine might be necessary. We tested IL-1 (with and without IL-4), IL-2, IL-4

alone and IL-6 as candidate interleukins. In our hands only IL-6 induced a loss of IgD, leading to the speculation that *in vivo* the combination of sig cross-linking and IL-6 induces the classic peritoneal $CD5^+$ phenotype. IL-5 receptors are reported to be induced on B cells following sigD cross-linking (although CD5 induction was not seen in this study) (*Allison* et al., 1991).

It is now generally accepted that the frequency of N insertions into rearranged heavy chains differs in B cells of foetal/neonatal and adult origin. The differences between the two populations are not absolute, and N-less segments have been found in cells of adult origin, although with low (2-10%) frequency, while only rarely were N insertions seen in foetal B cells (0.5-2%) (*Feeney*, 1992). This difference in N insertion pattern correlates with differences in the expression of terminal deoxynucleotidyl-transferase (TdT) (*Desiderio* et al., 1984).

The finding that the addition of IL-6 to sig cross-linked B cells induces many of the features of the CD5 B cell is provocative because it means that B cell activation in the absence of T cells can result in the induction of the CD5 phenotype. IL-6, which is produced by T helper-2 cells, is also made by fibroblasts (*Zilberstein* et al., 1986), macrophages (*Aarden* et al., 1985), plasma cells (*Kawano* et al., 1988) and endothelial cells. Therefore, *in vivo* $CD5^+$ B cells might be generated by multiple epitope (repeating unit) antigens, the thymus-independent type 2 (Tl-2) antigens, in association with IL-6. Because antibody responses to Tl-2 antigens require macrophages it is reasonable to hypothesise that CD5 cells result from Tl-2 antigen presentation by macrophages or other IL-6 producing cells.

B cells activated by anti-lgM and IL-6 also lost their surface CD23 and had decreased amounts of CD45(B220) IgD (*Ying-Zi* et al., 1991), both features of peritoneal $CD5^+$ B cells. Apparently then, CD23 loss could result from activation. We thought that we might be able to find examples of B cells at a point after activation *in vivo* at which they expressed CD5 but had not yet lost their CD23. It was likely that cells with this phenotype might be more prevalent in the young mouse, at a time when there are many splenic CD5 cells. We reasoned that these would be newly activated cells that might not yet express a mature phenotype. Indeed, when we examined small $CD5^+$ B cells from young mice we found them to be $CD23^+$ (*Rabin* et al., 1992a). We think it is reasonable to propose that many B cells of foetal/neonatal origin are stimulated by Tl-2 type antigens to enter the CD5 pathway, subsequent ligation of IL-6 (or an equivalent cytokine) inducing the loss of IgD and CD23 and the characteristic secretion of IgM and IgG_3.

We also noted that losses of CD23 (*Kikutani* et al., 1986) and IgD (cf, *Kroese* et al., 1990) together with a decrease in CD45RB (*Birkeland* et al., 1988) are seen in B cells within germinal centres where CD5 is not expressed. Activation of B cells within germinal centres is a T cell dependent process. This suggests that T cell dependent activation of B cells might produce cells with a characteristic surface phenotype. We were encouraged to think along these lines by our observation that activation with LPS failed to induce CD5 expression but did cause increased CD44, as well as the loss of CD23 and IgD. Interestingly, LPS induced a dramatic decrease in the expression of the heat stable antigen (HSA) as detected by the monoclonal antibody J11d. We had not seen J11d loss after treatment with anti IgM, even with added IL-6, nor was J11d low on unmanipulated peritoneal $CD5^+$ B cells.

Table 2: Surface markers of pre-B cells

Molecule	Foetal	Adult	Reference
CD45	yes	yes	*McKearn* et al., 1985
CD45RA	No/yes	yes	*Kincade* et al., 1981 *Landreth* et al., 1983 *Paige* et al., 1985
CD45R(B220)	yes	yes	Many, cf. *Strasser*, 1988
Qa-2	no	yes	*Kincade* et al., 1981
CD72 (Lyb-2)	no/yes	yes	*Kincade* et al., 1981 *Paige* et al., 1984, 1985
PB76	yes	yes	*Strasser*, 1988
AA4.1	yes	yes	*Paige* et al., 1985 *McKearn* et al., 1984
GF1	yes	yes	*McKearn* et al., 1984, 1985
BP-1	yes	yes	*Strasser*, 1988 *Cooper* et al., 1986
CD2	?	yes	*Yagita* et al., 1989a,b
HAS	yes	yes	*Hardy* and *Hayakawa*, 1991, 1992 *Hardy* et al., 1991
[Heat stable antigen]			(unpublished)
CD43	yes	yes	*Hardy* and *Hayakawa*, 1991, 1992 *Hardy* et al., 1991

We then tested the idea that B cells could be activated by two distinct induction pathways. One, the CD5 inducing pathway has been discussed above. The other, we thought, would occur after T dependent *activation without the need for slg cross-linking*. We (David Parker and Henry H. Wortis) are currently using a model system involving activation with purified rabbit anti mouse IgM. We stimulate high density resting splenic B cells in one of two ways: with $F(ab')_2$ of rabbit anti mouse IgM antibody; or with Fab of the same antibody together with equal numbers of a T helper cell line specific for rabbit Fab. Our early, preliminary results indicate that both treatments result in activation as measured by greater forward and size scatter and increased CD44 expression. Only the cells stimulated by F(ab')2 became $CD5^+$. Only the cells stimulated by Fab plus T helper cells became $J11d^{low}$. Control groups cultured with Fab or T helper cells alone did not become activated nor altered in surface phenotype.

Previously David Parker demonstrated that B cells can be activated by cognate interaction with helper T cells even in the absence of sig cross-linking (*Tony* et al., 1985). Subsequent work has amply confirmed this idea. This finding suggested the possibility that there are two distinct signals for B cell activation, each inducing a unique differentiation pathway. Current evidence is entirely consistent with the idea that $CD5^+$ B cells are cells that have responded to Tl-2 antigen stimulation but not definitive. Features distinguishing TD and Tl-2 responses are summarised in Table 3.

It is possible that not all B cells are able to enter the TD pathway. Norman Klinman suggests that only B cells with a $J11d^{low}$ phenotype can enter the memory pool and undergo affinity maturation (*Linton* et al., 1989). Since then it is was shown that low J11d can be in-

Table 3: Differences in B cell responses to thymus dependent and thymus independent-2 stimuli

	Type of stimulation		
Feature	TD	Tl-2	Reference
Surface	$CD5^-$	$CD5^+$	Proposed
Phenotype	$J11d^{low}$	$J11d^{high}$	Proposed
Early activation gene *egr*-1 expressed	no	yes	*Klaus* and *Parker*, 1992
Isotypes	IgM, IgG^1, IgG^2 IgA, IgE	IgM, IgG^3	*Slack* et al., 1980
In germinal centers	yes	no	*De Sousa* et al., 1969 *MacLennan* et al., 1982
Somatic mutation	common	rare	cf. *Rajewsky* et al., 1987
Memory	yes	no	*Howard* and *Courtenay*, 1974

duced by IL-4 (*Yin* and *Vitetta*, 1991; and our own unpublished observations) or by LPS IgD (*Ying-Zi* et al., 1991). Therefore, low J11d must not be a definitive marker for a predetermined a lineage of memory B cells. Nevertheless, Klinman has shown that only transferred $J11d^{low}$ cells generate high affinity antibodies (*Linton* et al., 1992). He argues that since these cells are found in nude mice they are unlikely to originate only from an initial interaction with helper T cells. Recently it was shown that both T helper 1 and 2 cells can induce affinity maturation (*Rizzo* et al., 1992). It is not known whether Th1 cells induce expression of the $J11d^{low}$ phenotype.

If CD5 B cells do not generate somatic mutations (but see *Taki* et al., 1992), there are important implications for our understanding of the origin of pathogenic autoantibodies. Early B cells express a repertoire that recognises conserved epitopes including self- antigens. Since this is a germ-line encoded repertoire (not requiring junctional diversity) it has been selected over evolutionary time. The precise selective advantage conferred by this repertoire is uncertain. It may provide for a first line of defence against pathogens, substitute for regulatory molecules, or regulate the antibody response. None of these are mutually exclusive properties.

That the foetal repertoire is skewed toward binding many epitopes, but with low affinity, makes it likely that early B cells will be activated by self-antigens. The reason for this is shown in Figure 1. Here it can be seen that sig cross-linking will be readily induced by repeating unit antigens. On the other hand, unique epitopes, such as those found on most protein antigens will fail to accomplish this. Finally, if a cell surface presents multiple unique epitopes which can all be recognised by the polyreactive antibody of a given B cell, that B cell might be induced by sig cross- linking to become $CD5^+$ and secrete IgM antibody. This makes it highly likely that the emerging foetal/neonatal repertoire will be activated by contact with self-antigens. Adult B cells, expressing antibodies with high affinities for unique epitopes would not be likely to be triggered in this manner.

Some time ago the ability of $CD5^+$ B cells to provide help for antibody responses was reported (*Okumura* et al., 1982; *Sherr* and *Dorff*, 1984). The finding that CD5 B cells can produce IL-10, a viability factor for B cells, and

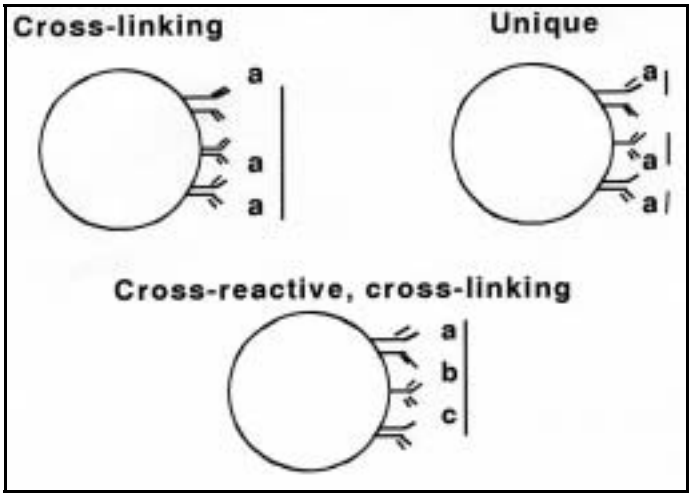

Figure 1: Patterns of B cell binding: upper left, binding to repeating unit antigen; upper right, binding to unique epitope antigen; bottom, polyspecific binding to multiple epitopes.

that B cells can produce other interleukins, such as IL-6, as well as TNF-α and -β (*O'Garra* et al., 1991) suggests a mechanism for B-B help. The finding that the ligand for CD5 is CD72 (Ly-b2) (*van de Velde* et al., 1991), itself a molecule expressed on B but not T cells suggests that CD5-CD72 ligation might play a role in this help (*Kamal* et al., 1991). Indeed, there is evidence that antibodies to CD72 block Tl-2 as well as TD antibody responses (*Subbaro* and *Mosier*, 1982). In any case, Ig-lg binding together with CD5-CD72 binding might provide the basis for B-B help.

We have proposed that $CD5^+$ B cells are Tl-2 responding cells rather than the product of a predetermined CD5 lineage. This "specificity" hypothesis is tested by immunoglobulin transgenic mice. It predicts that transgenic mice constructed to express an immunoglobulin with a specificity common to naturally occurring $CD5^+$ cells will contain a high frequency of $CD5^+$ cells. Confirmation was obtained in experiments utilising a transgene encoding a heavy chain including the V_H 11, D and J_H gene segments from a peritoneal B cell producing antibody with anti bromelain treated mouse red blood cell specificity (*Hardy* and *Hayakawa*, 1991; *Hardy* et al., 1991). A striking result was obtained with a heavy/light chain transgenic expressing an NZB derived anti mouse red blood cell autoantibody (*Okamoto* et al., 1992). Essentially all peripheral B cells are lost and only the peritoneal B cells remain. (However, the peritoneal B cells have not been directly tested for CD5 expression.) The proposed explanation of this result is that there is autoantigen induced deletion of the peripheral B cells and the few cells that make it to the peritoneum live on in a sequestered environment. An alternative interpretation of these experiments is that the accumulated CD5 lineage cells derive from foetal/neonatal precursors. This does not appear likely because there do appear to be transgene encoded antibody producing B cells in the marrow. A direct test of the specificity hypothesis would be a transfer of B cell precursors from the adult bone marrow of transgenic mice into SCID, RAG-1 or RAG-2 knockout mice.

The distortion of the endogenous

immunoglobulin repertoire seen in some immunoglobulin transgenics also provides evidence for the repertoire hypothesis. In the M54 mice the transgene encodes a heavy chain expressing a member of the J558 V_H family (17.2.25) (*Weaver* et al., 1986). The repertoire of B cells expressing endogenous genes is skewed toward Q52 and 7183 family members (*Iacomini* et al., 1991) with a high frequency of autoantibody production (*Thereza Imanishi-Kari*, personal communication). There appears to be activation and positive selection of these cells (*Grandien* et al., 1991; *Rabin* et al., 1992b). Further, there is an increased frequency of $CD5^+$ B cells within this B cell population (but not in those B cells expressing the transgene). Foetal cells do not appear to be the source of these B cells, as judged by transfer experiments and the presence of N insertions in DJ_H junctions (*Iacomini* and *Imanishi-Kari*, 1992). Therefore, in the M54 mouse the presence the 17.2.25 transgene causes a distortion of the endogenous repertoire favouring D proximal V_H gene utilisation. Autoantigens activate these cells causing an increase in the CD5 population.

An interesting phenomenon was observed when the 17.2.25 transgene was introduced into mice expressing *X-linked immune deficiency (XID)* (*Rabin* et al., 1992b). This defect causes a deficit of CD5 cells and a failure to respond to Tl-2 antigens. XID transgenic mice have normal numbers of transgene expressing cells but lack cells expressing endogenous immunoglobulin. Another transgenic mouse, which carries genes encoding the M167 immunoglobulin, produces B cells with specificity for PC (*Kenny* et al., 1991). This particular specificity occurs frequently in the anti PC repertoires of normal mice but is never seen in XID mice. Introduction of the M167 transgene into XID mice results in progeny that lack peripheral B cells expressing the transgene. This is not due to a failure to produce B cells as mature cells can be found in the bone marrow. Kenny and co-workers propose that the cells are lost to a deletion mechanism following activation via ligand binding. These results appear to confirm a model of B cell deletion in XID mice proposed many years ago by *Klinman* et al. (1983).

Proof of the predetermined lineage hypothesis may rest on the identification of a stable lineage marker. Until then, the weight of evidence supports the specificity hypothesis of CD5 B cell formation.

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THE ORIGIN OF IMMUNE REPERTOIRES BY MEANS OF NATURAL SELECTION

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INTRODUCTION

Biological systems are the result of opposing forces between conservation and flexibility. Both evolutive choices share their submission to the very same natural selection constraints, together to random genetic drift of nearly neutral mutations (*Allen*, 1991). In the immune system, between the presumed genetic potentiality to generate variable (V) regions (its random element) and individual V region repertoires, there are discriminating filters and driving forces. They should focus our interest if we want to get some insight of the "immunological topography" (*Jerne*, 1960), physical substrate of what has recently been called the "intentionality" of the system (*Cohen*, 1992). Interspecies evolutive selection from bony fishes to man (although not necessarily moving in an ascending way) results in a Darwinian pressure to increase, through gene duplication, variability of immune receptors, acting specifically over their (hypervariable) CDR regions (*Tanaka* and *Nei*, 1989; *Schroeder* et al., 1990; *Tutter* and *Riblet*, 1989; *Schwager* et al., 1989; *Ghaffari* and *Lobb*, 1991). Throughout the ontogeny of the individual, there are germline-encoded, developmentally controlled patterns of expression of particular V genes (*Yancopoulos* et al., 1984). In vertebrate development, and in the critically initial conditions, the cellular fates use to be consequence of continuing cell-to-cell interactions in defined times and micro-environments, more than the result of intrinsically programmed cell lineages developing in mosaic patterns (*Wilkins*, 1986; *Raff* et al., 1991). They adjust the genome potentiality to basic levels of variability (its norm of reaction) fitted to the requirements of each moment, their early products, however, frequently persisting for the whole lifespan of the individual as it is the case for perinatal lymphoid subsets in mouse. In a third level, ligand-dependent selection (superantigens, epitopic fine specificities) is required for the full development (*Era* et al., 1991) and unique picture of each V-repertoire, at its most unpredictable component. To summarise, between what is possible and the real V-region repertoire, there are: 1) the evolutionary filter, 2) the constraints of tightly regulated gene expression in development, and 3) mechanisms of somatic selection imposed by both self and non-self environments. To know what repertoire modifications take place at each level of selection (evolutive, developmental, somatic Ag-dependent) is relevant to elucidate the pathogenic mechanisms underlying immune-mediated diseases, as well as the eventual possibilities of immunomodulation. We

also consider that this is the only way of having some insight into the physiological function of this biological complex. And, although not definitive, these analyses might provide (semi-quantitative) information about the antigenic world which "interests" more the immune system, be self, foreign or any other unclassified pattern.

EVOLUTION, GENOMIC ORGANISATION AND STRUCTURE OF THE V-REGION SYSTEM

Immunity (that is, resistance to pathogenic encounters) and self-non-self discrimination exists both in invertebrates and in vertebrates (*Theodor*, 1970). The structural bases of these properties, however, completely differ between the two subphyla, and vertebrates utilise the diversity of variable receptors (Ig, TCRs), built from rearranging DNA fragments, as well as they rely on the very polymorphic MHC locus. V receptors are multidomain proteins, evolving through gene duplication (the Ig superfamily) from primordial receptors of intercellular adhesion (CAMs), which are characterised by homophilic binding and auto-affinity (*Edelman*, 1987). Each one of these domains, being encoded by a different exon, is supported by intra-segment interactions defining its local functionality, and it is able of autonomous selection (*Simon* and *Rajewsky*, 1990). The phenotype emerging from these "supergenes" is somatically impinged by the products of the polymorphic MHC locus, genetic substrate of individuality (*Holmberg* et al., 1984; *von Boehmer* et al., 1978). V-regions represent the compromise between evolutive forces of positive selection for variability (*Tanaka* and *Nei*, 1989) *versus* the conservation of basic requirements for binding to the ligand. These two alternatives are distributed inside the same Ig V region (hypervariable CDR 1-3 *versus* framework regions) and they also distinguish different V_H families, some of which are highly conserved inter-species (*Ghaffari* and *Lobb*, 1991; *Meek* et al., 1991), some others represent evolutive newcomers. While the same VL is frequently used to form Abs with different specificities, this is rarely true of V_H, suggesting that the Ab-forming system rely much more on V_H than on V_L (*Kabat* and *Wu*, 1991). This could also apply to TCR Vβ versus Vα, but formal data are lacking. It is suggestive for this point to note that both Ig V_H and TCR Vβ are first rearranged, and expressed alone in cellular stages, which support very high turnovers and stringent selection mechanisms. V genes are classified in families, defined by the internal homology of their components; they display very different complexity sizes between them. Also, on the basis of the conservation of solvent-exposed, FR1 intervals, V_H families have been clustered in clans, which are maintained through evolutionary barriers (*Kirkham* et al., 1992). The loop region of FR3 determinants differentiates the families within a clan. As it has been revealed for Vβ-homologous determinants (*Choi* et al., 1990; *Cazenave* et al., 1990), these conserved residues could be involved in the initial recognition of clan/family-specific ligands in H-chain expressing precursors, previously to the full development of H-L Ig clonal specificity. These genes are distributed throughout the H chain locus either in clusters (mice) or more interspersed (humans) (*Meek* et al., 1990; *Schroeder* et al., 1990; *Walter* et al., 1990; *Shamblott* and *Litman*, 1989; *Amemiya* and

Litman, 1990; *Schwager* et al., 1989)

The selection of V genes (evolutive, developmental, somatic) passes in all cases through their products, by means of encounters with complementary ligands. However, V genes only account for the less variable CDR1 and CDR2 regions, and some residues of CDR3, which is the most relevant site to define Ag binding. Concerning the dominant H, β and γ chains, this CDR3 region is completed by combinatorial rearrangement to D and J segments, by exonuclease-dependent nucleotide nibbling, and the addition of non-templated P (complementary to the last two bases at the end of the coding joints) and N nucleotides (these latter only in H, β and γ, but no or very little in L, α and δ chains). All together, these mechanisms are probably more relevant in the building of target determinants for the ongoing selection of Ig repertoires. The former ones (use of particular V gene families, conservation of framework determinants, etc.), are based on stringent genetical constraints, and are probably devoted to assure the best starting of the system. Besides the described junctional diversity, V-D-J rearrangements in H and β chains give potentially rise to three DH reading frames. In most of adult B cells, only one, RF1, is evolutive and somatically selected; early B cells show, however, a more diversified usage of RFs (*Gu* et al., 1990), and no preferences are evident among TCR β chains (*Prochnicka-Chalufour* et al., 1991). In chicken Igs, whereas there is no DH RF selection in spleen and bone marrow, this is very strong when B cells home the bursa (*Reynaud* et al., 1991). The preferential generation of aminoacid residues (Gly, Ser, Pro, Tyr) implicated in loop formation within the CDR3 has been advanced as responsible factor for these DH RF biases (*Abergel* and *Claverie*, 1991).

Finally, the most recently evolved mechanism for diversity generation is Ag-triggered, somatic hypermutation, acting over rearranged Ig V segments and their flanking sequences (10^{-3}/bp/cell generation, 10^3-10^4-fold the basal levels taking place in the rest of the genome) (*Lebecque* and *Gearhart*, 1990). In the course of immune responses, it multiplies the offering of V regions for the best Ag fit (*Rajewsky* et al., 1987; *Milstein*, 1990; *French* et al., 1989). Besides their unbiased usage of DH RFs, TCR genes also differ from Ig genes in their lack of somatic hypermutation. Significant biases from the neutral replacement/silent (R/S) ratio of mutations (*Shlomchik* et al, 1987) towards the R component demonstrate the positive selection of these clonal specificities. Negative selection for aminoacid changes, either in order to maintain global Ig structure (in FR regions) or because germline Ig sequences already displayed ideal binding for certain Ags (e.g. bacterial polysaccharides) reduces R/S ratios. Also, there is the possibility that early B-cell populations and most of selected natural antibodies disregard this mechanism of diversity (*Forster* et al., 1988; *Rajewsky* et al., 1989). Amphibia, for instance, although able to hypermutate, do not select these mutants. Consequently, they do not have significant affinity maturation in ongoing responses, either because the long lifespan and advantage of initial cells, or due to the lack of germinal centres where intraclonal competition of Ab mutants happens in mouse (*Wilson* et al., 1992; *Jacob* et al., 1991). In selected situations and in species as chicken, rabbits and probably humans, gene conversion mechanisms also diversify rearranged Ig sequences (*Reynaud* et al., 1989; *Becker* et al., 1990; *Sanz*, 1991).

These mechanisms of V-region diversity generation plus the unrestricted

pairing of H and L chains (*De Lau* et al., 1991), are all devoted to construct Ig molecules which will bind ligands through energetic interactions, at least able to displace water molecules. From this basic constraint to the high-affinity, highly specific, Ag-Ab complex, a dynamic process of physical selection will take place between the two components of the complex. The optimal interaction is not necessarily the strongest one, if only because this might disturb complex dissociation, reduce its half-live by increasing elimination and, consequently, limit the global efficacy. Crystalographic work has revealed that all the possible human Igs ascribe to a reduced number of canonical structures, defining the CDR fold (mostly, CDR1 and CDR2) on the nature of a small number of conserved residues and the length of the loop (*Chothia* and *Lesk*, 1987). They can provide a certain insight of the presumed ligands for the Ab, as e.g. protein-binding Abs tend to have flat surfaces, while hapten and other Ag-specific Abs show deep grooves or pocket-like binding sites. It is intriguing to note that these canonical structures contain very different sequences from several unrelated V_H families (*Chothia* et al., 1992), posing a question to the analysis of V_H distribution as representative of Ig selection for fine Ag specificity. These restrictions in Ab conformation further underline the value of additional (junctional) mechanisms of diversity, and eventually, reduce previous calculations about repertoire sizes based in maximal genetic potentialities.

This genetic potential has a tissue-specific expression in haematopoietic cell lineages that, in contrast to other systems, conserve a germline pool of immature stem cells (HSCs). This means that V-region repertoires can be (more or less) continuously renewed from this source of novelty, what generates the necessity for stringent mechanisms of somatic selection throughout the lifespan of the individual. The rules (cellular and genetic) governing the entry of new V-region specificities in early ontogeny versus those displayed in the adult are probably rather distinctive. For example, many more TCRs emerge from the very active postnatal thymus, while adult T lymphocytes are mostly maintained by clonal expansion of mature lymphocytes in the periphery.

WHY V-REGION REPERTOIRES OF EARLY ONTOGENY DIFFER SO MUCH FROM THOSE USED IN THE ADULT

The expression of the mature Ig V_H repertoire in the adult mouse usually does reflect the genomic complexities of V_H families, with small mouse strain-dependent variations (*Wu* and *Paige*, 1986; *Zouali* and *Theze*, 1991). Very soon, however, it was observed that there is a preferential rearrangement and expression of the most J-proximal, 3' V_H genes early in ontogeny, in pre-B cells and in continuously, newly-emerging lymphocytes (*Yancopoulos* et al., 1984; *Perlmutter* et al., 1985; *Schroeder* et al., 1987; *Schroeder* and *Wang*, 1990; *Malynn* et al., 1990; *Freitas* et al., 1990; *Decker* et al., 1991; *Berman* et al., 1991). These genes are little polymorphic (*Sanz* et al., 1989) and highly conserved elements between species (*Ghaffari* and *Lobb*, 1991; *Meek* et al., 1991), classical features attributed to biologically relevant molecules. This ontogenic pattern does not completely apply to VL genes, which shows non-stochastic biases early in development, that are not related with chromosomal position (*Kaushik* et al., 1989; *Teale* and *Morris*, 1989; *Kalled*

and *Brodeur*, 1990; *Gulgou* et al., 1990). Both H and L chains family preferences result in the fact that nearly half of all mature, resting B cells in perinatal stages are accounted by only 6 V_H + Vκ family pairs (*Kaushik* et al., 1990). Concerning the TCR-forming chains, Vγ genes also appeared in a developmentally-controlled series of waves, probably due to targeted rearrangements, which are selected and home particular environments (mostly, epithelial layers) (*Haas* and *Tonegawa*, 1992). There is also experimental support that 3' Vα and 5' Jα segments predominate in foetal and neonatal mice (*Roth* et al., 1991), and we lack of clearly positive evidences concerning Vβ genes. Even in the case that more data appear about these V family distributions, it seems that developmental biases linked to chromosomal position are more remarkable among early V_H, and Vγ genes. They remind of the genetic organisation of enzymes responsible for integrated metabolic pathways, which are also codified in gene clusters, or the regulated expression of developmentally crucial genes on the basis on their order in broad units of transcription in the chromosome (*Edelman* and *Jones*, 1992).

The change from D-proximal to global V_H family usage is clearly influenced by somatic selection pressures, be external encounters (*Bos* and *Meeuwsen*, 1989) and/or natural Igs (*Freitas* et al., 1991). It is surprising that, while the usage of V_H families in the mature compartments is well defined in each mouse strain and roughly related to their size, individual V_H genes are very strongly selected in each individual, from the primary organs to the periphery (*Gu* et al., 1991). It seems an open question to us whether the mechanisms implicated in family usage (or normalisation?) follow different rules (*Kirkham* et al., 1992) from the Ag-dependent selection of individual genes.

As exposed above, junctional diversity is a relevant component of the paratopic specificity at the CDR3. The length of this domain increases progressively with time, substantially expanding the diversity of a single V(D)J rearranged gene. While exonuclease nibbling and P nucleotides addition are constant throughout life, there is a progressive increase in the addition of non-templated N nucleotides to H, β, and γ D-J and V-D junctions, probably due (but not only) to TdT enzymatic activity (*Feeney*, 1990, 1991; *Gu* et al., 1990; *Itohara* and *Tonegawa*, 1990; *Bogue* et al., 1991; *Bangs* et al., 1991; *Rellahan* et al., 1991; *Meek*, 1990). Perinatal repertoires, already restricted at the level of V family utilisation, are further limited in junctional variability, and this restriction is tightly controlled by both developmental and somatic selection forces (*Ikuta* and *Weissman*, 1991; *Bogue* et al., 1991; *Carlsson* et al., 1992). Finally, most of early Ig repertoires do not experiment the diversity potential of somatic hypermutation, and maintain their germline character. In mouse, but not so clearly in humans, this is also true of the majority of ligand-selected peripheral B cells (*Gu* et al., 1991). It thus seems that somatic mutation is only triggered by a particular signalling pattern whose constraints (Ags, T-cell stimuli, germinal centre microenvironments, etc.) are not fully elucidated.

From the point of view of the molecular components of early V-region repertoires, it can be concluded that they are few -in terms of diversity- (much less than in adult times), they are very conserved, and they do not utilise further diversification mechanisms appearing in the adult. Although it can be argued that this design is due to system "immaturity", the starting V-repertoires are fully functional in terms of recogni-

tion and response, and they actually persist for the whole lifespan of the individual. Their pattern of recognition is devoted to self-binding (autobodies), V-to-V region connectivity, self-Ag recognition and multireactivity (*Kearney* and *Vakil*, 1986; *Holmberg* et al., 1986; *Carlsson* and *Holmberg*, 1990; *Lehuen* et al., 1992). Subsequent to this, they connect apparently unrelated immune pathways, and modifications in their dynamics induce dramatic changes in adult immune behaviour (*Vakil* et al., 1986). Probably due to both expanding environments and driving internal complementarities, these initial cell compartments are highly activated. They will be quantitatively dominated in adult times by clones prepared to react with non-self new encounters. Concerning the biological value of these findings, our current experimental tools should allow us to analyse: 1) the implication of each one of the selective levels defined before (evolutive, developmental, somatic) in the building of these very different repertoires, and 2) the eventual dispensability of each component and mechanism, due to system redundancy or use of parallel pathways, for the global integration and physiology of the immune system.

B CELL POPULATIONS, COMPARTMENTS, LINEAGES, REPERTOIRES. WHAT IS THE MATTER?

Mouse B lymphocytes are divided, on the basis of surface Ag patterns, in clearly distinguishable cell groups (*Herzenberg* et al., 1992). As it was shown for erythrocyte and macrophage populations, haematopoietic stem cells (HSC) from different sources and/or timings tend to differentiate *in vivo* to particular sets of B lymphocytes (*Kantor* et al., 1992). The earliest HSCs detected in the embryo produce, upon cell transfer/graft, perinatal B cells (B1a, B1b), while bone marrow-derived HSCs differentiate preferentially to adult (B-2) cells. These differentiation events happen in a sort of wave-like fashion, as, when advancing in time, the maturation to previous cell subsets goes down or is even exhausted, while the next one expands and dominates (*Marcos* et al., 1991). Although it was postulated the existence of intrinsically different progenitors (*Herzenberg* and *Herzenberg*, 1989), we consider more plausible the view that, while maintaining their totipotentiality, HSCs undergo selected fates after distinctive intercellular regulation in development. Alternatively (and not in an exclusive way), a sort of HSC clock counting the past number of cell divisions versus the timing where the HSC is thrown in a pathway of cell commitment by asymmetric division, might also be considered (*Holliday*, 1991). Emerging from the same HSC pool, various experimental set-ups (*in vitro* culture, cell transfer, graft) reveal different mature populations, in some cases, preferentially revealing HSC potentiality (*in vitro*), in others, more close to their actual cell fates (grafts). Our current experiments and views support that branching decisions for different B-cell populations are developmental, although a lot of work remains to be done, in order to elucidate the variables implicated in these processes (Table 1).

Together to the disappearance of embryonic haematopoietic potentials, there is an arrest in the entry of novel B cell and V-region specifities in the primordial cell populations, which, however, persist throughout life. They need to be maintained by clonal survival and/or expansion, and they are submitted to the

Table 1: Generation of B lymphocytes throughout mouse ontogeny

PROGENITOR CELLS		B-CELL PRODUCTION		
Sites	Timing	*In vitro*	Cell transfer	Graft
Yolk sac	8-10 d	++	+/-	- -
Emb. (below diaphragm)	8-10 d	n.d.	++	n.d.
Embryo	9,5 d	++	n.d.	n.d.
Para-aortic Splanchnopleura	8,5-9 d (10-18 som.)	++	??	++ (B1a)
Omentum	13 d	++	n.d.	++ (B1a>B1b)
Liver	11-19d	++	++	n.d. (B1b>B1a>B2)
Spleen	15-5 d post-birth	++	++	n.d. (B1b>B2>B1a)
Bone marrow	adult	++	++	n.d. (B2>B1b>>B1a)

POTENTIALITY → CELL FATE
Microenvironment
Cell interactions
Short-range growth factors
Genetic programs

Earlier HSC asymmetrical divisions
More germline constrained
Lower repertoire size; less open to novelty

Latter HSC asymmetrical divisions
Full display of genetic diversity

The data summarised in this Table came from: *Paige* et al., 1979; *Tyan* and *Herzenberg*, 1968; *Ogawa* et al., 1988; *Godin* et al., 1992; *Solvason* et al., 1992; *Velardi* and *Cooper*, 1984; *Kantor* et al., 1992; and our unpublished material.
n.d.: not done, to our knowledge.
??: ongoing experiments.

same kind of selective pressures than other peripheral B cells. This long-term Ag selection obviously can result in oligoclonality (*Stall* et al., 1988). Although perinatal B lymphocytes are relatively "diluted" throughout life by adult populations, this is probably not so much the case for one of their most relevant aspects, that is, Ab secretion by plasma cells: Experimental findings from different sources all agree with the fact that a big fraction of adult plasma cells and natural Abs in the serum came from these early waves of differentiation (*Forster* and *Rajewsky*, 1987; *Kroese* et al., 1989; *Marcos* et al., 1992; *Godin* et al., 1992). And it is clear that, in a cell-per-cell basis, the initial B cells are more prone to plasma cell differentiation than the latter ones. It can be concluded that they are not only selected to persist as available components of the system, but also that they are positively driven to "occupy" a very central role in the B-cell compartment. In this way, critical information about the primordial conditions of immune starting is maintained throughout life in the functional core of the system. Recent studies of *in situ* hybridisation for V_H family usage in Ig-congenic mice, and in PBL cDNA from adult human beings (*Viale* et al., 1992; *Braun* et al., 1992) extend that notion to adult V-region repertoires. Together to any other kind of Ag pattern, the products of these long-lived B lymphocytes could impinge definitively the selection of novel specificities arising from the H chain-expressing, late pre-B cells in the adult bone marrow (*Marcos* et al, 1991; *Freitas* et al., 1991; *Sundblad* et al., 1991). Besides the implication of early-born, long-lived B-1 cells to "remember" perinatal repertoire patterns (*Jeong* and *Teale*, 1990; *Gu* et al., 1990), these

lymphocytes also go through great life periods of cellular selection. This (and not preferential rearrangements) results in the increased expression of certain V_H /V_L chain combinations and specificities only inside these populations, and not in the others, even if located at the same sites (coelomic cavities) (*Andrade* et al., 1989; *Carmack* et al., 1990; *Conger* et al., 1991; *Hayakawa* et al., 1990). The distinctive significance of each one of these parameters (time and selective microenvironment of emergence, cell lifespan and turnover, somatic selection by certain Ags, etc.) in the maintenance of perinatal B-1 cells in the adult is still a matter of debate and current research.

SOME REMARKS ABOUT IMMUNE DISEASE

Behind any abnormal immune functionality, there are disturbed processes at one or several of the selection levels that we have discussed above. Although many accompany the disease, probably, the control of a few critical ones is really relevant to re-establish physiology. We need to distinguish between what necessarily determines a phenotype, and what only reinforces it, or is merely an epiphenomenum. Thus, most of current debates in present-day autoimmunity and lymphoproliferative diseases need to be approached from these perspectives. Several representative examples can be mentioned:

- If we want to understand how BM-uncommitted, lymphoid progenitors can either transfer or cure autoimmune diseases (*Kawamura* et al., 1990), we need to know what epigenetic decisions they have already passed through, in order to be already settled as either normal or diseased.
- Even considering the former statement, autoimmune disease is Ag triggered and Ag-dependent (*Shlomchik* et al., 1987).
- Many Ab specificities, which accompany disease development, reveal the markers of strong Ag selection, and it has been speculated that they could represent a pathogenic component. However, and in the very same individuals, people frequently detect self-reacting germline Abs, preferentially codified by those genes dominant in early ontogeny (*Möller*, 1992). What is the real meaning for pathology of both types of clonal anti-self specifities? The latter ones, are they pathogenic, are they accompanying and irrelevant epiphenomena, or are implicated in some kind of homeostatic, regulatory behaviour? It is possible, for instance, to genetically segregate many of these traits, as it is the case with the increased levels of serum Igs and autoimmune pathology, with recombinant inbred mouse lines (*Datta* et al., 1982). Depending of the answer, one can imagine how much different will be the therapeutical approach to the patient.
- More and more, it has been confirmed the preferential expansion of perinatal lymphocytes in the context of immune disease (*Marcos* et al., 1988). We only have some guesses of the putatively disrupted filters, which drive these populations out of their ontogenical timing.
- Although with contradictory data, there are clear descriptions of TCR Vβ restricted usage in some autoimmune conditions. Is this because the stringent immune response, either to some kind of "superantigens" or to fine MHC-peptide specificities, or are there some more basic restrictions to the whole display of TCR diversity?
- Depending of the Ig V-repertoire they

express, the spectrum of B-cell neoplasias extends from clinically indolent, early germline Ig-expressors (B-chronic lymphocytic leukaemia, monoclonal gammapathy of undefined significance, Waldenström's macroglobulinaemia, etc.) to more aggressive tumours, bearing a representation of all Igs, which are frequently mutated (follicular lymphoma, multiple myeloma, etc.). We consider that these patterns of B-cell neoplasia's clonotypes can provide relevant information about the lymphoproliferative origin, and their future evolution under somatic selection pressures (*Friedman* et al., 1991; *Marcos* et al., 1991).

This list is obviously unfinished, and we only wanted to underline with it how important we consider the study of basic principles of V-region repertoire building for the better approach to clinical problems. The behaviour of our immune system is simply the result of complementary solutions (*Coutinho*, 1989) to the challenge of natural selection, interspecies, in the developmental uncoiling of DNA information, and confronted with a world of distinctive patterns and novelty.

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T CELL REPERTORE SELECTION BY SUPERANTIGENS

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SUMMARY

Superantigens are substances of bacterial or viral origin that interact specifically with the β-chain of the T cell receptor. During development, immature T cells encountering superantigens are physically eliminated in the thymus by a mechanism of programmed cell death (apoptosis). In contrast, mature T lymphocytes in the periphery respond to superantigens by proliferation and acquisition of effector functions. This initial response is often followed by inactivation and/or death of the superantigen-reactive T cells. Thus superantigens provide a useful model system to investigate the fate of T cells encountering neo-self antigens at different stages of development.

INTRODUCTION

The recognition of foreign antigens by T lymphocytes is mediated by the T cell receptor (TCR), which is a heterodimeric molecule composed of α and β chains (*Davis* et al., 1988; *Marrack* and *Kappler*, 1987). Like the immunoglobulins, TCR α and β chains contain variable (V) junctional (J) and constant (C) domains that are encoded by distinct genetic elements that recombine during ontogeny. In the mouse there are 20 Vβ elements and 12 Jβ elements whereas the number of Vα and Jα elements is estimated at ~100. This large genetic pool allows a considerable permutational variability in the specificity of the TCR, which is necessary to cope with the large number of foreign antigens and pathogens that can be encountered by a given individual.

In contrast to immunoglobulins, TCR recognize a complex ligand formed by the binding of a small (processed) peptide to molecules encoded by the major histocompatibility complex (MHC) (*Bjorkman* et al., 1987). Two types of mature T cells can be distinguished on the basis of their ligand specificity: $CD4^+$ cells recognize peptides bound to MHC class II molecules whereas $CD8^+$ T cells recognize peptide: MHC class I complexes (*Swain*, 1983).

During development in the thymus, T cells are positively selected by interaction with self MHC molecules (*von Boehmer*, 1986). In this way mature $CD4^+$ and $CD8^+$ T cells are subsequently able to recognize foreign antigens (processed peptides) only in association with appropriate self MHC class II or I molecules, respectively. (This process is frequently referred to as MHC restriction). At the same time, developing T cells are subjected to negative selection events to ensure that cells with autoreactive TCR are functionally eliminated from the repertoire (*Marrack* and *Kappler*, 1987; *von Boehmer*, 1990). This latter process (also referred to as immunological tolerance) will be discussed in detail in this review.

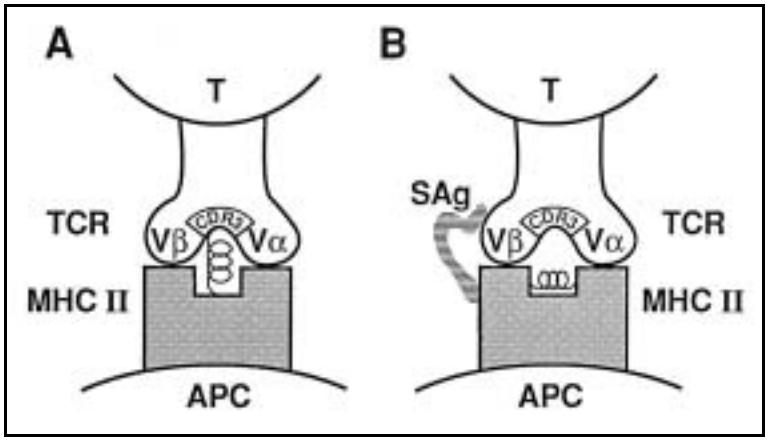

Figure 1: T cell receptor interaction with conventional (peptide) antigens or superantigens.

A. Processed peptides (▮) bound to MHC class II molecules on antigen-presenting cells (APC) interact specifically with the hypervariable CDR3 region of the TCR.

B. Superantigen (SAg) binds (either simultaneously or sequentially) to both MHC class II and a site on the outer surface of TCR Vβ. This binding does not require a specific peptide: CDR3 interaction as in A.

SUPERANTIGENS

As mentioned above, T cell recognition of conventional (peptide) antigens associated with MHC molecules is mediated by the TCR. In general the specificity of this type of recognition is dependent upon the contribution of most (or all) elements that confer TCR variability (Vα, Jα, Vβ, Jβ). An apparent exception to this generalization is found with a recently characterized group of substances referred to collectively as "superantigens". The recognition of superantigens by the TCR seems to depend only upon the Vβ domain, with no apparent contribution from any of the other variable TCR elements. However, like conventional antigens, superantigens require presentation by MHC molecules. To date only MHC class II molecules have been shown to bind and present superantigens to T cells. A schematic comparison of TCR recognition of conventional peptide antigens and superantigens is shown in Figure 1.

Two classes of superantigens are well characterized. The first group is exemplified by the exotoxins of *Staphylococcus aureus*. These substances, which have been known for some time as potent stimulators of T cells, have more recently been shown to act as superantigens in that they bind MHC class II and interact with particular TCR Vβ domains (*White* et al., 1989). The second group of "superantigens" was originally identified as genetic elements that encode a strong T cell stimulating capacity in the mouse (*Festenstein*, 1974). These so-called minor lymphocyte stimulatory (Mls) determinants were subsequently shown to behave as Vβ-specific superantigens (*Kappler* et al., 1988; *MacDonald* et al., 1988). Very recently it has further been shown that most (if not all) Mls-like determinants are encoded by endogenous copies of mouse mammary tumor virus (MMTV). The MMTV gene product responsible for this Mls activity is located in an open reading frame (orf) in the 3' long terminal repeat (LTR) of the virus (*Acha-Orbea* and *Palmer*, 1991).

Table 1: Summary of best characterised murine Vβ-specific superantigens

Superantigen	TCR Vβ
SEA	1, 3,10,1 1, 17
SEB	7, 8.1, 8.2, 8.3,17
SEC1	8.2, 8.3,11,17
SEC2	8.2,10, 17
SEC3	7, 8.1, 8.2
SED	7,8.1,8.2,8.3,11, 17
SEE	11,15, 17
TSST-1	3,15, 17
Mls-1 (Mtv-7)	6, 7, 8.1, 9
Mls-2 (Mtv-13)	3
Mls-3 (Mtv-6)	3
Mls-4 (Mtv- 1)	3
Etc-1 (Mtv-9)	5.1, 5.2, 11

For further information see: *Abe* and *Hodes* (1989), *Herrmann* and *MacDonald* (1991) and *Janeway* (1991)

A summary of known superantigens and their corresponding TCR Vβ specificities is given in Table 1.

Details of the presumed trimolecular interaction between superantigens, MHC class II molecules and TCR Vβ domains remain to be defined. For bacterial enterotoxins (which are readily available in pure form) it is clear that high affinity binding to MHC class II molecules occurs (*Marrack* and *Kappler*, 1990); however no direct enterotoxin; TCR Vβ binding has yet been demonstrated. The situation with Mls determinants is much less clear, since the MMTV orf protein has only recently been shown to encode the functional superantigen. Nevertheless site-directed mutagenesis experiments have provided indirect evidence that residues in TCR Vβ outside of the presumed peptide: MHC binding domain are involved in contacting both bacterial enterotoxins and Mls (MMTV) gene products (*Choi* et al., 1990; *Dellabona* et al., 1990; *Pullen* et al., 1990).

T cell responses to superantigens differ from conventional antigen responses in several aspects (Table 2). First, because of their Vβ specificity, superanti-

Table 2: Comparison of T cell responses to antigens and "superantigens"

Property	Antigens (peptides)	Superantigens	
		Mls	Enterotoxin
Frequency	low ($< 1/10^4$)	high (1/10)	high
T cell receptor	unique (α/β)	Vβ restricted	Vβ restricted
MHC requirement	class I or class II	class II (E>A)	class II (not species specific)
Phenotype	$CD4^+$ (class II) $CD8^+$ (class I)	$CD4^+$ and $CD8^+$	$CD4^+$ and $CD8^+$

gens elicit a response from a high proportion (up to 20%) of T cells in a primary response. In contrast, the frequency of T cells responding to peptide: MHC complexes are usually undetectable without prior immunization. Second, the presentation of superantigens by MHC molecules is distinct from conventional antigen presentation. All superantigens characterized to date are presented by MHC class II molecules, whereas both class I and II MHC molecules can bind and present antigenic peptides. Third, both mature T cell subsets ($CD4^+$ and $CD8^+$) respond to superantigens, despite the fact that only $CD4^+$ cells respond to peptide antigens presented in association with MHC class II.

Because of their specificity for TCR Vβ domains, superantigens are powerful tools to study the selection of the T cell repertoire *in vivo*. Since a given Vβ domain is expressed by a significant proportion of T cells (on average 5% in the mouse), it is feasible (with the aid of Vβ-specific monoclonal antibodies) to follow the fate of potentially autoreactive T cells in an unambiguous way. The following sections describe in more detail how this property of superantigens has been exploited experimentally.

CLONAL DELETION DURING INTRATHYMIC DEVELOPMENT

During development, the thymus is colonized by stem cells originating in the fetal liver (or adult bone marrow). These early stem cells of the T lineage have an immature $CD4^-$ $CD8^-$ phenotype (although they appear to transiently express low levels of CD4, at least in the mouse) and have not rearranged their TCR genes. Once in the thymus, $CD4^-$ $CD8^-$ cells sequentially rearrange and express their TCRβ and TCRα genes. In parallel they acquire surface expression of both CD4 and CD8. The majority population in the thymus, located primarily in the cortex, belong to this $CD4^+$ $CD8^+$ TCR^+ subset. At this stage of development, $CD4^+$ $CD8^+$ cells become committed to the CD4 or CD8 lineage, and those cells with appropriate TCR specificity for self MHC are positively selected for survival. This complex (and still poorly understood) series of events results in the generation of mature $CD4^+$ and $CD8^+$ T cells restricted to MHC class II or I, respectively. These mature cells are located primarily in the thymic medulla and are destined to emigrate to the periphery. A summary of these events is given in Figure 2.

The fate of autoreactive T cells during thymic development was first directly investigated by exploiting the correlation between TCR Vβ specificity and the presence of endogenous (MMTV-encoded) superantigens. These studies showed unequivocally that most autoreactive T cells are clonally deleted as they mature in the thymus (Kappler et al., 1987). Similar results were obtained by injecting exogenous superantigens (Staphylococcal enterotoxins) in neonatal rnice, resulting in clonal deletion of the appropriate Vβ subset (*White* et al., 1989). These conclusions were subsequently confirmed by independent experiments involving transgenic mice bearing TCR α and β chains of defined MHC antigen specificity. Again clonal deletion in the thymus was observed when developing T cells encountered self antigens (*von Boehmer*, 1990).

By employing superantigen: Vβ correlations as well as transgenic TCR models, the process of clonal deletion in the thymus has been intensively investigated over the last several years. From such studies, it is clear that clonal deletion can occur at several different stages

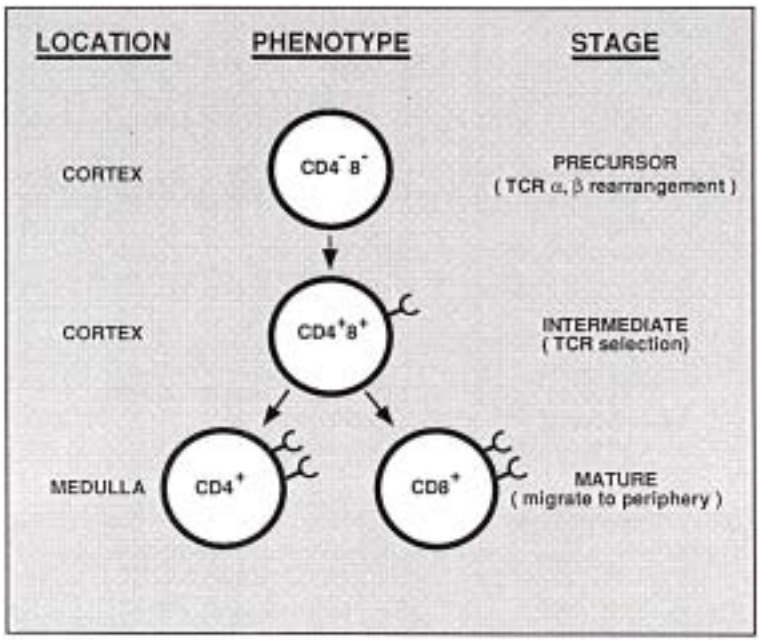

Figure 2: Stages of intrathymic development.
$CD4^-8^-$ precursor cells give rise to mature $CD4^+$ and $CD8^+$ T cells via a $CD4^+8^+$ intermediate stage. Following TCR α and β gene re-arrangement, the TCR (⊥) is first expressed at low levels on the cell surface at the $CD4^+8^+$ stage. Positive and negative selection then takes place, leading to the survival of mature $CD4^+$ and $CD8^+$ cells expressing high TCR levels. For further details see: *Fowlkes* and *Pardoll* (1989).

of intrathymic development. Depending on the self antigen encountered, clonal deletion may occur either very early ($CD4^+$ $CD8^+$ stage) or relatively late ($CD4^+$ or $CD8^+$ stage). The factors responsible for this variability in stage of deletion remain to be determined. One obvious explanation would be that clonal deletion occurs whenever there is sufficient TCR avidity for the self ligand. Since TCR avidity increases during development with increasing TCR surface density, it would follow that the threshold required for clonal deletion could be reached at different developmental stages for different self ligands. Alternatively it is possible that interaction with specialized thymic stromal cells (or production of specific co-factors) contribute in some way to the deletion event.

It is now widely accepted that the mechanism of death of autoreactive thymocytes is closely related to the programmed cell death (also referred to as apoptosis) that occurs in many developmentally regulated systems (*Cohen*, 1991; *Golstein* et al., 1991). Apoptosis involves rapid morphological changes (including conspicuous condensation of nuclear chromatin) followed by degradation of internucleosomal DNA (*Wyllie* et al., 1980). This type of death can be prevented (or delayed) by inhibitors of de novo RNA or protein synthesis. The nuclease responsible for DNA cleavage has not been identified, nor has it been determined whether DNA degradation per se is a causative (or secondary) event in apoptosis.

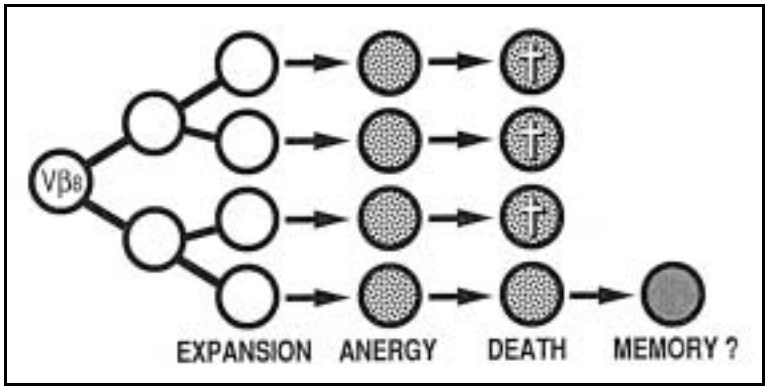

Figure 3: Sequence of events following *in vivo* injection of superantigens.
Administration of SEB (specific for $V\beta8^+$ T cells) results in an initial clonal expansion of $V\beta8^+$ cells in peripheral lymphoid tissues followed by cell death and anergy of the surviving $V\beta8^+$ cells. The relationship of this process to the generation of memory cells is unknown. A similar sequence of events occurs when other superantigens are injected, except in this case other TCR Vβ are involved (see Table 1).

PERIPHERAL TOLERANCE

Despite the potential efficiency of clonal deletion of autoreactive T cells, it is evident that not all self antigens can be expected to be expressed in the microenvironment of the developing thymus. For example, antigens expressed in specialized tissues (as well as antigens expressed late in ontogeny) would be expected to encounter mature T cells after they have left the thymus. For these reasons it would be anticipated that tolerance mechanisms distinct from clonal deletion should exist.

In the context of the superantigen models discussed here, peripheral tolerance can be investigated by injecting adult mice with superantigens and monitoring the subsequent behavior of reactive T cells expressing appropriate TCR Vβ domains. Such experiments have recently been carried out both for bacterial enterotoxins (*Kawabe* and *Ochi*, 1991; *MacDonald* et al., 1991; *Rellahan* et al., 1990) and Mls antigens (*Dannecker* et al., 1991; *Rammensee* et al., 1989; *Webb* et al., 1990). In both cases, injection of superantigens leads to an initial clonal expansion of T cells with reactive Vβ domains in lymph node and spleen. This expansion involves both $CD4^+$ and $CD8^+$ subsets, as would be predicted from the *in vitro* behavior of superantigens. Following this expansion period there is a rapid decline in the number of T cells expressing these Vβ domains. *In vitro* studies indicate that this decrease is due (at least in part) to selective death of reactive T cells; however it has not been excluded that other phenomena (such as altered migration patterns *in vivo*) may also contribute to this decrease. The mechanism of superantigen-induced peripheral T cell death is controversial; evidence both for (*Kawabe* and *Ochi*, 1991) and against (*MacDonald* et al., 1991) an apoptotic mechanism has been reported.

Despite extensive cell death, many T cells with superantigen-reactive Vβ domains survive *in vivo*. These remaining cells exhibit a tolerant (or anergic) phenotype inasmuch as they specifically fail to proliferate when re-challenged with the relevant superantigen *in vitro*. This

failure to proliferate correlates with a lack of interleukin-2 (IL-2) production by the tolerant cells. The molecular basis of this anergic state remains to be elucidated; however it is clear that IL-2 mRNA accumulation (as assessed by Northern blot) is greatly reduced in anergic T cells stimulated by superantigens. Further studies should reveal whether this represents decreased rates of IL-2 transcription and, if so, whether this in turn can be related to a deficiency in any of the known transcription factors acting on the IL-2 promoter. A summary of events occurring upon stimulation of peripheral T cells with superantigens is shown in Figure 3.

Few comparable studies on the mechanism of peripheral tolerance have been carried out in other model systems. Nevertheless recent data with TCR transgenic mice suggest that the same sequence of events (i.e. clonal proliferation, death and anergy) occur upon exposure of peripheral T cells to conventional antigens as well (*Rocha* and von *Boehmer*, 1991).

CONCLUDING REMARKS

Superantigens have proven to be a very useful paradigm for elucidating the fate of T cells confronting self (or foreign) antigens at different stages of development. The unique TCR Vβ specificity of these reagents allow (in combination with the relevant monoclonal antibodies) unambiguous conclusions with regard to clonal expansion, death or anergy of reactive cells in various experimental systems. These data complement studies with TCR transgenic mice, where similar conclusions have been reached in many instances. However in contradistinction to the transgenic models, superantigen: Vβ correlations present certain experimental advantages, not the least of which are the possibility to study unmanipulated animals and to use unreactive Vβ subsets as appropriate negative controls.

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POSTTHYMIC DEVELOPMENT OF CD4$^+$ AND CD8$^+$ T CELLS IN RATS

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INTRODUCTION

Peripheral T cells in rats differ in their expression of the membrane determinants CD4, CD8, CD45RC, RT6 and Thy-1 (*Brideau* et al., 1980; *Ely* et al., 1983; *Spickett* et al., 1983; *Thiele* et al., 1987). By combining these markers it becomes possible to identify a large number of phenotypically different subsets of peripheral T cells (*Kampinga* et al., 1992, 1997).

The presence or absence of CD4, CD8, CD45RC or RT6 expression have been associated with differences in T cell functions (for references see *Kampinga* et al., 1997). Such an association has not been found for Thy-l (*Golub*, 1988).

Besides indicating differences in function, these determinants have also been proposed to mark different T cell lineages or different stages of maturation (*Mojcik* et al., 1988; *Powrie* and *Mason*, 1988; *Thiele* et al., 1987). Relating to this subject numerous studies have been performed with CD45RC. So far however, they have generated rather confusing and conflicting data. Some authors claim that the loss of CD45RC is a uni-directional event and marks a subset of memory cells developing from naive CD45RC T cells after activation by antigen (*Powrie* and *Mason*, 1989). Others suggest that the expression of CD45RC is bi-directional and that this marker identifies T cells that exist transiently in different functional or activated stages (*Bell* and *Sparshott*, 1990; *Sparshot* et al., 1991). A similar situation exists for Thy-1 and RT6, for which it is also still not clear whether cells expressing these markers represent different T cell lineages and/or different functions, and whether losing, respectively gaining of these markers is a uni-directional maturation and/or differentiation effect or just reflecting temporary stages of activation (*Mojcik* et al., 1988).

Theoretically, based on the different possibilities of presence or absence of Thy-1, RT6 and CD45RC expression on T cells, 8 different subsets can be created. This number is increased to 27 when also the level of expression intensity (i.e. dull and bright is taken into account. In previous experiments (*Kampinga* et al., 1992, 1997) we have shown that at least 11 of these latter possibilities can be identified in the peripheral lymphoid organs. To establish the relationships between these 11 different subsets, we used the method of vascular thymus transplantation in RT7 congenic rats (*Kampinga* et al., 1990a), and followed the phenotypic changes of donor thymic emigrants during their sojourn in the lymphoid organs. Based on these and additional short-time thymectomy experiments a putative pathway of postthymic T cell development was postulated (*Kampinga* et al., 1997).

In this article we propose a similar, but more simplified diagram, of post

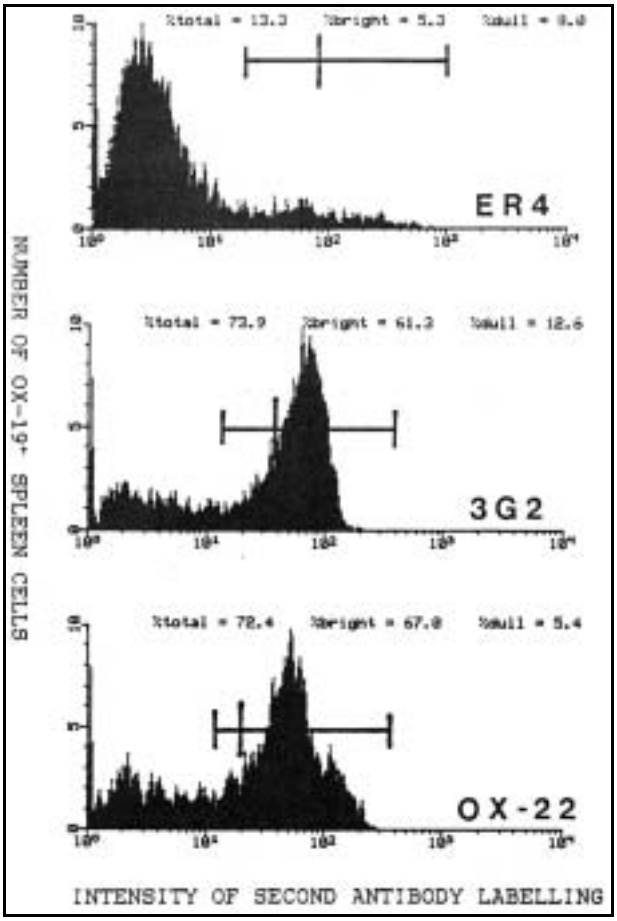

Figure 1: FACS analysis of different T cell subsets (Experiment I).

thymic T cell development and provide additional data, which further strengthens the validity of our hypothesis on the relationships of the different T cell subsets. In addition, new data will be presented showing that $CD4^+$ T cells follow the developmental pathway as originally postulated for all T cells, but that $CD8^+$ T cells take a slightly different route.

MATERIAL AND METHODS

Experiment I

Using standard labelling procedures (*Kampinga* et al., 1997), spleen cells of two SPF 13 wk old male PVG rats were double labelled with FITC conjugated OX-19 (anti-CD5, used as marker to define T cells; *Dallman* et al., 1984) in combination with the biotinylated subset monoclonal antibodies (mab's) ER4 (anti-Thy-1; *Vaessen* et al., 1985), 3G2 (anti-RT6; *Butcher*, 1987) and OX-22 (anti-CD45RC; *Ely* et al., 1983) respectively. The same was done with biotinylated 3G2 and FITC conjugated ER4 and OX-22 respectively. In addition, allo-phyco-cyanine conjugated

Table 1: calculation method used for establishing the percentages of total T cells for each of the 15 different T cell subsets found by OX-22 labelling

Subset phenotype	% of total T cells in spleen	
	FACS	Microscope
Thy-1$^+$ T cells:		
Thy-1^d/RT6$^-$/CD45R$^-$	4.0 (1.2)	4.5 (1.7)
Thy-1^d/RT6^d/CD45R$^-$	1.9 (0.1)	<1
Thy-1^d/RT6^b/CD45R$^-$	1.8 (0.7)	0
Thy-1^b/RT6$^-$/CD45R$^-$	3.4 (0.4)	3.2 (0.6)
Thy-1^b/RT6^d/CD45R$^-$	0.7 (0.1)	0
Thy-1^b/RT6^b/CD45R$^-$	1.1 (0.1)	0
Thy-1-/RT6+ T cells:		
Thy-1$^-$/RT6^d/CD45R$^-$	2.1 (0.5)	1.2 (1.1)
Thy-1$^-$/RT6^d/CD45R^d	0.7 (0.1)	1.9 (1.1)
Thy-1$^-$/RT6^d/CD45R^b	8.3 (0.7)	10.8 (0.9)
Thy-1$^-$/RT6^b/CD45R$^-$	4.5 (0.3)	1.5 (0.3)
Thy-1$^-$/RT6^b/CD45R^d	3.1 (0.1)	2.9 (0.9)
Thy-1$^-$/RT6^b/CD45R^b	51.3 (1.2)	61.2 (2.6)
Thy-1-/RT6- T cells:		
Thy-1$^-$/RT6$^-$/CD45R$^-$	8.0 (0.1)	9.8 (1.7)
Thy-1$^-$/RT6$^-$/CD45R^d	1.5 (0.2)	0
Thy-1$^-$/RT6$^-$/CD45R^b	8.1 (2.8)	4.1 (2.8)

OX-19 was used in combination with FITC conjugated ER4 and biotinylated OX-22. R-PE conjugated Streptavidin was used as a second-stage reagent. For analysis a FACS 440 (Becton Dickinson), equipped with an argon and helium-neon laser, in combination with Lysis (Becton Dickinson) software was used. Lymphocytes were gated according forward and sideward scatter profiles.

Based on the shape of the histograms of both labelled spleen cell populations, and the experience of similar previous FACS studies, three arbitrary gates were established for each of the subset mab's separately (Figure 1). In this way OX-19$^+$ cells were divided in negative, dull and bright labelled cells, for each of the subset mab's respectively. The same gates were used for the double labelling with 3G2 in combination with ER4 and OX-22 respectively. T cells with bright 3G2 staining were gated and within these gated cells the percentage cells with bright ER4 respectively bright OX-22, and dull ER4 respectively dull OX-22 labelling was established. This was repeated for dull 3G2 stained cells. For the triple staining, OX-19$^+$ cells positive for ER4 staining were gated and checked for OX-22 labelling.

The calculation method used for establishing the percentages of total T cells for each of the 15 different T cell subsets found by this labelling procedure is mentioned in Table 1.

Experiment II

Spleen cells and Peyer's patches (Pp's) cells (pooled from 4 Pp's of each rat) obtained from two SPF 13 wk old male PVG rats were labelled as in experiment I. Analysis of the cells was as in experiment I, but cells were now only checked for the presence or absence (and not the intensity) of labelling.

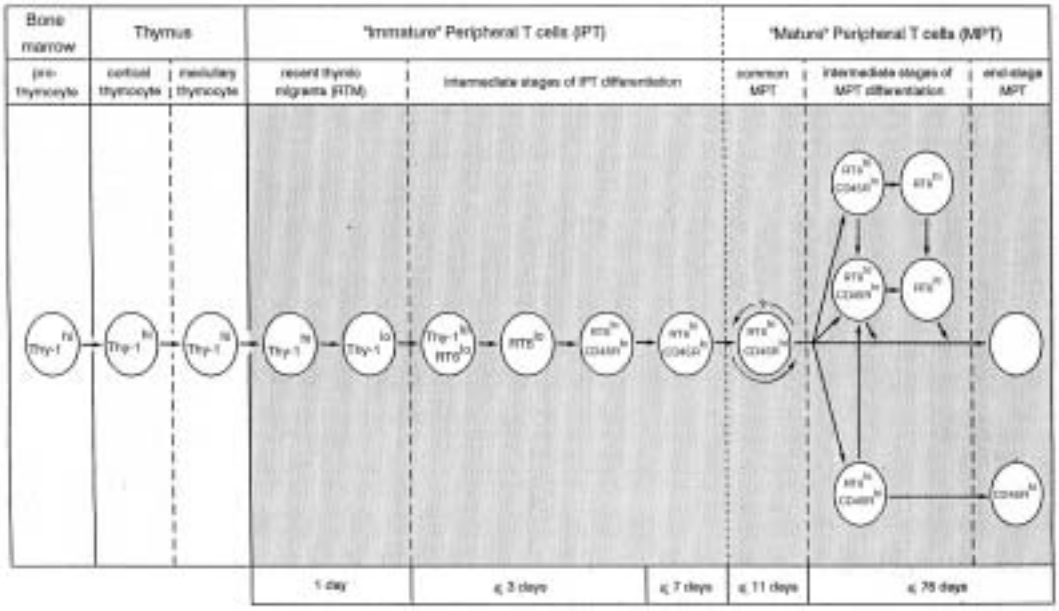

Figure 2: Postthymic T cell differentiation.

Experiment III

Cervical lymph node (CLN) cells (pooled from 4 nodes of each rat) were obtained from two 112 wk old male PVG rats. These rats were kept under SPF conditions for the first 8 wk of life, whereafter they were thymectomised and kept under conventional conditions. Cells were labelled with the same mab combinations as mentioned in experiment I, but instead of using FITC conjugated antibodies, now non-conjugated mab's were used in combination with isotype specific second-stage reagents (*Kampinga* et al., 1997). This method gives a stronger fluorescence signal, which makes it easier to define the difference between negative and weakly positive stained cells. This was especially important, because large numbers of cells lacking Thy-l, RT6 and/or CD45RC expression were expected in this experiment. Pilot experiments had shown that the results of these two staining methods did not differ much from each other when used for T cell subset analysis in young adult rats. Analysis of the cells was as in experiment II.

Experiment IV

Blood leukocytes obtained from 8 wk, 17 wk and 75 wk old male PVG rats (each group n=2) were isolated, using standard procedures, from blood obtained from the tail vein or heart. The same was done for 29, respectively 42 wk old male PVG rats (each group n=2), that were thymectomised at 6 wk after birth (housing conditions for all rats were similar as in the above experiments). Leukocytes were triple labelled using allo-phyco-cyanine conjugated to OX35 (anti-CD4; *Brideau* et al., 1980), respectively OX8 (anti-CD8; *Brideau* et al., 1980) together with the combination of biotinylated 3G2 and FITC-conjugated ER4, and the combination of biotinylated 3G2 and FITC-conjugated OX-22 respectively. For analysis an Elite cell flow cytometer (Coulter), equipped with an argon and helium-neon laser, in combination with Coulter's analysis software was used.

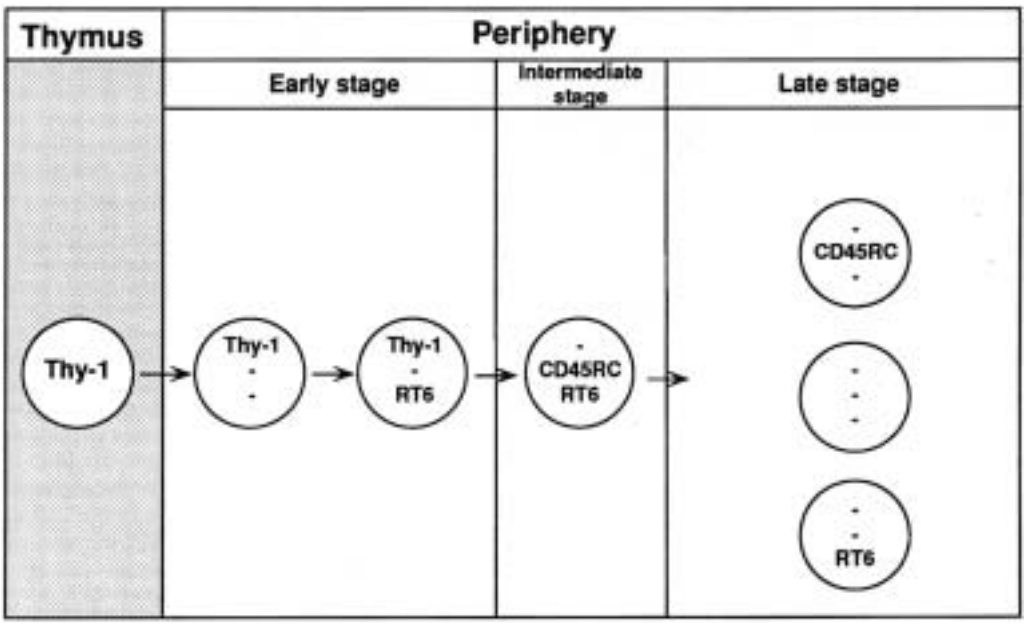

Figure 3: The hypothesis of postthymic T cell development in a more simplified form.

Lymphocytes were gated. In addition, $CD4^+$ respectively $CD8^+$ cells were gated, and within these gated T cell lineages the frequency of the different subsets were analysed for both combinations of subset mab's.

RESULTS AND DISCUSSION

T cell subset analysis by FACS supports the previous hypothesis about postthymic T cell development

The previous hypothesis (see Figure 2) on postthymic T cell development was based on labelling studies using immunofluorescence microscopy (*Kampinga* et al., 1997). With this method 11 different T cell subsets were identified (Table 1). However, counting cells under a microscope may not be very accurate, because the number of cells that can be checked is rather limited and the judgement of whether a cell has a bright or dull labelling is not very objective. Therefore, it was decided that for future studies FACS analysis should be used. However, before doing so it was felt that repeating part of the previous study was needed to see whether the FACS data are comparable with the microscope data, and to find out whether the previous hypothesis still holds despite changing the method.

The results of experiment I (Table 1) show that all subsets identified by microscopy were also found by FACS analysis, and that no major differences in the percentages were found. With FACS analysis 4 additional "new" subsets could be identified. Their respective percentages however, were less than 2% and therefore around the limits of the accuracy of the used method for FACS analysis. But even when they really exist, their presence does not interfere with the validity of the original hypothesis.

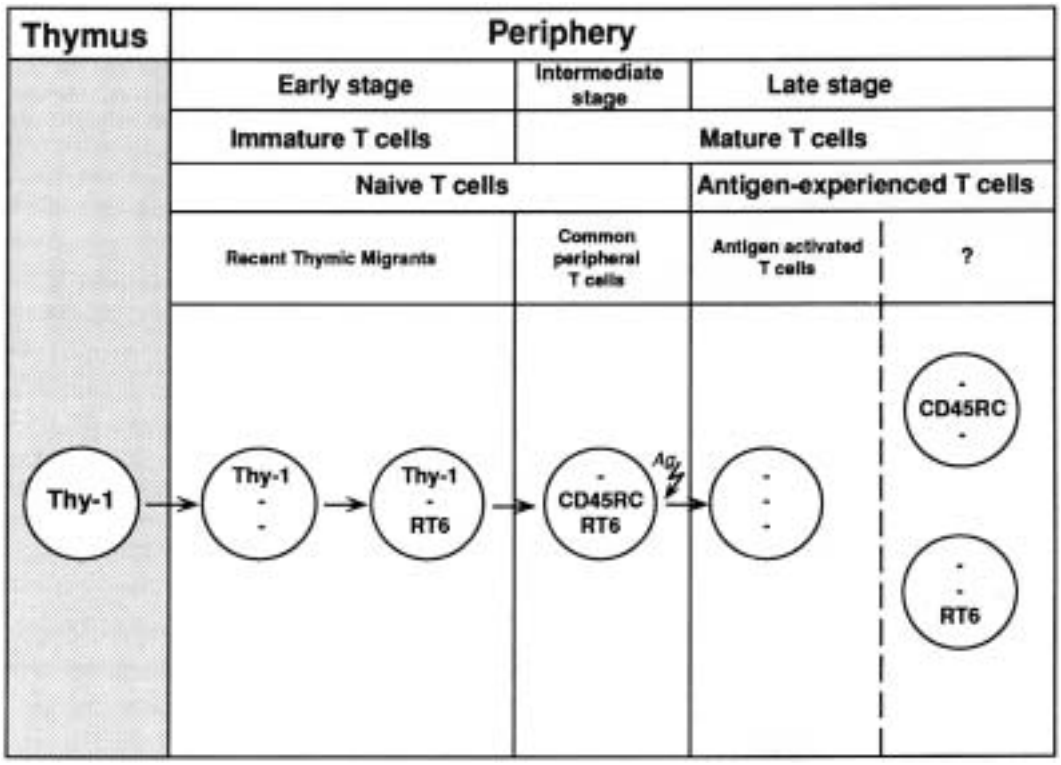

Figure 4: Both Thy-1$^+$ and Thy-1$^-$/RT6$^+$/CD45RC$^+$ T cells are not part of the late stages of postthymic T cell development.

The hypothesis of postthymic T cell development in a more simplified form (Figure 3)

The large number of different T cell subsets shown in Figure 2 makes it very complicated to further unravel the process of T cell development. From our previous experiments (*Kampinga* et al., 1997) it has become clear that distinguishing dull from bright labelled cells is not essential. Although their presence in the diagram of postthymic T cell development gives a certain refinement, they do not seem to be necessary for understanding the important steps in the pathway of postthymic T cell development. All the different T cell subsets mentioned in Table 1 can be accommodated within the following 6 main subsets:

Thy-1$^+$/RT6$^-$/CD45RC$^-$,
Thy-1$^+$/RT6$^+$/CD45RC$^-$,
Thy-1$^-$/RT6$^+$/CD45RC$^+$,
Thy-1$^-$/RT6$^+$/CD45RC$^-$,
Thy-l$^-$/RT6$^-$/CD45RC$^+$, and
Thy-1$^-$ /RT6$^-$/CD45RC$^-$.

Using the previous hypothesis, the relationships between these main subsets can be drawn in its most simplified form as shown in Figure 3. The validity of this basic diagram will be tested and the contents extended with the following experiments.

Both Thy-1$^+$ and Thy-1$^-$/RT6$^+$/CD45RC$^+$ T cells are not part of the late stages of postthymic T cell development (Figure 4)

In the previous study (*Kampinga* et al., 1997), it was shown that all thymo-

cytes that leave the thymus express Thy-1 in the absence of CD45RC and RT6 expression. Already within one week the majority of these recent thymic migrants (RTM's) become Thy-l$^-$ and start to express both maturity markers CD45RC and RT6. These latter cells are called common peripheral T cells (CPT's). After a while, CPT's start to loose one or both maturity markers, and become part of the last stages of postthymic T cell development.

One way of explaining this route is that all RTM's "automatically" and quickly develop into CPT's (as part of an extra-thymic and last maturation step for thymocytes), but that CPT's have to wait for getting triggered by antigen recognition, before they differentiate into the late-stage compartment.

This explanation would predict, that (i) when the production of thymocytes is stopped for a short period of 2 wk, Thy-1$^+$ T cells should be nearly absent, whereas the frequency of CPT's should be hardly influenced. Furthermore, this would predict (ii) that in the absence of thymocyte production for a long period of time the CPT frequency should severely decrease. The first prediction has been shown to be true in our previous experiments (*Kampinga* et al., 1997). More recently we have shown that 2 years after thymectomy the frequency of CPT's has been halved compared to the adult situation.

Antigen-activated and proliferating T cells are Thy-1$^-$/RT6$^-$/CD45RC$^-$, and antigen recognition is likely to be involved in the transition from CPT to late-stage T cells (Figure 4)

In contrast to spleen, Pp's will contain large numbers of (gut) antigen-activated and proliferating T cells. So, comparison of the subset frequencies between spleen and Peyer's patches should reveal the phenotype of activated/proliferating T cells. Furthermore, if our idea that CPT's have not experienced previous antigen contacts is true and that, in contrast to the postulated maturation step between RTM's and CPT's, transfer to the late-stage compartment only happens after activation by antigen, the expected increase of activated T cells in Pp's should be accompanied by a decrease of a similar magnitude within the CPT population, but should hardly effect the frequency of RTM's. We have shown that activated/proliferating T cells are likely to be Thy-l$^-$/RT6$^-$/CD45RC$^-$, and that the enormous increase of the frequency of this subset is almost exclusively at the expense of the CPT frequency. Our proposed phenotype for activated/proliferating T cells *in vivo* is in agreement with studies showing that antigen or mitogen activated T cells loose CD45RC and RT6 expression *in vitro* (*Bell*, 1992; *Powrie* and *Mason*, 1989, 1990).

Thy-1$^-$/RT6$^+$/CD45RC$^-$ and Thy-1$^-$/RT6$^-$/CD45RC$^+$ T cells are likely to be resting memory T cells (Figure 5A and 5B)

Memory T cells with specificity for a certain antigen originate from T cells that have been previously activated by the same antigen. Since further development (besides a temporary state of [re-]activation when a memory T cell encounters the same antigen) of memory T cells is unlikely, memory T cells are seen as the end-stages of postthymic T cell development (*Freitas* et al., 1986; *Jerne*, 1984; *Stutman*, 1986).

All antigen-activated/proliferating T cells are likely to be Thy-l$^-$/RT$^-$/CD45RC$^-$ T cells (see above). In addition, if one conversely assumes that all Thy-l$^-$/RT6$^-$/CD45RC$^-$ T cells are in a state of activation, than only the remaining two other phenotypes of the late-stage compartment can be consid-

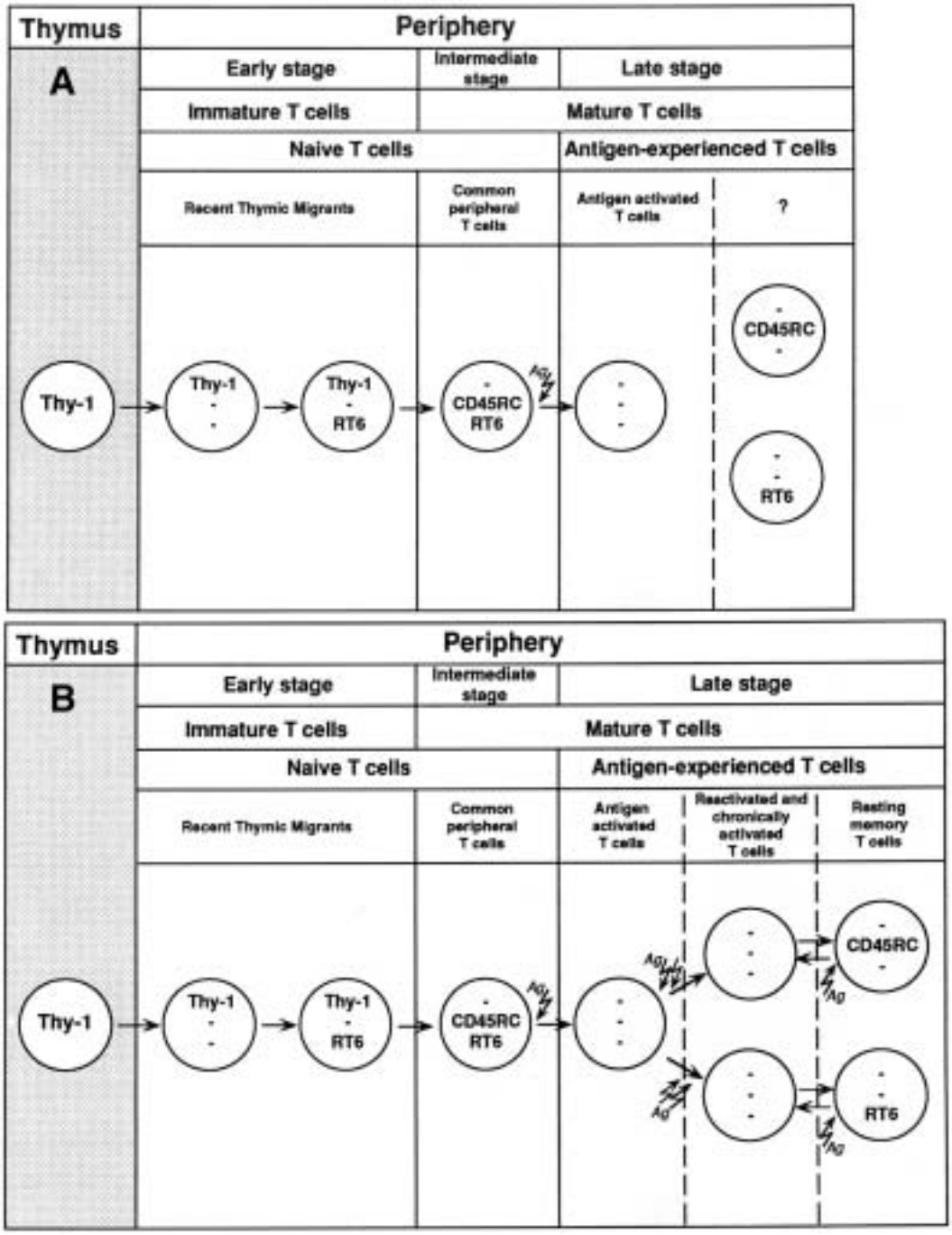

Figure 5: Thy-1$^-$/RT6$^+$/CD45RC$^-$ and Thy-1$^-$/RT6$^-$/CD45RC$^+$ T cells are likely to be resting memory T cells.

ered as being resting memory T cells. A consequence of this idea is that, similar to the findings of the group of Bell (*Bell* and *Sparshott*, 1990; *Bell*, 1992), the

loss of CD45RC (and RT6) is reversible, and that for the majority of T cells the presence or absence of CD45RC may only indicate whether a T cell is resting or activated respectively.

Powrie and *Mason* (1989) have found that memory T cells that help B cells in a secondary response are $CD45RC^-$. According to our diagram, these memory T cells are likely to be of the $Thy\text{-}1^-/RT6^+/CD45RC^-$ phenotype. Whether cells with the $Thy\text{-}1^-/RT6^-/CD45RC^+$ phenotype are involved in different memory T cell functions as, for instance, DTH reactions ($CD4^+$ T cells) and/or secondary graft rejections ($CD8^+$ T cells) is not clear from the literature.

Besides acutely antigen-activated T cells also chronically antigen-activated T cells may exist. It has for instance been suggested that *in vitro* activated Th0 cells, that have developed from naive T cells due to a single addition of antigen, can further differentiate into activated Th1 or Th2 cells when they are chronically stimulated by the same antigen (*de Jong* et al., 1992; *H. Savekoul*, personal communication). Rather than directly developing from activated Th0 cells, we feel it to be more likely that resting memory T cells develop from these chronically activated Th1 and Th2 cells. Both acutely and chronically antigen-activated T cells are likely to be $Thy\text{-}1^-/RT6^-/CD45RC^-$. In the human situation acutely and chronically activated late-stage T cells (i.e. $CD45RO^+$) differ in their expression of CD27 (*Loenen* et al., 1991), a marker that unfortunately is not available in rats. In addition, *Gray* (1991) has suggested that for the maintenance of a particular memory T cell repertoire, repeated reactivation (possibly due to the proposed continuous presence of a small amount of antigen left in the body) of these memory T cells is necessary. So it may well be that chronic activation not only leads to the initial forming of memory T cells, but that this is also a prerequisite for maintaining them. So we would expect a high turnover of T cells in the late-stage T cell compartment. Recent observations of the group of Beverly (*Michie* et al., 1992), showing that, in contrast to CPT's, late stage T cells are short-lived, fit nicely with this prediction.

Possibilities for maintenance of the CPT pool after thymectomy (Figure 6)**:**

An unexpected result from the long-term thymectomy experiment was that CPT's, although strongly decreased in percentage, were still present. One possible explanation is that the remaining CPT's have still not encountered their antigen trigger for further development. Although this can not be excluded, it is unlikely since we waited two years after thymectomy before analysing the cells, which should be enough to enable a rat housed under conventional conditions to encounter a vast number of different antigens. In addition, according to the work of *Stutman* (1986), it is likely that the remaining CPT's that have not received such an antigen-trigger during this period would have died as part of a peripheral repertoire selection process. Our finding that large numbers of T cells die soon after they have left the thymus (*Kampinga* et al., 1988, 1997) fits nicely with *Stutman*'s idea.

Another explanation, which we favour, is that CPT's might have self-renewal capacity. From other studies (*Freitas* et al., 1986; *Stutman*, 1986) it has become clear that within the T cell pool more cells die than are emigrating from the thymus. *Stutman*'s experiments suggest that the peripheral T cell pool as a whole is maintained by the progeny of a special subset of T cells with precursor activity (the so called postthymic T cell precursors [PTP's]

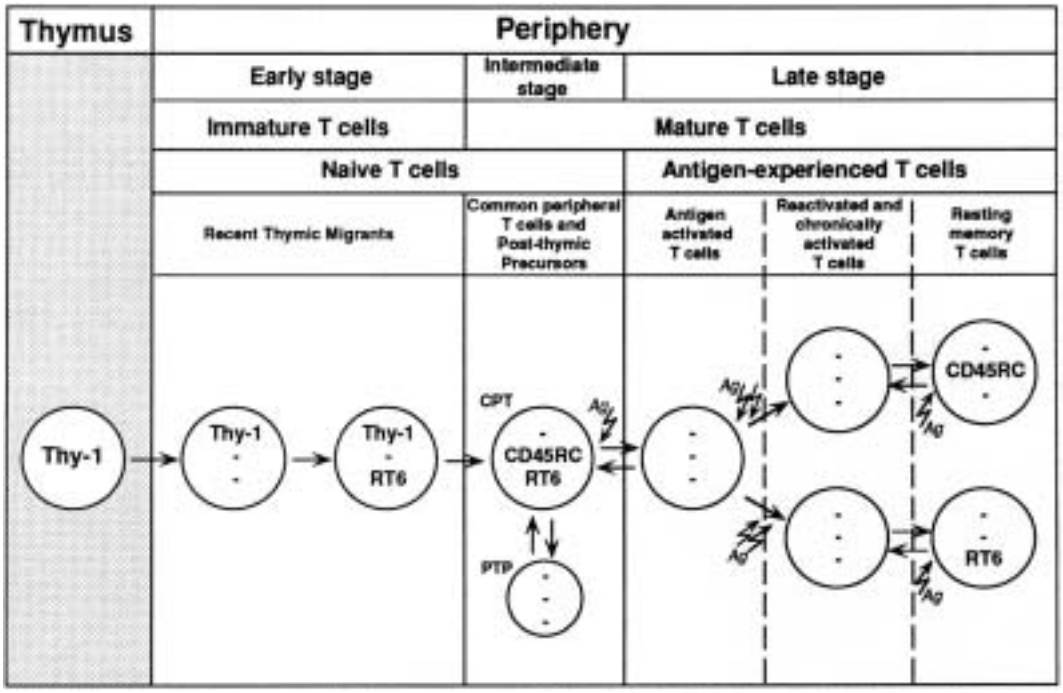

Figure 6: Possibilities for maintenance of the CPT pool after thymectomy.

that proliferate in the absence of an antigen trigger; for references see: *Stutman*, 1986). If one assumes that the number of T cells in the peripheral T cell pool is solely maintained by PTP's, and the unexpected large number of remaining CPT's after thymectomy is a result from the self-renewing capacity of these PTP's, than at least part, if not all, of the PTP activity must derive from cells situated in the intermediate stage. In the absence of thymic output one might expect that a substantial part of these PTP's are proliferating. From the results of experiment II one can expect that proliferating PTP's lack both RT6 and CD45RC expression. In conclusion, our data suggest that active PTP's are Thy-1$^-$/RT6$^-$/CD45RC$^-$ and resting PTP's are Thy-l$^-$/RT6$^+$/CD45RC$^+$. This also fits nicely with the work of *Sparshott* and colleagues (1991), who showed that potential PTP capacity is present in both CD45RC$^+$ and CD45RC$^-$ T cell subsets (when transferred to athymic nude rats, small numbers of both these cell populations were shown to expand very rapidly by an apparently non-antigen driven mechanism, resulting in normal T cell numbers after a few months). Whether partial PTP activity is also present amongst cells of the late-stage compartment can not be excluded from our experiments. *Stutman*'s experiments, however, strongly suggest that PTP activity is only present in the early stages of T cell development (*Stutman*, 1986).

A third explanation, favoured by the groups of Bell (*Bell*, 1992) and Rocha (*Freitas* et al., 1986; *Rocha*, 1987), is that cells from the late-stage compartment can return to the intermediate stage. The idea is that all T cell divisions necessary to maintain the peripheral lymphocyte pool are antigen driven. This would mean that part of the antigen activated Thy-l$^-$/RT6$^-$/CD45RC$^-$ T cells

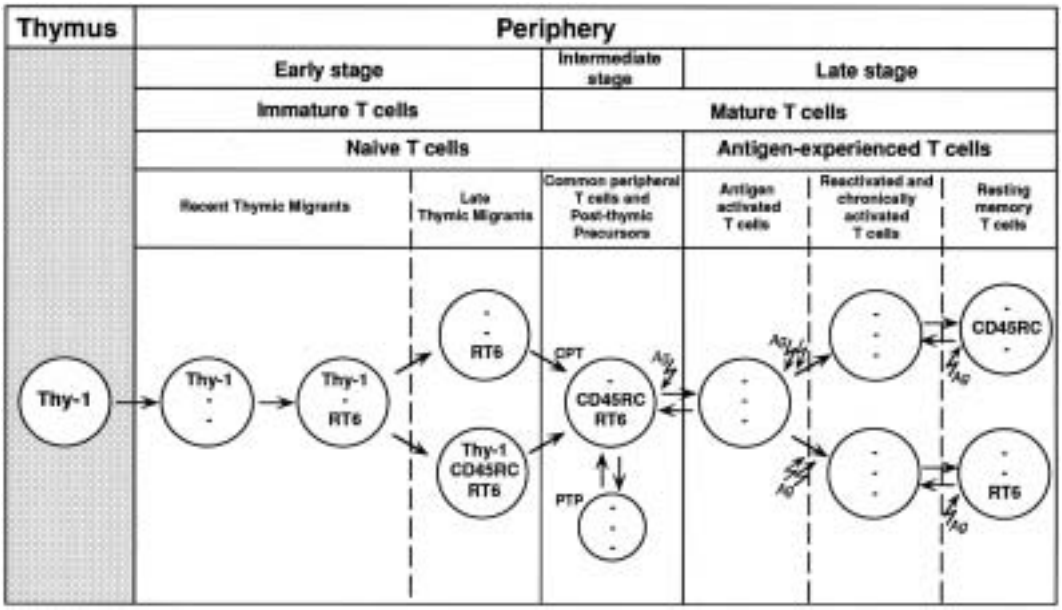

Figure 7: Additional maturation steps in the early-stage compartment.

will return to a resting state and become Thy-1$^-$/RT6$^+$/CD45RC$^+$ again. Although such a mechanism explains why the vast majority of CD45R$^-$ rat T cells rapidly changes in CD45RC$^+$ T cells when maintained *in vitro* in the absence of antigen (*Bell*, 1992), but does not explain why memory T cells have not been found amongst CPT's. A possibility that might explain both observations is that only activated Th0-like cells are able to return to the intermediate-stage compartment, whereas cells that are already further differentiated into chronically activated T cells have changed in such a way that they have lost this capacity and irreversibly will become memory T cells when they enter the resting state. Conversely, this change or commitment might be such that upon reactivation of resting memory T cells, the cells can also not enter the intermediate stage anymore. By definition it is of course not possible to regard cells that have been activated by antigen and returned to the Thy-l$^-$/RT6$^+$/CD45RC$^+$ phenotype as being "antigen naive". Functionally however, they can be regarded as being naive, since, at least so far, only primary responses have been found amongst Thy-1$^-$/RT6$^+$/CD45RC$^+$ T cells.

In conclusion, one could argue that in the absence of thymic output the pool of CPT's is partially maintained by both antigen and non-antigen driven cell divisions. Since it is assumed that the non-used naive repertoire vanishes as part of a peripheral selection process, both antigen and non-antigen driven proliferation eventually will lead to a severely decreased diversity of the intermediate-stage compartment's repertoire, and the remaining naive repertoire is likely to become oligoclonal in nature upon ageing.

Additional maturation steps in the early-stage compartment (Figure 7)**:**

In the previous experiments using the model of vascular thymus transplantation (see Figure 1) it was suggested that Thy-l$^+$/RT6$^+$/CD45RC$^-$

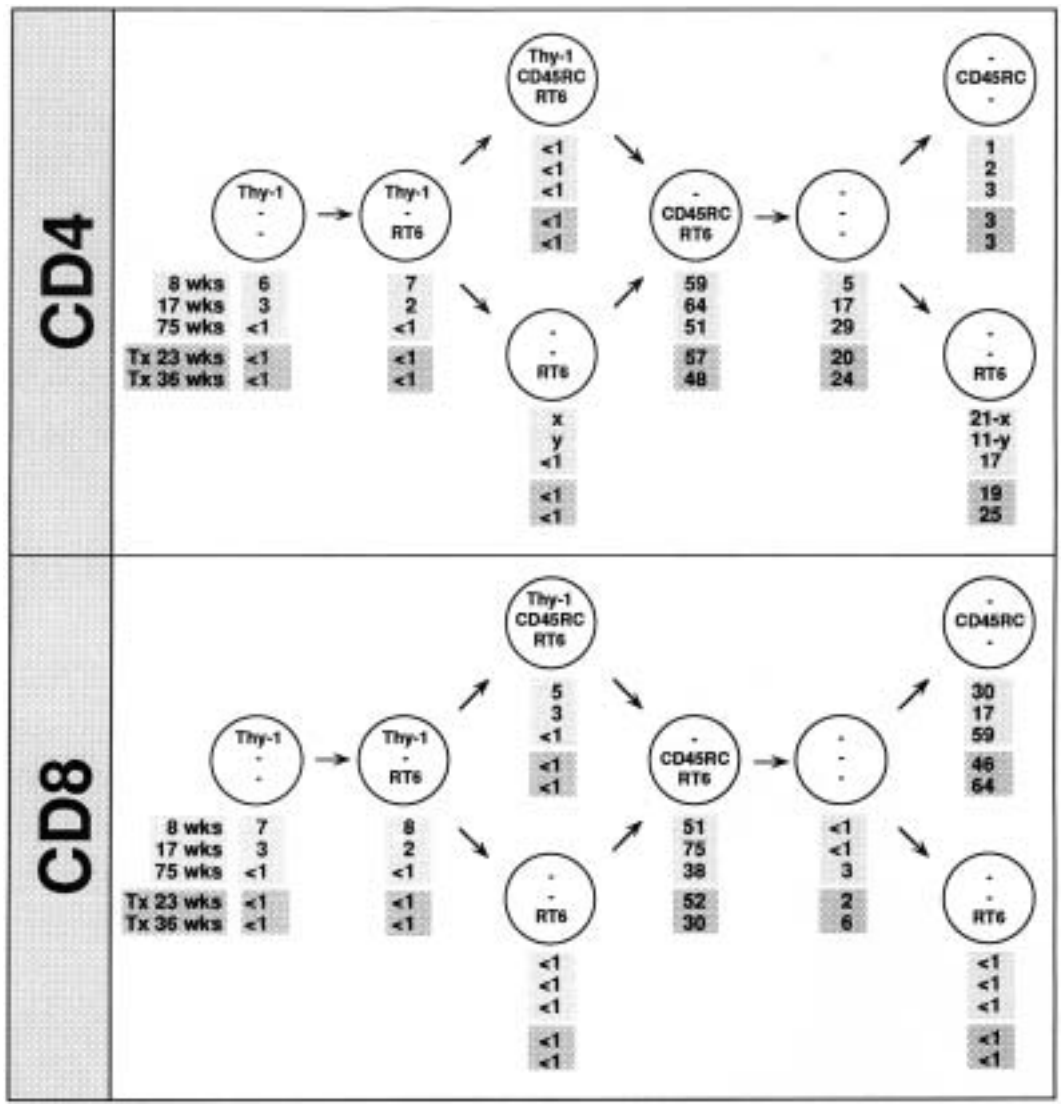

Figure 8: Postthymic T cell development of $CD4^+$ and $CD8^+$ T cells (1).

RTM's before maturing into CPT's did pass a stage in which they had lost Thy-1 expression, but had not yet gained CD45RC on their membrane (phenotypically this subset of late thymic migrants does not differ from the Thy-1^-/$RT6^+$/$CD45RC^-$ resting memory cells!). Recently, by analysing T cell subsets in neonatal rats, we have found more evidence for the existence of this intermediate subset. In the first two weeks after birth high percentages (up to 40%!) of Thy-l^-/$RT6^+$/$CD45RC^-$ T cells could be found, whereas during this period the frequency of Thy-l^-/$RT6^+$/$CD45RC^+$ T cells was very low (<10%). These data are in agreement with recent studies of *Thiele* and colleagues (1987). Although, formally we can not exclude the possibility that all of these Thy-l^-/RT^+/$CD45RC^-$ are in fact resting memory T cells, it is very unlikely that such a large number of T cells would have had time enough in the first two weeks

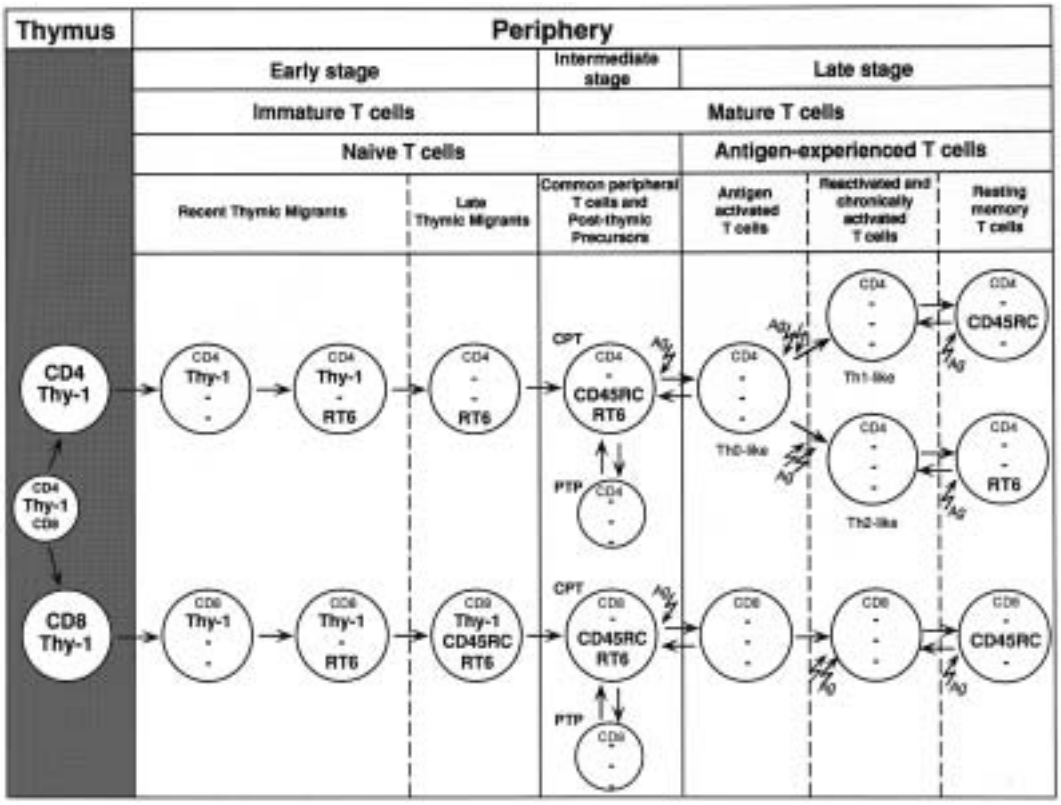

Figure 9: Postthymic T cell development of $CD4^+$ and $CD8^+$ T cells (2).

of life to follow the sequence of maturing into CPT's, getting activated by antigen, passing the chronically activated stage and ending as resting memory T cells. Therefore, we feel that in neonatal and young adult animals, which both have a high output of recent thymic migrants (see Figure 8), a large part, if not all, of the $Thy\text{-}1^-/RT6^+/CD45RC^-$ subset are in fact thymic migrants rather than resting memory T cells.

Although not found in adult and aged rats, occasionally a subset of T cells with the $Thy\text{-}1^+/RT6^+/CD45RC^+$ phenotype has been noticed in very low frequencies (around 1%) in young adult rats, and has been found to be increased in neonatal rats (around 3%). Due to the expression of Thy-l it is likely that these T cells are late thymic migrants, which are just about to become CPT's.

In conclusion, apparently two different subsets of T cells seem to be located between RTM's and CPT's. Because it is difficult to see in which sequence these more mature thymic migrants should be drawn in our diagram of postthymic T cell differentiation, they have been located above each other (Figure 7).

Postthymic T cell development of $CD4^+$ and $CD8^+$ T cells (Figures 8 and 9):

So far, our present hypothesis on postthymic T cell differentiation is based on data from the total population of T cells. Whether the T cell developmental pathways are similar or different within de $CD4^+$ respectively $CD8^+$ T cell lineages is subject of our present studies, from which the data of a first set of pre-

liminary experiments will be shown here.

In PVG rats about 80% of the peripheral T cells are $CD4^+$. So it is likely to expect that the majority, if not all, of our findings for the development of the total T cell pool will also apply for $CD4^+$ cells. Experiments from *Mason*'s group (*Powrie* and *Mason*, 1988), showing that all $CD8^+$ T cells are nearly found to be $CD45RC^-$ is already an indication that a similar sequence is not necessary the case for $CD8^+$ T cells.

Previous experiments from our own group have shown that both $CD4^+$ and $CD8^+$ T cells are Thy-l^+ when they leave the thymus, and that the RTM's of both lineages are likely to pass the intermediate Thy-l^-/$RT6^+$/$CD45RC^+$ stage before entering the late-stage compartment (*Kampinga* et al., 1997). In addition, *in vitro* activation of both subsets resulted in the loss of CD45RC and RT6 (*D. Mason*, and *H-G. Thiele*, personal communication). Based on these previous findings it looks like as if the major steps of postthymic maturation and differentiation are similar for both subsets. Therefore, it was decided to use the present hypothesis as a temporary template to analyse the development of $CD4^+$ and $CD8^+$ T cells separately.

Using triple-staining, it was first analysed whether all the 7 phenotypically different subsets mentioned in Figure 7 could also be found within $CD4^+$ respectively $CD8^+$ blood T cells, derived from young adult (8 wk) and adult (17 wk) rats. As shown in Figure 8, it was found that within $CD4^+$ T cells only the Thy-l^+/$RT6^+$/$CD45RC^+$ phenotype could not be identified, whereas within $CD8^+$ T cells only the Thy-1^-/$RT6^+$/$CD45RC^-$ phenotype was lacking. This strongly suggests that $CD4^+$ RTM's mature into CPT's with Thy-1^-/RT^+/$CD45RC^-$ as an intermediate step, whereas $CD8^+$ RTM's seem to use Thy-1^+/$RT6^+$/$CD45RC^+$ as an intermediate step. Furthermore, the complete absence of Thy-l^-/$RT6^+$/$CD45RC^-$ cells suggests that, in contrast to $CD4^+$ cells, $CD8^+$ cells might only have one phenotype (i.e. (Thy-1^-/$RT6^-$/$CD45RC^+$) indicating resting memory T cells.

Another test for showing that the proposed sequence of postthymic development for the total T cell pool is, at least for the most part, also applicable for the $CD4^+$ and $CD8^+$ T cells separately, is to compare within these two lineages the frequencies of the different phenotypes during ageing of the T cell pool. It is generally assumed that aged rats have hardly any thymic function left (e.g. *Greenstein* et al., 1987; *Ezaki* et al., 1990). Therefore, the prediction would be that, compared to 17 wk old adult rats (young adult rats can not be used, since the presumed [see above] presence of high numbers of late thymic migrants in these rats makes the interpretation of the data very complicated), 75 wk old aged rats should have a severely decreased frequency of phenotypes that mark thymic migrants and CPT's, whereas an increase is to be expected for T cells expressing markers that are supposed to be indicative for resting memory T cells. Furthermore, since repeated activation is associated with the maintenance of the memory T cell pool (see above), an increase in the frequency of memory T cells should be accompanied with an increase of activated T cells exhibiting the proposed Thy-l^-/$RT6^-$/$CD45RC^-$ phenotype. Similar findings are to be expected by comparing 17 wk old rats with adult rats that have been thymectomised at 6 wk of age, and by comparing rats that differ in the length of the period after thymectomy. Figure 8 clearly show that all of these expectations are met.

Based on these results a new diagram of postthymic T cell development can now be drawn for the separate $CD4^+$ and $CD8^+$ T cell lineages (Figure 9).

CONCLUSIONS AND FUTURE EXPERIMENTS

The above experiments have further strengthened the key parts of our previous hypothesis on postthymic T cell development. Furthermore, some minor alterations have been added, and suggestions have been put forward to explain how the CPT pool maintains a reasonable size during ageing. In addition, a new hypothesis has been postulated about the sequence of postthymic T cell development separately for $CD4^+$ and $CD8^+$ T cells (Figure 9).

This new hypothesis is mainly based on indirect evidence, as changes in the frequencies of the different subsets during ageing and after thymectomy. Additional experiments, in which the different T cell subsets are sorted, transferred to congenic (nude) rats and checked for phenotypic changes at different intervals (*Bell* and *Sparshot*, 1990; *Sparshot* et al., 1991; *Fowell* et al., 1991), are needed to formally proof the postulated relationships. Furthermore, new ageing and thymectomy experiments are needed to investigate whether the changes in phenotype we have found upon ageing are due to some kind of special feature of recirculating T cells in blood, or whether they are representative for the whole T cell pool.

Another point that need being studied is our assumption that all ($Thy\text{-}l^-$) T cells that lack both RT6 and CD45RC expression are always activated/ proliferating T cells. Besides classical BrdU incorporation studies, activated/ proliferating cells can also be identified using HIS45, an antibody against QCA-l, because this antigen is thought to be only present on non-activated, non-proliferating quiescent cells (*Kampinga* et al., 1990b). This marker might therefore be a useful tool to investigate whether within the $Thy\text{-}l^-/RT6^-/ CD45RC^-$ subset a small non-dividing "new" subset is hidden. Similar studies are planned using OX39 and OX-40, which are antibodies specific for different activation molecules (*Fowell* and *Mason*, 1993; *Paterson* et al., 1987).

In the diagram, we not only postulate a sequence of phenotypic changes, but also connect purely speculative, certain phenotypes with certain T cell functions. Sorting of, for instance, the proposed Th1-like and Th2-like resting memory T cells followed by activation *in vitro* is needed to establish their expected cytokine production profiles (Th1: IL-2 and IFN-γ; Th2: IL-4, 5 and 10) (*Fowell* et al., 1991). In addition, functional studies will be needed to investigate whether the proposed Th1-like memory T cells are involved in typical functions as DTH, help for CD8 cytotoxic T cells, macrophage activation and direct cytotoxicity, and whether the proposed Th2-like memory T cells are involved in typical functions as B cell help, initiation of IgE production and regulation of eosinophils/mast cells (for references: see *Swain* et al., 1991).

Factors suggested to be involved in influencing activated Th0-like cells in deciding whether to differentiate in either Th1-like or Th2-like subsets are, amongst others, type of APC, concentration of presented antigen and it's structure, route of antigen challenge, and the presence of certain cytokines made by other leukocytes (for references: see *Swain* et al., 1991).

One can imagine that these circumstances may differ from one rat strain to the other, resulting in rats that preferable react on antigens in a Th1-like and others in a Th2-like fashion, and it is likely that such preference can be indirectly read from the ratio between the frequencies of the two resting $CD4^+$ memory T cell subsets. As a consequence of these differences, the former

rats might be prone to get spontaneous or easily induced cellular autoimmune diseases, whereas the latter rats might be prone for humoral autoimmune diseases and IgE based allergies. In a first pilot experiment, we have indeed found some indications for this idea. BB and adult Lewis rats were found to lack completely our proposed Th2-like resting memory (Thy-l$^-$/RT6$^+$/CD45RC$^-$) T cell subset, and these animals are known to be models for cellular autoimmune diseases like diabetes, thyroiditis, multiple sclerosis and rheumatoid arthritis (for references: see *Groen* et al., 1989). On the contrary, adult BN rats were found to lack completely our proposed Th1-like resting memory (Thy-l$^-$/RT6$^-$/CD45RC$^+$) T cell subset, and these animals are used as an autoantibody induced nephritis model and known for their increased production of IgE antibodies (for references: see *Aten*, 1992). Control rat strains like AO and PVG, that are not prone to develop autoimmune diseases, were found to contain both of the proposed resting memory T cell subsets. Obviously these intriguing findings need confirmation, and a large number of different rat strains should be incorporated in the follow-up studies. When these findings are confirmed, one might try to explore the possibility of developing new therapies for auto-immune diseases based on influencing the factors involved in directing activated Th0-like cells in their differentiation to Th1- or Th2-like cells, an idea already previously suggested by Mason's group (*Day* et al., 1992).

Pilot studies have shown that our results in thymectomised rats do not markedly differ from euthymic 2,5 year old rats. This not only further confirms that aged thymi do not produce RTM's in traceable numbers anymore (see Figure 8), but also suggest that the presence or absence of thymic hormones do not seem to be of importance in regulating the pathway of postthymic T cell development. Furthermore, our data indicate that a large population of T cells in aged rats are already antigen experienced, and that the diversity of repertoire amongst the remaining (and during ageing continuously further declining number of) naive CPT's is severely decreased. Due to the absence of input from thymic derived "fresh" repertoire, holes in the peripheral T cell repertoire will eventually develop and the naive repertoire will become oligoclonal in nature. As a consequence of this, aged rats are in a situation in which they may encounter antigens for which highly specific T cell receptors are not available anymore. This situation might, at least partly, be responsible for the impairment of immune functions found in ageing immune systems (for references: see *Makinodan* and *Kay*, 1980). Regeneration of thymic function in aged persons might solve this problem. Such a therapy has become a realistic option for the near future due to the fact that growth hormone, which has a potent rejuvenating effect on aged thymi (*Kelley* et al., 1986), has become readily available in a recombinant format (*Vance*, 1990).

An important issue about animal studies is whether the data generated from such experiments reflect the human situation. Unfortunately, it is unknown whether human T cells express RT6 antigens, although the genetic code seems to be present (*H-G.Thiele*, personal communication). In addition, CD45RC has not yet been identified in humans. However, the expression pattern of the human CD45RB antigen has been suggested to be similar to the rat's CD45RC (*Fowell* et al., 1991). Based on our new hypothesis, we have started to perform a few initial experiments on analysing human CD4$^+$ T cell subsets in the late-stage compartment (i.e. CD45R0$^+$/CD45RA$^-$). Using additional

markers for "acute" activation ($CD38^+$) and chronic activation (CD27) (*Loenen* et al., 1991), preliminary results show that in the late-stage compartment the following $CD4^+$ T cell subsets can be identified: $CD27^+/CD38^+/CD45RB^-$ (possibly representing Th0-like activated cells), $CD27^-/CD38^{+/-}/CD45RB^-$ (possibly representing chronically activated Th1- and Th2-like cells), $CD27^-/CD38^+/CD45RB^-$, and $CD27^+/CD38^-/CD45RB^-$. To our opinion, the latter two are likely to represent the proposed rat analogues for resting Th1- and Th2-like memory T cells respectively. Similar ageing and thymectomy (Myasthenia Gravis patients!) studies, as have been performed in rats, are presently in the process of being carried out in humans. These and additional studies on cytokine profiles of sorted human T cell subsets will help to further unravel the phenotypes of human memory (and other important) T cell subsets, and might also answer the question whether experiments in animals are useful to understand the complexity of the human immune system.

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THE DEVELOPMENT OF THE SPECIFICITY REPERTOIRE OF THE IMMUNE SYSTEM: REVIEW OF THE INTERNAL DISCUSSION

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INTRODUCTION

Before we can attempt to understand the complex interactions between the immune system and the host's microflora, we have to understand the dynamics of the contacts between both from the first day after birth e.g. the ontogeny of the immune system. In other words we need information about the immune system before and after exposure to external antigens.

During a one-and-a-half day internal discussion between the speakers of the 6th Old Herborn University Seminar some elements of the ontogeny of the immune system have been discussed. This report focuses on 5 different topics: Early B cell development, B cell repertoire selection during development, function and development of CD5 B cells, T cell repertoire selection during development, and finally tolerance and self assertion. Discussion of each topic was introduced and chaired by one or the speakers invited (names between brackets). This report reflects the important issues raised.

EARLY B CELL DEVELOPMENT
(M.D. Cooper)

Ontogeny of the B cell system

Humans have a very long gestational period of 40 weeks. The first B cells are seen around 7 weeks of gestation. B cells of all isotypes, as detected by conventional antisera, reach adult frequencies around 10 to 12 weeks of foetal life. First IgM plasma cells are seen around week 12. IgG plasma cells appear somewhat later, around week 20, and finally IgA plasma cells appear at week 32. Most plasma cells are located in spleen and bone marrow.

Maternal IgG peaks around birth (week 40). These antibodies gradually disappear and are not detectable one year later. Serum IgM appears late in gestation (before birth) and reaches adult levels at one year of age. Foetal serum IgG and IgA appear later, around birth. IgG reaches adult levels at 5 to 6 years of age, whereas serum IgA reaches a mature level at puberty.

Factors involved in the functional delay of B cells

Development in foetus and new-borns

Although B cells of all isotypes are present early in the development of the foetus, serum immunoglobulins appear

very late. Apparently a delay in the (terminal) differentiation of the B cell population is present. This has clinical significance e.g. the immune response against pneumococcal antigen is severely impaired during the first 1 to 2 years after birth.

A major question is how to explain this apparent delay. Possible explanations may include:
1. the inability of the B cells to respond,
2. T cell regulatory mechanisms, and/or
3. environmental factors.

Ad 1:

Neonatal/foetal B cells can be an immature population or different population of cells which cannot differentiate. Support for this is that non specific stimulation *in vitro* with mitogens or EBV transformation of newborn B cells (i.e. 20-30% of the lymphocytes in circulation) results in secretion of IgM, whereas almost no IgG production and even no IgA production is found (although initially 5% of the B cells are surface IgA positive). Other data have shown that marginal zone B cells in the spleen are not fully developed (CD2l down regulated) until 2-3 years after birth. During foetal life the B cell repertoire is limited/restricted: between 7 and 8 weeks of gestation exclusively V_H5/6 is present. Later (week 8) B cells also express V_H3 until close to birth when other V_H genes are expressed as well. It is not known whether these preferential rearrangements are due to selection mechanisms. N-sequence additions start very early during gestation and numbers of insertions gradually increase during foetal life.

Ad 2:

T cells in the new-born might be different from adult T cells. Indeed, virtually all Th cells are Th2 cells. Th1 cells appear to lack. This may result in differences in lymphokine profiles in newborns, which in turn may explain the relative absence of isotypes other than IgM. Furthermore, also CD45 R0 cells appear to be absent and 45% of cord blood T cells are CD45 RA positive. The vast majority of these RA^+ cells express the CD38 marker, which is associated with activation. CD45 RA cells in adults are CD38 negative. This particular subpopulation of CD48 RA and CD38 double positive cells is believed to have a specific suppressive effect.

Ad 3:

The different microenvironment of new-borns can also contribute to the delay in antibody production of various isotypes. The intestinal microflora in the neonate differs substantially from the adult. It is also possible that antigen presenting cells are functionally absent, e.g. it is known that mature FDC are absent in rat spleen until 2 weeks after birth (*Kroese*). However, transfer of adult B and T cells to neonatal mice results in normal adult level responses upon antigenic stimulation (*Coutinho*).

Development after bone marrow transplantation

After bone marrow transplantation (BMT) to supply haemopoietic stem cells (haematological malignancies), erythrocytes and myeloid cells repopulate quickly. However, it takes much longer to functionally regenerate the immune system. In this respect, the development of T and B cells after BMT is similar to the neonatal development of the immune system. BMT patients cannot generate adequate antibody responses of all isotypes upon antigenic stimulation one year after transfer. The 'cleaner' the preparation of haemopoietic stem cells (BM-graft) the longer it takes to regenerate the immune system. This is likely due to absence of outgrowth of mature (contaminating) lymphocytes.

B CELL REPERTOIRE SELECTION DURING DEVELOPMENT

(K. Rajewsky)

Relatively little is known of the establishment of the B cell repertoire. By contrast, for T cells there is good evidence for both negative (active deletion) and positive selection (active survival). Negative selection, for example, is seen as physical deletion of thymocytes after interaction with superantigens in the thymus. Positive selection occurs after interaction of T cell receptors on thymocytes with environmental (MHC class II) antigens or peripheral T cells that interact with classical exogenous antigens. Two levels of B cell selection can be distinguished:

1. Selection in the bone marrow (BM),
2. Selection during the generation in the germinal centres.

Here we will consider positive and negative selection at these two levels.

Selection of repertoire from progenitor B cell to mature B cell

Studies in immunoglobulin transgenic mice (e.g. *Nemazee*, *Goodnow*) have shown that negative selection can occur among BM B cells. Cells are deleted in the BM as soon as they express surface immunoglobulin specific for endogenous antigens. The mechanism for this cell death is not known and there is no evidence yet that this occurs by apoptosis. Possibly most important in this process may be the microenvironment of the BM.

Indications for positive selection of B cells come from experiments comparing the V_H gene repertoire of pre-B cells and peripheral IgM^+ IgD^+ B cells. Data show that in the stable pool of relatively long lived cells, the different numbers of the J558 family V_H genes are present at not random frequency. This selection is likely due to antigens as in germfree (GF) B6 mice the J558 V_H gene repertoire of mature splenic B cells is similar to that of pre-B cells. However, as mentioned by *Rajewsky*, one should keep in mind that GF B6 mice are only derived by caesarean section of conventional B6 mice after which the neonates are fostered by GF Balb/c parents. Another ligand that may play a role in selection of B cells is antibody itself. This was shown by experiments of *Kearney*, who treated mice from birth with anti-Id antibodies, which resulted in a significant chance of the repertoire.

Best evidence for positive selection of B cells is seen at the level of $CD5^+$ B cells (see discussion *Kearney*) as reflected by strong enrichment in V_H11 and V_H12 genes and specificities for autoantigens and antigens expressed on microorganisms.

Generation of high affinity antibodies in germinal centres

Germinal centres are clusters of B blastoid cells that develop upon antigenic stimulation in the centre of follicles. Within the germinal centre B memory cells are being formed. During this proliferation process B cells expand in an oligoclonal fashion while they undergo isotype switching and somatic mutations. B cells expressing immunoglobulins with high affinity for the antigen that induces this germinal centre reaction are rescued from cell death by positive selection. B cells with low or absent affinity are not selected and will die by apoptosis. Molecules involved are e.g. CD40 and BCL-2. In mice the most differentiated B cells have the highest level of BCL-2 compared to pre-B cells in the bone marrow that have >100 fold lower levels (*Coutinho*).

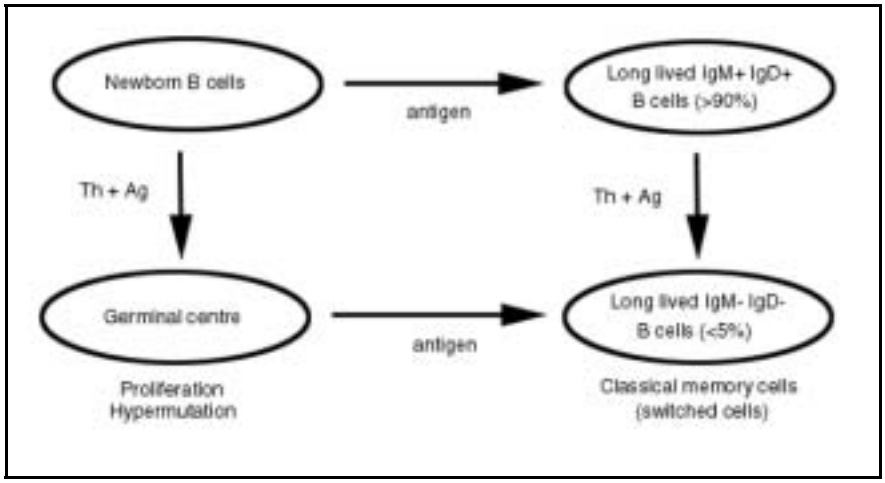

Figure 1: Hypothetical scheme of generation of peripheral B cell pool through cellular selection.

There is weak evidence for negative selection in germinal centres that might be needed in case the somatic mutations lead to autoreactivity. Signals involved in these selection phenomena might be well studied in the near future using the recently developed "*in vitro* germinal centres method".

Positive selection has also been clearly demonstrated for memory cells. Recently *Schittek* and *Rajewsky* analysed the J558 V_H gene usage of M^- D^- (memory) B cells of not deliberately immunised animals. Excitingly they show that same gene selection takes place among memory B cells as in M^+ D^+ mature B cells. Members of the J558 family used are similar to those from the pool of these M^+ D^+ B cells (see above). The only difference is that 80% of these V_H genes have somatic mutations.

Hypothetical scheme of B cell selection

During the generation of newborn B cells, cells may die if there is no good pairing of a rearranged heavy chain with a light chain. It is unknown what percentage of these cells finally produces a functional antibody and it is questionable whether this is a negative or positive selection process. It is also not known whether specificity selection occurs at the pre B cell level. However, it is known that the λ 5 light chain in combination with a μ heavy chain is expressed on pre B cells. According to one hypothesis, the role of expression of this complex is to test out the productive rearrangement of the μ heavy chain while it is not involved in a selection process. There is some evidence against negative selection at this level. Injection of anti λ 5 antibodies does not cause shifts in the repertoire. However, this might be explained by the presence of weak signal transduction mechanisms.

Data from *Holmberg* suggest that positive selection of D-proximal V_H-genes occur in perinatal life while the same genes are negatively selected in the adult BM. Another question is where the selection of newborn B cells takes place: In the BM or in the spleen or both. This question might be well addressed in rats but not in mice because only in the former species newborn B cells but not the IgM^+ IgD^+ B cells express Thy-1 allowing isolation of these cells for analysis (*Kroese*).

The generation of plasma cells is also

a selective process. Plasma cells producing isotypes other than IgM are derived from switched cells, an event that occurs in the germinal centre (GC). However, this does not imply that all switched cells must be derived from GCs. Indeed IgG and IgA producing cells are found in nude animals and antigen-free animals which lack GCs. Plasma cells in these animals, however, appear to make unmutated antibodies. In antigenfree mice the number of IgG and IgA plasma cells is 100 fold lower compared to conventional animals and their specificity repertoire resembles that of IgM plasma cells (*Bos*).

FUNCTION AND DEVELOPMENT OF B-1 CELLS (CD5 B CELLS)

(J.F. Kearney)

Lineage origin of CD5 B cells

There is still controversy about the lineage origin of CD5 expressing B cells. These B cells are now called B-l cells (previously called CD5 B cells or Ly-l B cells) and are distinct from conventional B cells in anatomical location, function (including repertoire), phenotype, and development. B-1 cells are enriched in the peritoneal and pleural cavities and produce multireactive antibodies directed to autoantigens and microorganism related antigens. The major point of discussion is whether CD5 expression on B cells defines a distinct B cell lineage with their own progenitor cells or that CD5 expression is the result of a distinct B cell activation pathway (thus, separate progenitors versus separate environments).

Transfer and transplantation studies by several groups have shown that B-1 cells are derived from progenitor cells found in foetal liver (*Herzenberg, Hardy, Kantor*) and exclusively from progenitor cells located in foetal omentum (*Kearney*), and paraaortic splanchnopleura from early (8.5-9 days) embryos (*Marcos*). Progenitor activity of adult bone marrow for B-1 cells is severely reduced (but not absent; in particular sister $CD5^-$ B-lb cells) and in the adult mouse B-1 cells maintain themselves by self-replenishment, although there are findings suggesting a pool of B-1 precursors in the adult (*Marcos*). B cells that belong to the conventional lineage are produced throughout animal life from progenitor cells located in the bone marrow. In the foetus, conventional B cells also derive from the foetal liver.

Wortis et al. speculate that B cells are not pre-committed to become B-1 cells. They claim that there are two B cell lineages, one foetal B cell lineage and one adult B cell lineage, which both can give rise to CD5 expressing B cells, after activation by certain antigens. One difference between the foetal and bone marrow lineage would be the number of N terminal sequences, inserted during the V_H gene rearrangement using the enzyme TdT. In lymphoid cells from foetal liver, TdT is absent while it is clearly present in bone marrow pro-B cells. Concomitantly, B cells derived from foetal liver almost lack N-terminal sequences. According to *Wortis*, activation of B cells from both lineages lead either to CD5 expressing B cells or B cells that have low levels of J11d ("classical memory cells"). Activation of B cells by crosslinking their sIg (e.g. by carbohydrates or anti-Ig), is thought to result in CD5 expressing B cells whereas activation of B cells in a T cell dependent fashion by non-crosslinking antigens results in J11d low cells (memory cells).

The CD5 B cell repertoire

Antibodies secreted by B-1 cells have a distinct and selected specificity repertoire. In general they produce multireactive antibodies which are enriched for auroreactivity and for epitopes expressed on microorganisms (like DEX). Antibodies produced by B-1 cells are strongly connected to each other and thus play an important role in the maintenance of the idiotypic network. At the DNA level they have a distinct V_H gene repertoire and exclusively use V_H11 and V_H12 genes and their V_H genes are usually not mutated. By these properties B-1 cells are believed to play a role in the first line of defence of the animals and to carry a kind of evolutionary memory to environmental antigens. Therefore they do not need high affinity antibodies encoded by mutated V_H genes. It is not known yet whether B-1 cells can participate in the germinal centre reaction, i.e. the sites where the somatic mutations are introduced.

The role of CD5 B cells in the mucosal immune system

Most plasma cells in the animal are IgA secreting cells, which are located in the gut wall. B-1 cells likely also contribute significantly to these IgA plasma cells as shown in transfer experiments (*Kroese*). In these studies peritoneal B cells are transferred to irradiated recipients, reconstituted with IgH-C congenic bone marrow. In these so-called lineage chimeras, only B-1 cells develop from the donor peritoneal cells and in the gut lamina propria many IgA plasma cells (up to 50%) have the allotype of the peritoneal cell donor. Similarly, SCID mice engrafted with foetal omentum, which results in development of B-l cells (but not conventional B cells), also produce IgA cells in the gut (*Kearney*). These type of experiments strongly indicate that IgA plasma cells can be derived from donor B-1 cells, in addition to conventional B cells that differentiate through Peyers patches. The relative contribution of these two different lineages in the production of gut IgA plasma cells in normal, untreated animals is not known. Furthermore whether these two IgA plasma cell populations have different repertoires and functions remains to be answered. In this context, monoclonal IgA antibodies (of unknown lineage origin) are the most polyreactive antibodies, even more than IgM antibodies (*Coutinho*).

T CELL DEVELOPMENT

(H.R. MacDonald)

Generation of T cells in the light of B cell development

Allelic exclusion of the T cell receptor

There is substantial evidence that the vast majority of B cells in normal animals express only one Ig heavy chain gene. This phenomenon is called allelic exclusion. However, data from transgenic mice suggest that endogenous genes may be expressed together with the rearranged m transgene, although controversy exists about the interpretation of these findings. Furthermore, for light chains 5-10% of hybridomas made from neonatal spleens (but not from adult spleens) simultaneously produce κ and λ light chains as demonstrated both at the protein and mRNA level (*Kearney*). These findings show that allelic exclusion for Ig light chains may not be complete in normal animals.

Recent studies with β gene transgenic mice show that in T cells allelic exclusion may also occur for TCR genes. In these mice endogenous β chain expression is inhibited by the transgenic β gene. No such allelic exclusion is found for α chains and approximately 30% of

murine T cells potentially produce two productive a chains. This has been demonstrated at the mRNA level but not yet at the protein level. Thus allelic exclusion does not seem to be absolute at the TCR level.

Regulation of rearrangement of the T cell receptor

Recombinase activity in B and T cells is associated with RAG1 expression. Although RAG1 is present during early B cell development, it is not known yet whether different levels of RAG1 can be found in different steps of B cell differentiation. Two waves of RAG1 expression are found during development of T cells in the mouse. Approximately 1% of murine thymocyte T cells has intermediate levels of RAG1 mRNA levels. These cells that express the IL2 receptor, are an early phase in T cell development and are associated with β and γ rearrangements. Later during development when the cells express CD4 and CD8, high levels of RAG1 mRNA are seen. This corresponds with rearrangement of the TCR α locus. At this phase of T cell development, further differentiation of T cells can be regulated by Ig ligating the TCR α chain, which leads to reversible staggering in development. It is not known whether there is a surface molecule expressed in conjunction with surface expression of the β chain on thymocytes early during development which parallels the expression of the λ 5 gene on pre B cells in the mouse.

T cell development

γδ T cells

With respect to the T cell receptor there are two distinct T cell populations. Two percent of the peripheral T cells express γδ TCR whereas all others express αβ. These T cells are not MHC restricted and therefore they may be generated at extrathymic locations. Indeed nude mice have γδ T cells.

Whether the two T cell populations are distinct lineages is not fully established. The low percentage of γδ T cells, present in a 1 to 50 ratio (with respect to αβ T cells), can be fully explained by the difference in the size of the coding genes (stochastic explanation). Knockout experiments show that α TCR chain knockout mice still express δ chains and vice versa δ knockout mice express α chains. Although α chains rearrange in the absence of δ chains (and vice versa), this does not need to imply that there is segregation of αβ and γδ T cells at the progenitor level.

Models for development of CD4 and CD8 T cells

An intriguing question is what determines whether a mature T cell becomes a CD4 or CD8 positive cell. At present there are two different models to explain this differential differentiation of T cells. First, according to the "instructional model" by *Von Boehmer* and others, all immature $CD4^+$ and $CD8^+$ T cells (cortical thymocytes) potentially have the capacity to differentiate either to single $CD4^+$ or $CD8^+$ cells. This final differentiation step is thought to be determined by interaction between TCR and ligand, i.e. MHC class I and II.

Secondly, in the "committed model" by *MacDonald*, T cells are already committed to become either single $CD4^+$ or $CD8^+$ cells at the $CD4^+CD8^+$ or even earlier at the $CD4^-CD8^-$ stage. The $CD4^+CD8^+$ cells only need the appropriate ligand to survive and become single $CD4^+$ or $CD8^+$ cells (experimental data by *MacDonald*).

According to which of the two models T cells differentiate is currently unknown. However, in both models T cells are positively selected upon interaction with MHC Class I or II antigens. Although most T cells are thus selected for interaction with class I or II anti-

gens, in normal mice a low percentage of the T cells appears not to be restricted heavily.

B cells in the thymus

B cells are always present in low numbers in the thymus of humans, rats and mice. Recent data indicate that thymic B cells may play a role in antigen presentation to thymocytes. This can be of functional importance for establishment of the T cell repertoire. There are experiments in mice that show that deletion of certain Vβ TCR T cell families occurs through MLS antigens expressed by thymic B cells harbouring MMTV (*Marcos*).

TOLERANCE AND SELF ASSERTION

(A. Coutinho)

Tolerance and the gut microflora

Tolerance is considered usually in the context of autoimmune diseases and allograft rejection. The majority of antigens that are encountered every day are self-antigens. If we take into account that there are 10 times more bacteria than eukaryotic cells in the human body (*van der Waaij*) the interaction of the immune system with intestinal antigens may not be overlooked and should be viewed in the light of tolerance as well. When we are speaking of tolerance one should be aware of the fact that tolerance depends upon the read out system and parameters tested. Apparent unresponsiveness of the immune system is not a passive state but is maintained through an active and dynamic process.

Intestinal bacteria are important for physiological functions of the human body (vitamin K production, colonisation resistance, digestion of food products, differentiation of the gut epithelium, mucus induction, haematopoiesis, etc.). For further information on this topic see Old Herborn University Seminar Monograph 1: Microecology of the Human Digestive Tract. Meanwhile it is of crucial importance that strong immune responses against gut microflora antigens do not occur. The bacteria live in symbiosis with the host's immune system. On the other hand the immune system should be able to induce specific immune responses to (potential) pathogenic bacteria.

Patients with agammaglobulinaemia are believed to have a normal microflora, which may suggest that there is no role for IgA in the gut with regard to regulation of the microflora. However, despite this statement, 90% of the IgA plasma cells are found in the intestinal tract.

The important role of the intestinal microflora is also illustrated in GvHD models. Lethally irradiated mice engrafted with allogeneic bone marrow cells may suffer GvHD if the recipients are conventional, whereas GvHD is absent when recipients are germfree (GF) or have been totally decontaminated (*Heidt*, *Veenendaal*). Furthermore, GF mice have shown to respond differently to mouse related microflora compared to mouse unrelated (human) microflora. After oral association with microflora, mice have been found to respond with higher antibody levels to a significant higher percentage of bacteria present in the human derived flora as compared to those in the mouse derived flora (*Veenendaal*). However, it remains unclear whether selection of intestinal microflora organisms is mediated through the immune system.

With regard to autoimmunity the gut microflora also plays an important role. Neonatally thymectomised mice or nu/nu mice suffer lupus-like autoimmune disease when they are maintained

under conventional conditions. However when they are germfree, isolated and monoassociated with *E. coli*, or conventional but isolated, no lupus-like autoimmune disease will occur. Similar results have been found in NZB mice. These results suggest that environmental bacteria or antigens, which are absent in isolated animals, are most important in inducing lupus-like autoimmune disease in functionally thymus deficient animals, whereas the role of the autologous microflora may be of limited importance. On the other hand the intestinal microflora may also be protective for autoimmune disease as BB rats or Fisher rats become more susceptible for diabetes or arthritis respectively when they are germfree.

B cell tolerance

General remarks

Discussing B cell tolerance the following points should considered. It is obvious that the V_H gene repertoire is of importance. Other aspects that are related with tolerance are the control of effector function: Many immune activities are not destructive at the target e.g. even in the presence of autoantibodies target organs are not always affected. Finally, the dynamics of antigen and immune response play a role in tolerance induction. The immune system only responses to major changes in the antigenic concentration (including self). Indeed usually self-antigens are kept at a constant level. Moreover, when xenogenous erythrocytes are injected slowly over a 24-hour period no specific antibody response is seen. Autoantibodies in immune diseases are not at a constant level but fluctuate.

B cell repertoire and tolerance induction

Deletion of B cells and their immediate precursors occur at different levels. With respect to particular V_H gene antigens, cell kinetics data show that many cells die at the transition of pre B cell to newborn B cell and from newborn B cell to mature B cell. This deletion process is clearly associated with selection phenomena, which are either positive or negative. The observation that autoreactive B cells are always seen in the periphery implies that a presumed deletion process is not complete. There is however no evidence that this failure of deletion results in autoimmunity.

How to explain this presumptive deletion of autoreactive B cells (negative selection) in the BM together with production of autoantibodies in the periphery (positive selection to antibody secretion)?

1. It could well be that different ligand concentrations have different effects on the B cell:
   a. low levels of ligand may result in survival but no secretion of antibodies
   b. intermediate levels of ligands may result in activation of the B cell concomitant with differentiation to plasma cell formation leading to Ig secretion
   c. high levels of ligand may lead to activation of B cells without secretion. The highest levels may lead to cell death.

   According to this view, if low levels of ligand are encountered during the generation of B cells, this results in positive selection whereas the highest levels may lead to negative selection. Total absence of ligands leads to apoptotic cell death.
2. According to *Cooper*, cross-linking of B cells at different stages of their development may have different effects. This may well be due to differences in signal transduction mediated after binding of proteins at different cell stages after cross-linking with different ligands e.g. IgM. This explains why immature μ^+ B cells treated with anti-μ undergo capping, whereas

mature μ^+ δ^+ B cells will be activated. Whether capping at the stage of the immature B cells will result in apoptotic death has not been investigated so far. Thus peripheral B cells may require higher ligand concentrations to become stimulated compared to immature newborn B cells in the BM. Furthermore for a particular B cell high affinity ligation results in deletion whereas low affinity ligation stimulates the B cell. In this way high affinity autoreactive B cells are deleted in the BM whereas multireactive low affinity B cells are positively selected and subsequently become stimulated into plasma cells in case higher concentration of autoantigens are encountered in the periphery.

3. Another explanation for the production of natural autoantibodies is that a different lineage i.e. CD5 B cells is responsible for the production of these antibodies, while autoreactive conventional B cells are deleted.

IgG autoantibodies

It is now clear that autoreactivity is not restricted to IgM antibodies but can also be found among IgG. The V_H genes of these IgG autoantibody-producing cells are generally not somatically mutated. Thus probably these cells have not participated in a germinal centre reaction. The affinity of autoantibodies (which are predominantly of the IgG isotype) has no relation to the biological function. Furthermore, there is no correlation between the titre and the degree or even the presence of autoimmune disease. For these reasons one should avoid the term pathogenic antibodies. Instead they should be named "Disease Related Antibodies".

Intravenous IgG treatment of patients with autoimmune disease has a beneficial effect. Mechanisms to explain this may include:

- blocking of Fc receptors
- supply of Ig anti exogenous bacterial related antigens
- supply of antibodies against cytokines mediating inflammation
- direct effect on autoantibodies
- adjuvant effects
- etc.

Autoantibodies may play a role in the fine regulation of the endocrine system as well. Some antibodies mimic hormonal effects, whereas others inhibit. The key function of this system is to dampen down major hormone level deviations. SCID mice, which lack immunoglobulins, might for this reason be more susceptible to all kinds of environmental variations. In this way autoantibodies determine the robustness of the endocrine system.

T cell tolerance

Contact at the T cell receptor level takes the same amount of aminoacids (15-20) as compared to ligand binding of B cells. Still there are major differences between B and T cells: The thymus is more secluded than the bone marrow. Therefore tissue specific antigens might never reach the thymus. This implies that there must be extrathymic T cell tolerance.

Normally a full tolerance for epithelium exists, which is not based on deletion. MLR is mediated by skin antigens presented by bone marrow derived cells, but not by skin antigens presented by the skin itself.

There is no good evidence that defects in deletion of autoreactive T cells are related to autoimmune diseases. Still intra-thymic deletion mediated by superantigens is known to take place.

Anergy does not exist at the level of T cells or B cells (*Coutinho*). Intrinsic all cells are or remain able to respond and therefore one cannot speak of anergy, tolerance or absence of response in a definite way.