Phenotypic Analysis Of A Novel Murine X Chromosome-linked Mutation Affecting Haematopoietic Cells And Skeletal Muscle

by

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Abstract

The phenotypic effects of a novel X-chromosome linked mutation were studied in the offspring of male mice treated with the mutagen ethyl nitrosourea (ENU). Studies of X-chromosome inactivation patterns in females heterozygous for the mutation (ENU/+) has delineated four distinct cell lineages affected by the mutation, namely, B, pre-B and T lymphocytes, erythrocytes and skeletal myocytes. The penetrance of the mutation depended on the age of mice, the cell lineage affected by the mutation and the stage of maturation of the cell lineage. Studies of B cells in females heterozygous for the mutation and the X-linked mutation, xid that affects B but not pre-B cells, indicated that the two mutations were not allelic. Factors influencing differences in X-chromosome inactivation between cells and hybrids and their relationship to alleles of the X-chromosome controlling element (Xce) in the ENU-mutant and parental strains were studied. It was not possible to identify the effects of the mutation on the immune system in functional terms either by flow cytometric analysis of leukocytes or after sensitisation to oxazolone. Results imply the mutation renders the affected cell lineages susceptible to competition with normal cells in the heterozygote, rather than there being any fundamental defect in cell function and that the mutation may be in a gene encoding a component of the cell cycle or controlling a maturation step.

ABBREVIATIONS

ADP	= adenosine diphosphate
ALD	= aldolase
AMP	= adenosine monophosphate
AO/EB	= acridine-orange/ethidium-bromide
ATP	= adenosine triphosphate
DTE	= dithioerythritol
EDTA	= ethylene-diamine tetra-acetic acid
FACS	= fluorescence-activated cell sorter
FITC	= fluorescein isothiocyanate
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
GDH	= glycerol dehydrogenase
G6PD	= glucose-6-phosphate dehydrogenase
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide: thiazolyl blue
NAD	= nicotin amide adenine dinucleotide
NADH	= nicotin amide adenine dinucleotide, reduced form
NDF	= neutral density filter
PBS	= phosphate-buffered saline
PEI-TLC	= polyethylene-imine, thin layer chromatography
PGK-1	= phosphoglycerate kinase-1 (enzyme)
Pgk-1	= phosphoglycerate kinase-1 (gene)
PMS	= phenazine methosulphate
T-eth-HCL	= triethanolamine hydrochloride
Xce	= X-chromosome controlling element
X^M	= maternally-derived X chromosome
X^P	= paternally-derived X chromosome

KEY TO ABBREVIATIONS USED IN TABLES AND GRAPHS

BM = bone marrow

HS = subpopulation of high scattering cells

IC = subpopulation of inner cortical thymocytes

M = subpopulation of medullary thymocytes

NL = neonatal liver

OC = subpopulation of outer cortical thymocytes

WBC = leukocytes (de-blooded extract)

WHOLE = unpurified tissue extract

STAINING FOR SPECIFIC ANTIGENS
(+ and - means staining positively and negatively, respectively for a specific antigen)

B = B220+ve (B cells)

Bb = cells staining brightly for B220

Bd = cells staining dully for B220 (pre-B cells)

(B/T)+ = B220+ve and Thy1.2+ve cells, pooled

(B/T)- = B220-ve and Thy1.2-ve cells, pooled

L1+ = Ly1+ve cells (subset of T and B cells)

L1b = cells staining brightly for Ly1

L1d = cells staining dully for Ly1

L2+ = Lyt2+ve cells (cytolytic T cells)

L2b = cells staining brightly for Lyt2

L2d = cells staining dully for Lyt2

L4+ = L3T4+ve cells (helper T cells)

L4b = cells staining brightly for L3T4

L4d = cells staining dully for L3T4

T = Thy1.2+ cells (thymocytes/ T lymphocytes)

Tb = cells staining brightly for Thy1.2

Td = cells staining dully for Thy1.2

Contents

CHAPTER 1

Introduction

This chapter introduces several topics that are relevant to the study of a novel X-linked mutation which affects the haematopoietic and muscular systems in mutagen-treated mice.

The topics *haematopoiesis* in which some general points are discussed and *lymphocyte differentiation* which includes characterisation of B and T cell subpopulations, lymphopoiesis and immunopoiesis are followed by an overview of the haematopoietic and muscular *X-linked defects* in humans and mice and the techniques used in analysis of the X-linked defects. Finally, the aims of this study are presented.

Haematopoiesis

In this study, the term haematopoietic is used in the general sense and means relating to and the formation of blood cells.

In higher vertebrates haematopoietic stem cells originate from yolk sac mesoderm and are subsequently found in intraembryonic tissues (Metcalf and Moore, 1971). Whether intraembryonic stem cells arise independently or as a result of migration from the yolk sac is controversial. Some evidence suggests that lymphocytes in the avian embryo are derived de novo from intraembryonic tissues

(Moore and Owen, 1966; Dieterlen-Lievre, 1975; Dieterlen-Lievre and Martin, 1981).

In the mouse, haematopoietic stem cells (HSCs) are detectable in blood islands of the yolk sac by 8-9 days of gestation and have also been detected in the embryo (Cumano et al, 1993; Godin et al, 1993; Medvinsky et al, 1993). By day 8, these stem cells or colony-forming cells (CFUs) are capable of forming erythroid, megakaryocytic, granulocytic and mixed colonies. From day 10, CFUs are detectable in the fetal liver and are no longer detectable in the yolk sac at day 12 (Moore and Metcalf, 1970; van Rees et al, 1990).

Precursor B cells (Owen et al, 1974) and T cells are detectable (Williams et al, 1977) in the 11-day fetal liver. Within a few days of birth, the liver ceases to generate lymphocytes (Melchers et al, 1975; Rosenberg and Cunningham, 1975).

Sites of haematopoiesis are also found subsequently in the spleen and towards birth the bone marrow. Bone marrow stroma appears to be mesenchymal in origin and is formed prior to macrophage and lymphoid invasion. Lymphoid precursors are found associated with macrophages prior to birth (Tavassoli and Crosby, 1970; Heinen and Tsunoda, 1987).

Stem cells persist throughout adult life seeding either myeloid (Till and McCulloch, 1961; Fowler et al,1967;

Kitamura et al, 1981) or lymphoid cell lineages (Trentin et al, 1967; Nowell et al, 1970; Abramson et al, 1977; van Rees et al, 1990). It has been speculated that lymphoid stem cells exist as an intermediate stage, between multipotential stem cells and lymphocytes (Kincade and Phillips, 1985).

For effective haematopoiesis to occur, stem cells have to be supported by a functional stroma or haematopoietic inductive micro-environment (HIM) (Trentin, 1971; Dexter et al, 1977; Tavassoli and Friedens, 1983; Allen and Dexter, 1984). Several culture methods have been used to study the factors involved in haematopoiesis including long-term marrow culture systems for lymphopoiesis (Whitlock and Witte, 1982; Whitlock et al, 1984; 1985), megakaryopoiesis (Metcalf et al, 1975; Nakeff and Daniels-McQueen, 1976), erythropoiesis (Stephenson et al, 1971) and mixed colony formation (Johnson and Metcalf, 1978; Metcalf and Johnson, 1978; Fauser and Messner, 1979).

Long-term bone marrow cultures involve the formation of a multi-cellular layer and can be used to study the factors involved in haematopoiesis such as cell-cell interaction and production of soluble factors (Dexter and Lajtha, 1974; Dexter et al, 1977).

Soluble factors, stimulators and inhibitors of stem cells, have been detected in bone marrow (Lord et al, 1976, 1977) and bone marrow cultures (Tokzoz et al, 1980;

Cronkite et al, 1983). Some molecular factors have been shown to have co-stimulatory activities with stromal cells. Examples of these include interleukins, colony-stimulating factors, insulin-like growth factor (IGF-1) and stem cell factor which is the ligand for the tyrosine kinase receptor c-kit expressed on various haematopoietic cells (Zsebo et al, 1990; McNiece et al, 1991; Ogawa et al, 1991; Rolink et al, 1991a; Hirayama et al, 1992; Ikutu et al, 1992; Kodama et al, 1992; Landreth et al, 1992). Cytokines exhibit pleiotropy and redundancy (Paul, 1989). Specificity to particular differentiation events may involve regulation of autocrine production of progenitor cells by stromal cells as well as paracrine production by stromal cells (Nakayama et al, 1989).

Lymphocyte Differentiation

Lymphocyte differentiation can be divided into two stages. Firstly, lymphopoiesis whereby lymphocytes develop from haematopoietic stem cells, discussed above. Secondly, immunopoiesis whereby resting lymphocytes are activated following exposure to antigen and interactions with various cell types and cytokines.

The various stages of lymphocyte differentiation can be defined by flow cyometric analysis of cell populations on the basis of cell surface antigen expression and cell size and by markers defining nuclear and cytoplasmic events. For the purposes of this study, characterisation of the various stages of differentiation relates to

analysis of cell surface markers in defining lymphocyte subpopulations.

B Cell Differentiation

B Cell Subpopulations

Hardy et al, 1991 used multiparameter flow cytometry analysis to study the cell surface markers differentially expressed during B lymphopoiesis to define various B-lineage subsets within the bone marrow by identifying B220 (CD45) found on B-lineage cells in addition to other cell surface markers. Cells defined as $B220^{lo}$ and $CD43^{+}$, or $S7^{+}$ the murine homologue, are named pro-B cells and can be subdivided into three fractions, named A, B, C and C' according to the differential expression of the markers BP-1 and HSA such that the earliest pro-B cell fraction A does not express BP-1 and HSA; cells in fraction B express HSA but not BP-1; cells in fraction C express both BP-1 and HSA; and cells in fraction C' differ from those in fraction C by expressing higher levels of HSA. Pro-B cells subsequently give rise to pre-B cells, named fraction D, a process that is marked by the loss of CD43 expression with greater expression of HSA. In turn, pre-B cells which do not express surface IgM and IgD give rise to immature B cells, named fraction E, expressing surface IgM which become mature B cells, named fraction F, expressing IgM and IgD.

Other work demonstrated that c-kit, a receptor tyrosine

kinase and IL-7R, the IL-7 receptor are differentially expressed by the pro-B cell fractions defined above. It was shown that cells in fraction A did not express c-kit or IL-7R; cells in fraction B and C expressed both c-kit and IL-7R; and cells in fraction C' expressed IL-7R but c-kit expression is down-regulated. IL-7R expression remained until expression of surface IgM. It was discussed that the decrease in c-kit expression between the pro-B stages C to C' might be related to the expression of the μ-chain molecule and the decrease in expression of IL-7R between stage D to E might be related to the expression of surface IgM (Era et al, 1994). The observed differential expression of c-kit and IL-7R is related to the factors affecting B lymphopoiesis namely, stem cell factor which is the ligand for c-kit and IL-7, discussed below. It should be noted that various studies discussed below refer to c-kit expression on pre-B cells which belong to fraction D according to the model of Hardy et al, 1991. However, this discrepancy can be related to a variation in the nomenclature that defines the B-lineage compartments used by various laboratories.

The earliest stages of B cell differentiation are characterised by Ig heavy-chain rearrangement. Recent studies of the rearrangement of the Ig heavy chain genes at various stages of differentiation within the bone marrow have shown that the Ig genes of the earliest pro-B cell fraction A, were in germline configuration, whereas pro-B cells within fractions B-C showed increasing D-J

rearrangement but no V-D-J rearrangement. Complete V-D-J rearrangement was seen in pre-B and B cell fractions (Hardy et al, 1991; Ehlich et al, 1993; Li et al, 1993). Following light-chain V-J rearrangement, an intact immunoglobulin molecule is produced and expressed as IgM on the cell surface (Yancopoulos and Alt, 1986). Cells expressing membrane IgG and IgA are only detectable after birth (Abney et al, 1978); IgD is detectable 10-15 days after birth, its concentration increasing until it is the predominant surface immunoglobulin by 3 months of age (Vitetta et al, 1975; Vitetta and Uhr, 1977).

Characterisation of peripheral B cell subpopulations by cell surface phenotype has defined distinct subsets of splenic B cells according to the density of membrane immunoglobulin isotype such that population I has high levels of IgD and low levels of IgM; population II has high levels of IgD and IgM; and population III has low IgD, high IgM and population IV expressed neither IgD nor IgM (Hardy et al, 1982, 1984).

Similarly, B cell subpopulations have been defined by the level of cell surface expression of IgM and the Fc receptor whereby B cells that are IgM^-, FcR^-; IgM^+, FcR^- and IgM^+, FcR^+ represent distinct maturation stages (Chan and Osmond, 1979). B cells have been defined that have higher expression of B220 (Forster et al, 1989), expression of CD23 and IgD and lower levels of HSA expression (Hardy et al, 1991; Tarlinton, 1993). It has

been shown that differential Ia expression can define recirculating B cells which have higher levels of Ia expression from pre-B and immature B cells of adult bone marrow. It was also shown that pre-B cells expressed Ia and pro-B cells did not express Ia within the adult bone marrow (Tarlinton, 1993).

Other B cell subpopulations defined by their cell surface phenotype include B-1 cells, which were formerly Ly-1 B cells, and B-2 or conventional B cells. B-1 cells are identified by their distinctive expression of IgM, IgD and CD5 (Ly-1), where IgM levels are higher and IgD levels are lower than levels in B-2 cells, including them in population III, as defined above (Herzenberg et al, 1986).

Other markers have been used to further define B-1 and B-2 cells. For example, in the peritoneal cavity B-1 cells are $CD11^{+}$ (Mac 1) and $CD23^{-}$ whereas B-2 cells are $CD11^{-}$ and $CD23^{+}$. Also, CD11 is expressed on peritoneal and pleural cavity B-1 cells but is not expressed on splenic B-1 cells or B-2 cells; CD23 is expressed on peritoneal B-2 cells and on the splenic B-2 cell population expressing high levels of IgD but it is not expressed on B-1 cells (Waldschmidt et al, 1991; Stall et al, 1992). It has been shown that B-1 cells express higher levels of MHC class II than B-2 cells and also that B-1 cells express the activation antigen CD44 (pgp-1) (Murphy et al, 1990).

In addition to cell surface phenotype, B cell sub-populations can be defined on the basis of anatomical localisation. For example, in contrast to B-2 cells, B-1 cells predominate in neonatal spleen, peritoneal and pleural cavities but are rarely found in lymph nodes, Peyer's patches or peripheral blood and represent a small percentage of total B cells in the adult spleen (Hardy et al, 1984; Hayakawa et al, 1985; Antin et al, 1986; Marcos et al, 1989; Kroese et al, 1992).

Furthermore, cell subpopulations have been identified on the basis of the developmental lineage to which they belong. On this basis, three distinct B cell lineages have been defined. B-1a cells that are $CD5^+$ are derived from progenitors present in fetal omentum and fetal liver but not adult bone marrow ; B-1b cells that are $CD5^-$ are derived from progenitors present in fetal omentum, fetal liver and adult bone marrow and, B-2 or conventional B cells are derived from progenitors present in fetal liver, adult bone marrow but not fetal omentum (Solvason et al, 1991; Kantor et al, 1992a, b; Solvason et al, 1992; Kantor and Herzenberg, 1993).

Other evidence suggests B-1 and B-2 cells are derived from independent lineages as irradiated recipients of neonatal or embryonic haematopoietic cells generate both B-1 and B-2 cells but recipients of adult bone marrow rarely generate B-1 cells (Hayakawa et al, 1985; Hardy and Hayakawa, 1986; Herzenberg et al, 1986). In fact,

depletion of B-1 cells, early in development, produces permanent B-1 cell loss (Lalor et al, 1989a, b). Other studies confirm independent progenitor origins of B-1a and B-1b cells (Lalor et al, 1989a, b; Stall et al, 1992).

B Lymphopoiesis

B lymphopoiesis is the ordered progression of a stem cell through various stages of differentiation from pro-B, to pre-B , to immature B cell resulting in the production of a mature B cell. The process occurs in the fetal liver and subsequently in the neonatal and adult bone marrow. Within the fetal liver, pre-B cells can be detected by day 11. By day 12, Ig heavy chains are being synthesised. Following light chain synthesis, immature B cells expressing IgM are detectable by day 16-17. Within a few days of birth, the liver stops generating lymphocytes (Spear et al, 1973; Owen et al, 1974; Melchers et al, 1975; Rosenberg and Cunningham, 1975; Raff et al, 1976; Cooper, 1981).

Cell surface components which can be used to define the various stages of differentiation, as discussed above, may be important in cell-cell interactions or may function as receptors for signals from the micro-environment resulting in the activation or inactivation of genes that regulate cell differentiation (Witte et al, 1987).

Culture systems have enabled studies of B lymphopoiesis (Whitlock and Witte, 1982; Kincade et al, 1989; Dorshkind, 1990; Rolink and Melchers, 1991). It has been shown that the proliferation and differentiation of pro- and pre-B cells require cell contact with a micro-environment of stromal cells. During B lymphopoiesis in fetal liver there is a stromal cell-dependent phase at 13-16 days of gestation, followed by a stromal cell-independent phase at 17-19 days of gestation. Cells expressing surface Ig appear at 16-17 days of gestation and cells become reactive to antigen at 18-19 days of gestation (Strasser et al, 1989). This suggests that gene rearrangements of the Ig heavy and light chain loci occur at the early stromal cell-dependent phase and that B cells expressing surface Ig become antigen-reactive cells at the later stromal cell-independent phase.

Stromal cells derived from fetal liver and bone marrow can support long-term proliferation of defined sub-populations of pro- and pre-B cells. The stromal cell-derived factor, IL-7 has been identified as a co-stimulator of proliferation of these cells (Namen et al, 1988).

Studies have defined three stages of pro- and pre-B cell development namely, a first stage requiring stromal cells for proliferation to occur, a subsequent stage which requires both stromal cells and IL-7 for proliferation to occur and a late stage which requires only IL-7 for

proliferation to occur (Nishikawa et al, 1988; Hayashi et al, 1990). Sudo et al, 1989 suggested that in the intermediate stage, the stromal cell contact with pro- and pre-B cells may induce IL-7 production by stromal cells.

Rolink and Melchers, 1993 discussed that pre-B cells may be sub-divided into a pre-BI stage followed by a transitory pre-BII stage and that the former remain at their given stage of differentiation in the presence of stromal cells and IL-7 and the latter remain at their given stage in the presence of IL-7 alone. It was shown that removal of IL-7 from cultures of pre-BI cells results in differentiation with unproductive rearrangements of Ig gene loci, down-regulation of CD43 and c-kit, leading to a loss of stromal cell with IL-7 reactivity and apoptosis of surface Ig^- and Ig^+ cells (Rolink et al, 1991b). Rolink and Melchers, 1993 discussed whether crowding within the marrow can result in some pre-B cells being removed from contact with stromal cells and the site of IL-7 production resulting in differentiation of these cells.

Other molecular factors, introduced in the section on haematopoiesis above, that are likely to have co-stimulatory activities possibly with different types of stromal cells and on different stages and subpopulations of differentiating B cells include IL-6, IL-11, GM-CSF, IGF-1 and stem cell factor or KL, the ligand for the

c-kit tyrosine kinase receptor expressed on pro- and pre-B cells (Zsebo et al, 1990; McNiece et al, 1991; Hirayama et al, 1992; Landreth et al, 1992). Stem cell factor has been shown to synergise with Il-7 in inducing pre-B cell proliferation (Billips et al, 1992). It was indicated that pro-B cells also required the presence of both factors for proliferation and that each factor had differing effects (Era et al, 1994).

Various adhesion systems have been shown to be important in forming interactions between stromal cells and the developing B cell. These include fibronectin, CD44 and its ligand hyaluronate, also the integrin molecule VLA-4 and its ligand VCAM-1, N-cadherin and N-CAM, and the surface proteoglycan syndecan (Bernardi et al, 1987; Thomas et al, 1988; Miyake et al, 1990a, b; Miyake et al, 1991a,b; Sanderson et al, 1992).

B-cell formation can be limited by the action of negative regulators. For example, transforming growth factor (TGF-β) can inhibit processes at the pre-B cell stage (Lee et al, 1987, 1989). There are also examples of cytokines that are positive and negative regulators of B lymphopoiesis. For example, IL-4 can stimulate pre-B cell growth (Peschel et al, 1989) and promote differentiation of pre-B cells to B cells (King et al, 1988) and as a negative regulator, IL-4 can inhibit pre-B cell proliferation (Fanslow et al, 1993). Other cytokines include IL-1 which can stimulate pre-B lymphoma

development (Giri et al, 1984) and act as a negative regulator of B lymphopoiesis in culture (Billips et al, 1990). γIFN can stimulate Ig expression of pre-B cells (Weeks and Sibley, 1988), can arrest proliferation and can induce apoptosis of pre-B cells in culture (Gimble et al, 1993; Grawunder et al, 1993).

During B lymphopoiesis, cell migration within the marrow occurs from the subendosteal region towards the centrally located sinuses. It has been suggested that compartmentalisation of the bone marrow is important in providing distinct stromal cells that produce different signals such that the subendosteal cells provide factors that support the early lymphopoietic stages and the central regions provide factors that support the later lymphopoietic stages (Jacobsen and Osmond, 1990; Rolink and Melchers, 1991).

Following gene rearrangement, the resulting Ig heavy and light chain repertoire expressed on the B cell surface is exposed to selective processes. As a result of negative selection against self antigens, self-reactive B cells are either deleted or anergised (Goodnow et al, 1989; Nemazee and Burki, 1989). Immature B cells leave the bone marrow and a small fraction enter the peripheral B-cell pool. This is a selective process and may depend on exposure to antigen (MacLennan and Gray, 1986; Forster and Rajewsky, 1990; Gu et al, 1991). Within the spleen, B lymphocytes migrate from the

red pulp to the white pulp (Brahim and Osmond, 1970; 1976). It has been suggested that newly-formed B lymphocytes become immunologically responsive after 1-4 days within the periphery (Osmond, 1986).

B Immunopoiesis

The process of immunopoiesis occurs mainly in secondary lymphoid organs such as the spleen, lymph nodes Peyer's patches and tonsils. The first 3-4 days of a primary antibody response involves the rapid conversion of virgin B cells into relatively short-lived plasma cells secreting mostly IgM of low affinity. For T-dependent antigens, a second phase of antibody response after 4-5 days results in the production of relatively long-lived plasma cells secreting high-affinity, mostly IgG or IgA with some IgE antibody and also memory cells that differentiate into plasma cells following subsequent exposures to antigen (Siekevitz et al, 1987; van Rooijen, 1990; Berek, 1992; MacLennan et al, 1992).

B cell activation follows cross-linking of cell surface receptors or surface immunoglobulin receptors for antigen. The following discussion relates to B cell activation following antigen invasion resulting in cross-linking of (1) B cell surface CD40 by its ligand in a T cell-dependent response to antigen and (2) surface immunoglobulin triggered by antigen.

Dendritic cells from skin or mucosa trigger a specific

immune response by trapping antigen and migrating into the T cell-rich paracortical areas of the secondary lymphoid organs such as the spleen or lymph nodes (MacLennan and Gray, 1986; Kroese et al, 1990; MacLennan et al, 1990; Liu et al, 1992; Gray, 1993). In the T cell-rich areas the antigen-derived peptides bound to MHC class II antigens are presented by the inter-digitating dendritic cells to virgin or memory antigen-specific T cells triggering the extrafollicular reaction and adhesion molecules maintain the interaction between the dendritic and T cells. Cross-linking of surface CD40 on dendritic cells by its ligand on T cells enhances production of cytokines by dendritic cells which triggers activation, proliferation and differentiation of T cells. Similarly, the cross-linking of surface CD28 on T cells by its ligand on dendritic cells stimulates increased T cell cytokine production which may function as T cell growth and differentiation factors, in an autocrine manner or may in turn activate the dendritic cells or may induce the proliferation and differentiation of antigen-specific B cells. T cells interact with the antigen-activated B cells through surface molecules. For example, cross-linking of surface CD40 on B cells by the CD40 ligand on activated $CD4^+$ T cells switches on activation and differentiation and may act as a signal for the migration of B cells into primary follicles composed of follicular dendritic cells (Tew et al, 1990; Armitage et al, 1992; Noelle et al, 1992; Schriever and Nadler,

1992).

A massive proliferation of B cells initiates the germinal centre reaction (Kroese et al, 1987; Liu et al, 1991) and once a germinal centre has formed, proliferating centroblasts are identifiable within the dark zone where high-rate somatic mutations within the variable regions of Ig genes of B cells occur (Tonegawa, 1983; Berek et al, 1991; Jacob et al, 1991).

Subsequently, centroblasts mature into centrocytes that are non-proliferating in the light zone. Within the light zone, centrocytes are selected for their ability to bind to antigen deposited on follicular dendritic cells as immune complexes. Survival of these cells depends on positive selection by antigen receptor triggering. The antigen presented on follicular dendritic cells is processed by selected B cells and presented to activated antigen-specific T cells that produce IL-2, IL-4 and IL-10. Subsequently, as a result of interactions with surface CD40 on B cells and its ligand, proliferation of selected B cells occurs. Isotype switching within B cells takes place and selected B cells are induced to differentiate into recirculating memory cells or plasma blasts that migrate from the germinal centres to the bone marrow or to the mucosal lamina propria forming plasma cells (Gray et al, 1991; Howard et al, 1992; Butch et al, 1993; Lebecque et al, 1993). Plasma cells produce immunoglobulins the isotypes of which have specific

effector functions such as binding to Fc receptors which results in an array of activities (Ravetch and Kinet, 1991).

Cross-linking of CD40 on B lymphocytes induces tyrosine phosphorylation of four substrates. Activation of protein kinases is important for the transduction of CD40 signals. Cross-linking of CD40 results in phosphorylation of a subunit of PI3K (phosphatidyl inositol 3 kinase) resulting in increased PI3K activity which catalyses phosphoinositol phosphorylation. Ligation of CD40 induces phosholipase C phosphorylation (Kansas and Tedder, 1991; Uckun et al, 1991; Banchereau et al, 1994).

CD40 cross-linking has been shown to induce B cells to produce IL-6 and IL-10 and the presence of IL-4 with the CD40 ligand enhances the proliferation of B cells. Other factors such as IL-1, Il-2, IL-10 and IFN γ, in combination with CD40 cross-linking, enhance B cell proliferation. CD40 cross-linking activates immuno-globulin isotype switching, the specificity of which is dependent on the type of cytokine involved. IL-4 and IL-10, in combination with CD40 cross-linking induce B cell differentiation with the production of immunoglobulins of various isotypes (Clark and Shu, 1990; Banchereau et al, 1991; Rousset et al, 1991, 1992; Spriggs et al, 1992; Armitage et al, 1993; Burdin et al, 1993).

The importance of the ligand for CD40 has been demonstrated in studies of patients with X-linked hyper-

IgM syndrome, discussed below in the section X-linked defects. B cells produce IgM but almost no IgG or IgA and secondary antibody responses to T-dependent antigens are defective in these patients (Ochs et al, 1971; Allen et al, 1993; Aruffo et al, 1993; DiSanto et al, 1993; Korthauer et al, 1993).

Following antigen invasion resting B cells are activated by cross-linking of surface Ig. Although antigens are recognised by surface IgM and IgD, transmembrane signalling induced by interaction of antigen with surface Ig is mediated by other associated molecules. The B cell antigen receptor complex appears to be composed of surface Ig molecules which are associated with the molecules Ig-α and Ig-β which may be responsible for generating the signals that mediate activation (Campell et al, 1991; Reth, 1992). The activation process involves calcium mobilisation and activation of protein kinase C (PKC) following PIP_2 (phosphatidylinositol 4,5-bis-phosphate) breakdown into IP_3 (inositol-1,4,5-tris-phosphate) and DG (diacylglycerol) where IP_3 causes Ca^{2+} release and DG causes PKC activation (Berridge and Irvine, 1984; Nishizuka, 1984). The combined effect of two signals, namely cross-linking of Ig receptors and helper factors leads to growth factor receptor expression and hence activation. Cross-linking of Ig receptors activates B cells and induces the formation of receptors for IL-4 or IL-5 which are B cell growth factors.

Subsequently, IL-4 induces early activation of resting B cells and IL-5 stimulates the proliferation of these activated cells. Finally, IL-6 which is a B cell differentiation factor, brings about the final differentiation of B cells, to form Ig-secreting cells. IL-4, IL-5 and IL-6 have other roles in B cell differentiation. IL-4 induces expression of FcR, MHC class II antigens and production of IgE and IgG1. IL-5 induces expression of the IL-2 receptor, IgM, IgG and IgA; and IL-6 stimulates IL-2 production and cell growth (Paul and Ohara, 1987; Takatsu et al, 1988; Van Snick, 1990). IFN α and IFN β have both positive effects on B cell proliferation (Defrance et al, 1986; Peters et al, 1986) and negative effects on differentiation (Rabin et al, 1986; Kashiwa et al, 1987).

Cytokines are produced from different $CD4^+$ helper T cells. Studies of murine T cell clones have shown that IL-4, IL-5, IL-6 and IL-10 are produced by TH2 clones whereas IL-2 and IFN γ are produced by TH1 clones (Banchereau and Rousset, 1992). As a result of the different cytokines produced, TH2 cells induce IgG1 and IgE secretion and TH1 cells induce IgG2a secretion (Coffman et al, 1988). The combination and the relative concentrations of cytokines produced can determine the Ig isotype secreted (Croft and Swain, 1991).

T Cell Differentiation

T Cell Subpopulations

The process of thymocyte differentiation can be studied by analysis of cell surface markers that define distinct subpopulations of T cells.

T cell markers have been identified on bone marrow cells which have been shown to have the capacity to form T cell clones in culture. These include, the early T cell marker, CD7 and co-expression of the stem cell marker, CD34 with various T-lineage restricted markers such as CD7, CD2 and cytoplasmic CD3. Flow cytometric analysis of three cell surface markers on bone marrow cells has identified a cell subpopulation that reconstitutes T cell development following transfer into immunodeficient SCID mice (Benveniste et al, 1990; Bertho et al, 1990; Palacios et al, 1990; Terstappen et al, 1992). The extent to which commitment to the T-cell lineage occurs in the bone marrow is controversial. Although the above evidence suggests some degree of commitment to the T-cell lineage in the bone marrow, other studies have demonstrated the ability of HSCs to colonise an irradiated thymus and that cells from the thymus can produce cells of the B- and myeloerythroid-lineage under certain conditions which may imply that HSCs seeding the thymus are multipotential (Spangrude et al, 1988; Kurtzberg et al, 1989; Spangrude and Johnson, 1990; Spangrude and Scollay, 1990).

The earliest thymocytes are blast cells expressing low level Thy-1, receptors for Il-3, IL-4 but no expression of CD4 (murine L3T4), CD8 (Lyt2) nor TCR. Expression of low level CD5 (Ly1) follows, with the receptor for IL-2 and a loss of the receptor for IL-3 (Lobach et al, 1985; Adkins et al, 1987; Bluestone et al, 1987; Campana et al, 1989; Fowlkes and Pardoll, 1989; Terstappen et al, 1992).

At this $CD4^-$ $CD8^-$ or double-negative stage there is rearrangement of the TCR gene the nature of which determines the T cell pathway followed. Thus, cells eventually differentiate into either TCR$\gamma\delta$/$CD3^+$ T cells at day 14-15 that remain double-negative or later acquire $CD8^+$ only or TCR$\alpha\beta$/$CD3^+$ T cells that are single-positive, $CD4^+$ or $CD8^+$, 2-3 days later following a transient $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ stage and then a double-positive $CD4^+$ $CD8^+$ stage. In the transition from the double-positive to single-positive stage, cells increase their surface expression of CD3 and TCR and alter their expression of homing receptors to that seen on virgin peripheral T cells (Pardoll et al, 1987; Palacios and Pelkonen, 1988; Fowlkes and Pardoll, 1989; Terstappen et al, 1992).

Single-positive thymocytes appear during the medullary stage following positive selection for single-positive cells with productive TCR gene rearrangements and the expression of TCR marks the end of the proliferative

phase by day 18-19 of gestation (Kinnon et al, 1986; Kronenberg et al, 1986; Jenkinson et al, 1987; Sprent et al, 1988; Guidos et al, 1989).

Thymocytes can be further defined by their differential expression of R0 and RA isoforms of CD45 or T-200 antigen, a protein tyrosine phosphatase. For example, expression of isoforms that stain with CD45R antibodies is higher for thymocytes of the medulla than the cortex and $CD4^-$ $CD8^-$ thymocytes have been shown to be $CD45R0^+$ $CD45RA^-$ (Pulido et al, 1988; Janossy et al, 1989; Gillitzer and Pilarski, 1990; Okumura et al, 1992).

Both $CD4^+$ and $CD8^+$ T cells can be subdivided by CD45 isoform expression (de Jong et al, 1991; Inoue et al, 1993; Okumura et al, 1993). In humans, peripheral T cells can be divided into subsets according to the reciprocal expression of the RA and RO isoforms of CD45 such that virgin T cells are defined as $CD45RA^+$ $/RO^{-\text{ to lo}}$ and effector/memory T cells are defined as $CD45RA^-$ $/RO^{hi}$ (Sanders et al, 1988; Picker et al, 1993). These two cell populations can be further defined by their differential expression of adhesion molecules that facilitate cellular interactions with target cells, accessory cells and endothelium. For example, the $CD45RA^-$ $/RO^{hi}$ subset displays increased expression of β2- and β1-integrins, ICAM-1 (CD54), CD2, LFA-3 (CD58) and HCAM (CD44) (Sanders et al, 1988; Buckle and Hogg, 1990; Picker et al, 1990; Shimizu et al, 1990, 1991).

It has also been shown that $CD4^+$ memory/effector T cells can be divided into TH1 and TH2 subsets which differ in the type of cytokine produced and these cells arise from a common precursor population, TH0 (Abbas et al, 1991; Mosmann et al, 1991; Romagnani, 1991; Swain et al, 1991).

T Lymphopoiesis

Stem cells from the fetal yolk sac, fetal liver and adult bone marrow colonise the developing thymus at 11 days of gestation in the mouse (Moore and Owen, 1967; Owen and Raff, 1970; Stutman and Good, 1971). The extent to which commitment to the T-cell lineage occurs in the bone marrow is controversial, as discussed above. In vitro studies have shown that the chemotactic peptide, thymotaxin which is produced by thymic epithelial cells is involved in migration of stem cells to the thymus (Champion et al, 1986).

T lymphopoiesis involves interactions between lymphoid progenitors and cells within the microenvironments of the thymus. The cortex makes up to 90% of the thymus and the medulla comprises 10-15% (Scollay, 1983). Prothymocytes have been located in the thymic cortex and during development they migrate to the cortico-medullary junction and then to the medulla (Owen and Raff, 1970). During the cortical stage, immense proliferation of prothymocytes occurs as a result of interaction with thymic epithelial cells or marrow-derived accessory

cells. Phagocytic cells of the thymic reticulum regulate proliferation by producing IL-1, prostaglandins and deoxynucleosides which stimulate blast proliferation and protect prothymocytes from deoxyguanosine activity and hence, cell death (Papiernik et al, 1983; El Rouby et al, 1985; Penit and Papiernik, 1986).

It has been shown that phagocytic cells of the thymic reticulum induce prothymocyte proliferation in combination with IL-2 (Papiernik et al, 1987). It has been suggested that at 12-15 days of gestation proliferation and differentiation of prothymocytes is induced by IL-4 (Palacios and Pelkonen, 1988) and at 15-16 days IL-2 is used as cells capable of producing IL-4 are found at the former stage (Sideras et al, 1988) and IL-2 activity appears to be at the latter stage (Lugo et al, 1986; Ceredig et al, 1987). It has been suggested that early on in the cortical proliferative phase, IL-2 is important for cell surface expression of CD4 and CD8 (Tentori et al, 1988).

The thymic epithelium has been shown to produce IL-1, IL-3, IL-6, IL-7 and LIF and GM-CSF which affect the differentiation of thymocytes (Fujiwara et al,1990; Le et al, 1990; Carding et al, 1991; Dalloul et al, 1991). Thymic epithelial cells express surface adhesion molecules which may form adhesive associations with thymocytes and may function in the transduction of activation signals (Singer et al, 1990). Thymic

epithelial cells also produce peptide hormones and chemotactic peptides which are involved in induction of stem cell differentiation, induction of surface markers on thymocytes and activation of effector activity (Dardenne and Bach, 1988; Dargemont et al, 1989).

Developing thymocytes, with varying affinity levels for MHC, interact with MHC molecules on the cortical epithelium (Benoist and Mathis, 1989; Blackman et al, 1989). Cells are subsequently screened for low-intermediate affinity levels (MHC-restricted cells), are positively selected, or protected and the remaining cells die. Transgenic studies have shown that mature T cells expressing a particular MHC class-restricted TCR, only develop in mice of the appropriate MHC haplotype such that class I-restricted TCRs are found on $CD8^+$ cells and class II-restricted TCRs are found on $CD4^+$ cells, in general (Kisielow et al, 1988; Sha et al, 1988; Teh et al, 1988; Berg et al, 1989); this was also demonstrated in normal mice (MacDonald et al, 1988).

Other studies show that self-peptides, presented on the thymic epithelium along with class I MHC molecules, are involved in positive selection of T cells that are class I MHC-restricted (Nikolic-Zugic and Bevan, 1990). It has been shown that an unidentified minor antigen, in addition to MHC class II molecule is required for clonal deletion and its effect can be mimicked by bacterial enterotoxins (Janeway et al, 1989; White et al, 1989). T

cell migration to the cortico-medullary junction occurs where further screening or negative selection for high affinity (auto-MHC reactivity) levels for MHC by macrophage and dendritic cells takes place (Lo et al, 1986 ; Lo and Sprent, 1986). This appears to be specific to the medulla (Hengartner et al, 1988; MacDonald, 1989). It has been shown that T cells bearing an auto-reactive TCR die in culture and this cell death can be prevented by inhibitors of RNA and protein synthesis (MacDonald and Lees, 1990).

It has been suggested that positive signals from the cortical epithelium occur because TCR density of immature thymocytes is too low for high-affinity MHC binding and as medullary thymocytes have increased TCR levels and therefore higher affinity levels for MHC, negative signals from the medullary dendritic cells results in clonal deletion. Furthermore, remaining cells receive positive signals, are protected and survive to become mature $CD4^+$ or $CD8^+$ T cells (Sprent et al, 1988).

Other results have shown that cortical epithelial cells present molecules which differ from medullary molecules (Marrack et al, 1989). A monoclonal antibody detecting an MHC class II epitope has been described which stains the medulla but not the cortex (Murphy et al, 1989).

Quackenbush and Shields, 1988 suggested that those cells which fail positive selection do not die within the thymus, are exported and fail to survive outside. Some

evidence suggests this cell death takes place in the intestine (Joel et al, 1971) and this view is in keeping with a lack of histological evidence of intra-thymic cell death (Rothenberg, 1990).

Lymphocyte fibronectin receptors may be involved in the migration of T cells from the thymus (Cardarelli et al, 1988). Once lymphocytes are in circulation, their homing receptors or points of adhesion interact with ligands of high endothelial venules (HEV). The nature of directed migration depends on the lymphocyte involved, their developmental stage, state of activation and history of antigenic stimulation and may involve haptotactic and chemotactic gradients (Yednock and Rosen, 1989).

Evidence suggests that T cell maturation is not confined to the thymus and that post-thymic factors are involved (Stutman, 1978; Piguet et al, 1981). It has been shown that recent emigrants are larger than peripheral T cells (Kelly and Scollay, 1990) and express different antigens (Scollay et al, 1984).

T Immunopoiesis

Antigenic stimulation of T cells results in clonal expansion, generation of memory T cells and maturation into regulatory or effector cells. T cells require activation signals from the antigen presenting cell (APC) in the form of cell surface molecules which provide co-

stimulatory signals. The level of expression by APC may depend on the state of cell activation and the presence of cytokines. For example, CD28 on T cells is a receptor for co-stimulatory signals provided by the B7 molecule on APCs (Jenkins et al, 1988; Mueller et al, 1989; Geppert et al, 1990; Linsley et al, 1991a, b; Liu et al, 1992) and studies have shown that activation of T cells with anti-CD28 mAb and anti-CD2 leads to the production of various cytokines (Thompson et al, 1989; Ledbetter et al, 1990; Cerdan et al, 1991). Adhesion receptors such as CD2 on T cells which interact with LFA-3 on the APC are important in stabilising interactions between these cells (Springer, 1990).

In general, $CD4^+$ cells are termed helper T cells and $CD8^+$ cells are termed cytolytic T cells and the cell surface glycoproteins CD4 and CD8 on T lymphocytes have specificity for class II or class I MHC proteins, respectively. These proteins are involved in the differentiation of thymocytes resulting in the production of a mature T cell repertoire and have a role in the activation and effector functions of mature peripheral T cells (Swain, 1983; Parnes, 1989).

$CD4^+$ T cells recognise antigen bound to MHC class II molecules and $CD8^+$ T cells recognise antigen bound to MHC class I molecules (Swain, 1983). In accordance with the co-receptor model, the association between antigen-bearing APCs and T cells leads to the co-receptor CD4 or

CD8 binding the same MHC ligand as the TCR/CD3 complex which involves transmembrane signal transduction by association with the tyrosine kinase p56^{lck} (Kupfer et al, 1987; Kupfer and Singer, 1989; Veillette et al, 1988; Shaw et al, 1989, 1990; Turner et al, 1990). Combinations of cell surface molecules can modulate the signalling events induced via the TCR/CD3 complex. A role for CD45 in T-cell signal transduction has been implicated (Pingel and Thomas, 1989; Koretzky et al, 1990, 1991; Weaver et al, 1992). It was suggested that CD45 may regulate tyrosine phosphorylation of p56^{lck} associated with CD4 or CD8 (Janeway, 1992).

Activated or memory subsets of T cells expressing distinct CD45 isoforms associating with the TCR/CD3 complex and CD4 or CD8 on different subsets of T cells have been described (Volarevic et al, 1990; Dianzani et al, 1992). Furthermore, T cells that express different CD45 isoforms release different cytokines (Bottomly et al, 1989). Hence, distinct subsets of T cells appear to differ in the organisation of CD45 with other cell surface structures which may relate to differences in their activation characteristics and expression of different CD45 isoforms may induce different signals that result in the differentiation of cells that produce different cytokines (Janeway, 1992).

Stimulation of the TCR/CD3 complex results in hydrolysis of PIP_2 to produce diacylglycerol which activates protein

kinase C and IP_3 which causes an increase in intracellular Ca^{2+} (Imboden et al, 1985). These events occur in some but not all T cell subsets where it appears that there is utilisation of different TCR-associated signal transduction pathways by TH1 and TH2 clones. For example, stimulation of TH1 clones with concanavalin A or anti-TCR mAb results in an increase in intracellular Ca^{2+} and in inositol phosphate production. However, following stimulation of TH2 clones, these secondary messengers are not detectable. (Gajewski et al, 1990; Williams et al, 1991). Furthermore, TH2 clones can be induced to produce IL-4 following treatment with ionomycin while similar treatment induces anergy in TH1 clones (Mueller et al, 1989; Abbas et al, 1991). It has also been shown that CD8 clones utilise similar signal transduction pathways to those of TH1 clones (Schell and Fitch, 1989).

Other factors have been described that determine the specificity of the T cell subset activated. For example, TH1 and TH2 clones respond differently to different types of APC possibly by utilising different cell surface receptors and ligands (Magilavy et al, 1989; Gajewski et al, 1991; Liu and Janeway, 1991).

Furthermore, the nature of the cytokines produced can determine the specificity of the T cell subset activated. For example, TH1 $CD4^+$ cells secrete IL-2 and IFN γ but not IL-4, IL-5, IL-6 or IL-10 and TH2 $CD4^+$ cells secrete IL-4, IL-5 and IL-10 but not IL-2 or IFN γ. These cells

arise from a common precursor population, TH0 which has the potential to produce a variety of cytokines. Hence, the functions of TH1 and TH2 cells differ due to the varying patterns of cytokine secretion. TH1 cells are mainly responsible for production of delayed-type hypersensitivity through the release of IFN γ which activates macrophages and TH1 cells also lyse virus-infected cells and induce destruction of extra- and intra-cellular pathogens by macrophages. Through the secretion of IL-4 and IL-5 which are B cell growth and differentiation factors, TH2 cells act as helper cells in interactions between T and B cells. Secretion of some cytokines by TH1 and TH2 cells can have an inhibitory effect on the other subset. IL-4 and IL-10 secretion by TH2 cells can suppress TH1 cell function and IFN γ secretion by TH1 cells is an inhibitor of TH2 cells. $CD8^+$ cells have been shown to have a TH1-like phenotype in secreting IFN γ but not IL-4 or IL-5 and under certain conditions a TH2-like phenotype in secreting IL-4 but not IFN γ and the TH2-like $CD8^+$ cells can behave like TH2-like $CD4^+$ cells in being inhibitory for TH1 cells (Mosmann and Coffman, 1989; Abbas et al, 1991; Kelso et al, 1991; Mosmann et al, 1991; Romagnani, 1991; Scott and Kaufman, 1991; Swain et al, 1991; Bloom et al, 1992; Sher and Coffman, 1992).

Also, antigen concentration can determine which T cell subset is stimulated in an immune response (Gajewski et al, 1989).

Following stimulation, T cells release IL-2 initially and IL-4, IL-5, IL-6 and IFN γ appear several days later (Swain et al, 1991). The combination and the relative concentrations of the cytokines released determines the effect on B cells. For example, T cells that release high levels of IL-4, IL-5, IL-6 but low levels of IL-2 and IFN γ induce B cells to produce all isotypes. However, T cells that release IL-2, IL-6 and IFN γ induce B cells to produce lower levels of Ig which is mainly IgM (Croft and Swain, 1991).

In general, the generation of cytolytic T cell responses requires help from helper T cells. It has been shown that TH1 clones secrete IL-2 which induces $CD8^+$ cytolytic clones to proliferate and IL-4 allows $CD8^+$ cytolytic T cells to develop from resting T cells. Il-6 and IL-10 enhance $CD8^+$ cytolytic T cell proliferation (Glasebrook and Fitch, 1979; Nabholz and MacDonald, 1983; Trenn et al, 1988; Vink et al, 1990; Chen and Zlotnik, 1991). However, some $CD8^+$ T cells have been identified as helper-independent which can be cultured without added growth factors (Widmer and Bach, 1981).

Mechanisms exist that inhibit an immune response. Results of various studies have revealed several negative immunoregulatory mechanisms. These include various models of T suppressor cells that inhibit the responses of either other T suppressor cells (Germain and Benacerraf, 1981) or helper T cells (Bloom et al, 1992) by producing

soluble factors. It has also been suggested that immune responses can be suppressed through elimination of APCs by cytolytic T cells (Simpson, 1988; Lanzavecchia, 1989). A third mechanism involves suppression by inducing anergy or antigen-specific unresponsiveness without deletion of the cells (Jenkins and Schwartz, 1987). Suppression can also be mediated by cytokines. For example, TH1 clones can be rendered unresponsive to stimulation with antigen and the APC and fail to produce cytokines following exposure to IL-2 (Wilde et al, 1984; Otten et al, 1986).

X-linked Defects

Alleles of human and murine genes encoding a variety of defects or mutations have been mapped to the X chromosome. X-linked defects, relevant to this study, include immunodeficiency syndromes, haematopoietic disorders and muscular disorders. In this section, an overview of relevant human and murine X-linked defects or mutations is given. This is followed by a discussion of the characterisation of the X-linked defects using studies of X-chromosome inactivation patterns, gene mapping and flow cytometry. Finally, the aims of the present study are presented. A comparative human and mouse X-chromosome map, Fig. 1 is given at the end of Chapter 1.

X-linked Human Immunodeficiency Syndromes

X-linked Severe Combined Immunodeficiency (XSCID)

X-linked severe combined immunodeficiency is characterised by hypo- or agammaglobulinaemia in affected males, atrophy of the thymus and absent or depressed T lymphocytes without the severe form of lymphopenia, characteristic of autosomal SCID. Patients are susceptible to bacterial, fungal and viral infection and lack delayed hyper-sensitivity reactions (Rosen et al, 1966, 1968; Dooren et al, 1968; Yount et al, 1978). B lymphocytes are present in normal or increased numbers and are capable of normal antibody production in the presence of normal T cells. B cells have been shown to express p120 antigen, a cell

surface protein normally expressed only on pre-B cells (Gougeon et al, 1990; Conley, 1992). It appears that bone marrow-derived T cells are primarily affected by the genetic lesion since the T cell deficiency can be corrected by bone marrow transplantation and the thymus of XSCID recipients can support donor T cell development (Rosen et al, 1962; Gitlin and Craig, 1963; Cooper et al, 1973; Buckley et al, 1976, 1986; Seeger et al, 1976; Griscelli et al, 1978; Pahwa et al, 1980; Reisner et al, 1983; Friedrich et al, 1985; de Villartay et al, 1986).

Studies of X-chromosome inactivation patterns in XSCID carrier females can be used to identify cells affected by the genetic defect where non-random expression of the X chromosome in cells identifies those cells carrying the mutant gene which are at a selective disadvantage, as discussed in detail below. Cell types affected include T and B cells and in some cases granulocytes and natural killer cells. Studies have shown non-random expression of the X chromosome for T cells (Puck and Conley, 1987; Conley, 1992; Puck, 1993), random X-chromosome expression for immature surface IgM^+ B cells and non-random X-chromosome expression for more mature surface IgM^- B cells (Conley et al, 1988). Wengler et al, 1993 showed non-random expression of the X chromosome for B cells, $CD4^+$ and $CD8^+$ T cells and natural killer cells but random X-chromosome expression for neutrophils and monocytes. Goodship et al, 1991 demonstrated non-random X-chromosome

expression for B and T cells and granulocytes. Hendriks et al, 1992 suggested the existence of two XSCID defects as random expression of the X chromosome for granulocytes was seen in two pedigrees studied but in a third pedigree there was non-random X-chromosome expression.

XSCID has been described as being phenotypically heterogeneous (Mensink and Schuurman, 1987). One form of X-linked combined immunodeficiency, similar to but less severe than XSCID, termed thymic dysplasia with normo-gammaglobulinaemia, has been described by several workers (Nezelof et al, 1964; Fulginiti et al, 1966; Miller and Schieken, 1967; Cooper et al, 1973; Lawlor et al, 1974). It is characterised by T and B lymphocyte dysfunction, with normal or slightly low lymphocyte levels and presence of plasma cells and immunoglobulins.

There is also evidence of human XSCID due to a membrane defect (Kersey et al, 1977; Yount et al, 1978). Affected individuals have normal levels of B cells, T cells and immunoglobulins but they have abnormal responses to concanavalin A, which is characterised by an inability to "membrane cap" membrane glycoproteins (Gelfand et al, 1979).

Brooks et al, 1990 described a form of X-linked combined immunodeficiency which differed from XSCID. Susceptibility to infection differed and patients survived into adulthood. In contrast to XSCID, serum concentrations of

immunoglobulin isotypes were normal and the deficiencies in specific serum antibody responses were most pronounced for IgG. The deficiencies in the numbers of T cells were less marked than in XSCID.

Another X-linked combined immunodeficiency, suggested as being either an attenuated form of XSCID or corresponding to a separate X-linked locus has been described (de Saint-Basile et al, 1992). Patients had T and B cell dysfunction and X-chromosome inactivation patterns were identical to those in XSCID. However, unlike XSCID, patients had normal numbers of T cells.

XSCID was mapped to Xq11-q13 (de Saint-Basile et al, 1987) and then to Xq12-q21.3 by Puck et al, 1988 who suggested that heterogeneity might exist as the exact localisation remained uncertain. Mensink and Schuurman, 1987 indicated that there may be more than one genetic locus for XSCID because there is immunological heterogeneity. XSCID was further mapped to Xq13.1-q21.1 (Puck et al, 1989).

The cell surface protein, interleukin-2 receptor γ chain (IL-2R γ) has been shown to be encoded by the gene which when mutated results in XSCID. Genetic linkage analysis indicated the colocalisation of the Il-2R γ gene and the XSCID locus to Xq13 (Noguchi et al, 1993a).

It has been shown that IL-2R γ is an essential component of the IL-2, IL-4 and IL-7 receptor systems (Kondo et al, 1993; Noguchi et al, 1993b; Russell et al, 1993) and it

has been suggested that as a component of these receptor systems can affect B and T cells in XSCID. T cells are induced to synthesise and secrete IL-2 when activated by antigens (Smith, 1989; Waldmann, 1989; Taniguchi and Minami, 1993). Il-4 is a B cell growth factor (Boulay and Paul, 1992; Puri and Siegel, 1993) and IL-7 is also a growth factor for pre-B cells (Namen et al, 1988a, 1988b), a growth factor for thymocytes (Murray et al, 1989; Watson et al, 1989; Uckun et al, 1991) and a growth factor for T cells (Chazen et al, 1989; Morrisey et al, 1989; Londei et al, 1990).

Other forms of SCID, which are not X-linked, include Swiss type agammaglobulinaemia, characterised by cellular and humoral immunodeficiencies (Conley et al, 1990). Also, SCID due to ADA (adenosine deaminase) deficiency, where lymphocyte numbers and immunoglobulin levels may be normal to very low (Giblett et al, 1972). ADA is an important enzyme in the purine catabolic pathway. In ADA deficiency, the normal catabolism of purines is inhibited resulting in accumulation of metabolic substrates which are toxic to the cell. ADA deficiency is due to either point mutations or deletions of the ADA gene (Bonithron et al, 1985; Markert et al, 1987). Also, SCID due to PNP (purine nucleoside phosphorylase) deficiency, where T lymphocyte numbers are very low but B lymphocyte and immunoglobulin levels are usually normal. PNP catalyses the conversion of inosine to hypoxanthine and guanosine to

guanine. In PNP deficiency, intracellular accumulation of metabolic substrates results in inhibition of DNA synthesis (Markert, 1991). Point mutations in the PNP gene have been identified that result in PNP deficiency (Andrews and Markert, 1992).

Other cases of SCID, due to functional T cell deficiencies include a disorder described as a familial defect in expression of the TCR/CD3 complex where a defective CD3 subunit prevents transfer of the TCR/CD3 complex to the cell membrane. One sibling died of acute autoimmune haemolytic anaemia whereas the other was apparently healthy (Alarcon et al, 1988). Defective signal transduction of T lymphocytes has been described with failure in induction of IL-2 production where the disease is characterised by severe recurrent infections (Doi et al, 1988; Weinberg and Parkman, 1990). Also, SCID with Il-1 receptor deficiency of T cells (Chu et al, 1984) and IL-1 deficiency (Sahdev et al, 1989).

X-linked Agammaglobulinaemia (XLA)

Bruton's X-linked agammaglobulinaemia (XLA) is usually characterised by recurrent bacterial infections and paucity of lymph node tissue. Patients have very low numbers of circulating B cells and normal levels of T cells (Bruton, 1952; Geha et al, 1973; Lederman and Winkelstein, 1985; Conley and Puck, 1988) and immuno-globulin levels are low but not absent (Ochs and

Wedgewood, 1989). The numbers of pre-B cells in the bone marrow is normal indicating a defect in the transition from pre-B to B cell (Pearl et al, 1978). Conley, 1985 showed that the small numbers of peripheral blood B cells present in XLA patients exhibit an immature phenotype and appear arrested at an early stage of development. A major and minor XLA phenotype depending on the stage at which B cell maturation is arrested has been described by Schwaber and Chen, 1988. The major phenotype is arrested at the stage of pre-B cells and the minor phenotype is arrested at the stage of immature B cells. The defect for both is due to a failure of rearrangement of the Ig heavy chain variable region. Kwan et al, 1990 and Schwaber, 1992 also demonstrated that the arrest in the transition from pre-B to B cell was due to a failure of rearrangement of the Ig heavy chain (V(D)J) region and that this arrest was incomplete, as some B cells were identified in peripheral blood.

From studies of X-chromosome inactivation patterns, the defect was shown to be intrinsic to B cells as there was non-random expression of the X chromosome for B cells (Conley et al, 1986; Fearon et al, 1987; Conley and Puck, 1988; Hendriks et al, 1989).

The XLA gene was originally mapped to Xq21.3-Xq22 (Kwan et al, 1986) and the locus refined to Xq22 (Kwan et al, 1990; Lovering et al, 1993; Parolini et al, 1993).

Vetrie et al, 1993 identified a gene, now known as Bruton's tyrosine kinase, BTK which encodes protein tyrosine kinases, is expressed in B cells, is found to be mutated in XLA patients and is tightly linked to the XLA locus. This was also demonstrated by Tsukada et al, 1993 and it was also shown that the gene encoded a cytoplasmic tyrosine kinase and its mRNA, protein expression and kinase activity were all reduced or absent in pre-B and B cells of XLA patients.

Studies have shown that cytoplasmic tyrosine kinases when coupled with lymphocyte surface receptors act as signal transducers following specific ligand binding (Bolen, 1993; Weiss, 1993). In addition, cytoplasmic tyrosine kinases have been shown to modify the activity of other tyrosine kinases and down-regulate the signal trans-duction pathways in which they participate (Nada et al, 1991).

It is possible that deficient expression of BTK may affect pre-B cell development by modulating the processes of apoptosis or selection in XLA. Identification of molecules that interact with BTK might provide clues about the regulatory properties of BTK in the signalling pathways of B cells (Tsukada et al, 1993).

X-linked Immunodeficiency with Hyper-IgM (HIGM1)

X-linked immunodeficiency with hyper-IgM (HIGM1) is characterised by recurrent bacterial infections with low

or absent IgG, IgA and IgE and normal to increased IgM and IgD serum levels. HIGM1 patients often have neutropenia and are susceptible to opportunistic infections characteristic of a T cell defect. B cells expressing surface IgM and IgD are present in normal numbers but IgG- and IgA-bearing B cells are undetectable (Rosen et al 1961; Levitt et al, 1983; Notarangelo et al, 1992). An inability to switch from IgM/IgD to other Ig isotypes has been demonstrated (Geha et al, 1979; Brahmi et al, 1983).

There have been conflicting results from studies of X-chromosome inactivation patterns to identify the defective cells in HIGM1. Although random expression of the X chromosome was found for T and B cells (Hendriks et al, 1990), non-random expression of the X chromosome was described for B and T cells and neutrophils reflecting the involvement of several cell lineages in the defect (Notarangelo et al, 1991). Hendriks et al, 1990 proposed that the defect in isotype switching may lie in a factor produced by T cells and transferred to B cells.

A primary T cell defect has been suggested as the basis for HIGM1 following studies in which B cells are induced to produce IgG and IgA in the presence of T lymphoblasts from patients with Sezary Syndrome (Mayer et al, 1986).

HIGM1 was first mapped to Xq24-q27 (Mensink et al, 1987) and the locus further refined to Xq26 (Padayachee et al,

1992, 1993). It has been suggested that a defective gene which maps to Xq26 and encodes the T cell ligand, CD40 is the gene in HIGM1 (Lane et al, 1992; Noelle et al, 1992; Allen et al, 1993; Aruffo et al, 1993; Fuleihan et al, 1993).

Deletions or point mutations of the CD40 ligand, also named gp39, have been identified in HIGM1 (DiSanto et al, 1993). Point mutations of the TRAP gene which encodes a TNF-related activation protein or CD40 ligand on T cells resulting in defective expression of TRAP on the T cell surface were identified. It was suggested that the failure of TRAP to interact with CD40 on B cells is responsible for the defect in Ig switching in HIGM1. The mutation which produces defective expression of TRAP has been shown to vary in HIGM1 patients (Korthauer et al, 1993).

It was shown that HIGM1 B cells express functional CD40 but T cells do not express detectable levels of functional CD40 ligand on the cell surface and suggested that activation signals from T cells to B cells cannot be delivered using the CD40 ligand-receptor pair (Aruffo et al, 1993). Durandy et al, 1993 confirmed that HIGM1 B cells have an intact Ig isotype switch mechanism as B cells could be induced to differentiate into IgG-, IgA- and IgE-producing cells in the presence of CD40 monoclonal antibody, IL-4 and IL-10. Using this method, an intact isotype switch mechanism in HIGM1 B cells was also

demonstrated by Fuleihan et al, 1993. It was also shown that HIGM1 T cells failed to induce IgE synthesis in B cells and are unable to express the CD40 ligand although the mRNA for the ligand is expressed normally.

Results of studies suggest that the defective expression of the CD40 ligand on T cells underlies the failure of isotype switching in HIGM1 B cells

Wiskott-Aldrich Syndrome (WAS)

Wiskott-Aldrich syndrome (WAS) is an X-linked condition in which patients are susceptible to infection, eczema and thrombocytopenia due to a platelet abnormality. Immunological abnormalities are low serum IgM, elevated IgA and IgE and failure to make antibodies to carbo-hydrate antigens (Aldrich et al, 1954; Cooper et al, 1968; Spitler et al, 1975; Remold-O'Donnell and Rosen, 1990). T cell function is variable and tends to decline with age; there may be decreased proliferative responses to mitogens and poor production of cytotoxic T cells (Ochs et al, 1980).

Studies of WAS have demonstrated imbalances in X-chromosome inactivation patterns for several haemato-poietic cell lines. Non-random X-chromosome expression was seen for B and T cells and monocytes (Fearon et al, 1988; Greer et al, 1989). Other studies revealed that the defect was more profound for T cells and platelets, less profound for granulocytes and B cells and there was

random expression of the X chromosome for erythrocytes and neutrophils (Gealy et al, 1980). Notarangelo et al, 1991 showed that T cells had non-random X-chromosome expression but the X-chromosome inactivation patterns were balanced for granulocytes.

A lymphocyte surface glycoprotein, CD43, gp115 or sialophorin which is expressed in various haematopoietic cell lines (Carlsson et al, 1986) has been shown to be absent or abnormally glycosylated in WAS lymphocytes and platelets (Remold-O'Donnell et al, 1984; Reisinger and Parkman, 1987; Higgins et al, 1991; Piller et al, 1991).

The gene for CD43 has been mapped to chromosome 16 (Pallant et al, 1989) which is not the primary locus for the gene in WAS. This was mapped to a marker in Xp11.3-p11 (Kwan et al, 1988) and the locus was further refined to Xp11.2 (Kwan et al, 1991; Greer et al, 1989).

It has been suggested that WAS is associated with a defect in the coupling of surface Ig on B cells resulting in impaired signal transduction pathways (Simon et al, 1992).

X-linked Lymphoproliferative Disease (XLP)

X-linked lymphoproliferative disease (XLP) is characterised as a combined, variable, progressive immunodeficiency disorder with an inadequate immune response to infection with Epstein Barr Virus (Purtilo et

al, 1974, 1975; Hamilton et al, 1980; Grierson et al, 1991).

Studies of X-chromosome inactivation patterns in XLP carriers revealed random X-chromosome expression for T cells, B cells and neutrophils which suggests that the defect either resides in a subset of haematopoietic cells or that the defect is not detrimental to cell survival or proliferation (Conley et al, 1990).

Linkage analysis mapped XLP to Xq24-q27 (Skare et al, 1987) and to Xq25-q26 (Sylla et al, 1989). A deletion involving Xq25 in XLP patients (Sanger et al, 1990) and males with deletions which result in XLP (Skare et al, 1993) have been documented.

Miscellaneous X-linked Human Haematopoietic Disorders

In chronic granulomatous disease, CGD, defects in the cytochrome b complex result in the failure of NADPH oxidase in phagocytic leukocytes to generate superoxide needed for the killing of micro-organisms (Baehner, 1990). The gene for CGD, CYBB has been mapped to Xp21.2-p21.1 (Baehner et al, 1986). Various mutations in CYBB and deletions in Xp21 have been described (De Saint-Basile et al, 1988; Dinauer et al, 1989; Francke et al, 1990; Bolscher et al, 1991; De Boer et al, 1992).

Other deficiencies include the properdin deficiency which involves fatal bacterial infection due to impairment of

complement activation via the alternative pathway. The properdin locus, PFD has been mapped to Xp11.23-p21.1 (Goundis et al, 1989). Alpha galactosidase deficiency of leukocytes, which is found in patients with Fabry disease. The locus GLA has been mapped to Xq22-qter (Shows et al, 1978). Various glucose-6-phosphate dehydrogenase deficiencies affecting the haematopoietic system are characterised by erythrocyte and leukocyte dysfunction. The G6PD locus has been mapped to Xq28 (Pai et al, 1980). GPL-115 deficiency (115,000 Dalton surface glycoprotein) which is characterised by immature T lymphocytes and T lymphocyte dysfunction (see McKusick, 1994). Glycerol kinase-1 deficiency, of leukocytes, which is associated with muscular dystrophy. GK has been mapped to Xp21.3-p21.2 (Bartley et al, 1986). Phosphoglycerate kinase-1 deficiency which gives rise to haemolytic anaemias and deficiency in leukocytes (Valentine et al, 1969). PGK has been mapped to Xq13 (Willard et al, 1985).

Other disorders include haemophilia, with type A and B, which results from defects of clotting factors VIII and IX with loci HEMA or F8C at Xq28 and HEMB or F9 at Xq27 (Tantravahi et al, 1986; Chance et al, 1983). Sidero-blastic anaemia with locus ANH1 or ALAS mapped to Xp21-q21 (Cox et al, 1990). Proliferating defect of haemato-poietic cells; acute leukaemia; thrombocytopenia; malignant reticuloendotheliosis. Finally,

localisation of gene GF-1 to Xp21-11 (Zon et al, 1990), a transcriptional regulator, has important implications for hereditary persistence of fetal haemoglobin syndromes.

X-linked Human Muscular Disorders

There are several different types of X-linked muscular dystrophy in man: Duchenne; Becker; McLeod; Emery-Dreifuss; scapuloperoneal myopathy and myotubular myopathy (Swash and Schwartz, 1988). The Duchenne gene (DMD) has been mapped to Xp21-p22.3 (Murray et al, 1982) and the Becker gene (BMD) to the same region (Kingston et al, 1983, 1984; Brown et al, 1985; Fadda et al, 1985); the Emery-Dreifuss locus (EMD) and the myotubular myopathy locus (MTM1) are at Xq28 (Boswinkel et al, 1985; Thomas et al, 1987); the McLeod locus (XK) is at Xp21.2-p21.1 (Frey et al, 1988) which is close to the Duchenne/ Becker locus.

Linkage analyses have lead to the suggestion that the Duchenne and Becker genes may be allelic (Murray et al, 1982; Kingston et al, 1983, 1984; Bakker, 1985; Brown et al, 1985; Fadda et al, 1985; Wilcox et al, 1985). Both DMD and BMD have been shown to result from mutations or deletions of the gene encoding dystrophin (Lindlof et al, 1988; 1989; Read et al, 1988; den Dunnen et al, 1989; England et al, 1990; Nordenskjold et al, 1990; Norman et al, 1990; Kilimann et al, 1992; Lenk et al, 1993).

The dystrophin gene product is absent in muscle of patients with Duchenne muscular dystrophy (Bulfield et al, 1984; Hoffman et al, 1987a; Koenig et al, 1987) and, in the Becker form, the dystrophin molecule is truncated (Hoffman et al, 1987b). The concentration of a glycoprotein, which is an integral component of the dystrophin complex, has been shown to be greatly reduced in DMD patients. It is suggested that the absence of dystrophin may lead to the loss of this dystrophin-associated glycoprotein which, in turn, leads to molecular pathogenesis of muscle (Ervasti et al, 1990).

X-linked Murine Loci

Animals with mutations affecting normal function and developmental processes can be used to study the factors controlling development and as models for the study of genetic disease in man. Comparison of the genetic maps in man and mouse may indicate chromosome regions where the genetic order is conserved and may identify mouse genetic loci which may be homologous to human mutations causing disease (see the comparative human and mouse X-chromosome map, Fig. 1 given at the end of Chapter 1).

Only a few X-linked immunodeficiency syndromes have been identified in mice. The xid mutation in CBA/N mice has been related to XLA in humans (see below). More recently, the murine gene for the Il2r γ chain was mapped to a locus on the X chromosome consistent with the human IL2R γ locus

at Xq13 (Noguchi et al, 1993a) and it was shown that the defect in the gene is not responsible for the xid mutation (Cao et al, 1993). The murine X-linked scurfy mutant, sf resembles WAS in humans although sf differs from WAS in that sf males are hypogonadal. The human and murine loci lie in homologous segments of the X chromosome although in different positions relative to other gene loci (Lyon et al, 1990).

Studies of the xid mutation allow analysis of the genetic factors affecting B lymphopoiesis and B cell function. Xid is characterised by a deficiency in responsiveness to type III pneumococcal polysaccharide antigens (Amsbaugh et al, 1972) and some type II thymus-independent antigens (Scher et al, 1975). It is also characterised by hypo- and unresponsiveness to different B cell mitogens (Scher et al, 1975; Goodman et al, 1978) and defects in B cell colony production, following antigenic stimulation (Kincade, 1977).

Particular B cell subpopulations are absent. These are $BLA\text{-}1^{+}$ cells which are normally detectable from two weeks of age (Hardy et al, 1983), $BLA1^{+}$, $BLA\text{-}2^{-}$ cells (Hardy et al, 1984) and $Lyb\text{-}5^{+}$ cells which are normally detectable 2-3 weeks after birth (Ahmed and Scher, 1976). Other absent B cell subpopulations include those cells that belong to Population I with high IgD, low IgM (Hardy et al, 1982) and members of Population III with low IgD,

high IgM (Hardy et al, 1984) and also, those cells expressing minor lymphocyte-activating determinants (Ahmed and Scher, 1976).

Studies of X-chromosome inactivation patterns have revealed that the defect in xid is intrinsic to B cells and that T cells, pre-B cells, other haematopoietic cells and non-haematopoietic cells are unaffected by the xid mutation (Nahm et al, 1983; Forrester, 1986).

Although xid, like XLA, is characterised by a B cell lesion, the defect is less severe than that seen in XLA patients. In contrast to XLA, xid mice do have peripheral B cells and immunoglobulins of different isotypes (Scher, 1982).

The xid locus has been mapped to the F1 region of the X chromosome which shares homology with the human XLA locus at Xq22 (Copeland and Jenkins, 1991; Hillyard et al, 1992). The murine Btk has been mapped to the xid locus and sequencing of the Btk gene identified a missense mutation which altered a single amino acid residue in the Btk protein (Rawlings et al, 1993; Thomas et al, 1993) indicating that xid and XLA share a similar molecular lesion despite exhibiting different disease phenotypes. In contrast to XLA, Btk mRNA expression and protein kinase activity in vitro were shown to be unaltered in the B cells of xid mice (Rawlings et al, 1993) which may

relate to the variation in phenotype of XLA and xid.

Several murine lymphoid loci, with no known human homologues have been characterised. These include the X-linked lymphocyte regulated sequence family, XLR which was originally identified from a lymphocyte-specific cDNA library (Cohen et al, 1985a). A cDNA was found to encode a protein expressed exclusively in the nucleus of B and T cells (Garchon and Davis, 1989).

Mapping studies have revealed that XLR sequences, Xlr-1 and Xlr-2 localise to two separate positions in region XA and that these loci do not map to the xid locus (Mullins et al, 1990) although it had been demonstrated previously that some members of the XLR gene family are closely linked to xid (Cohen et al, 1985a) and that the xid mutation disrupts XLR gene expression (Cohen et al, 1985b).

Other murine lymphoid loci include the lymphocyte membrane antigens, Lyx-1, Lyx-2, Lyx-3 which are controlled by immune response (Ir) genes and are found to be antigenically distinct such that Lyt-X relates to T cells and Lyb-X relates B cells (Lyon and Searle, 1989).

Murine loci which are homologous to haematopoietic loci in man have been mapped to the short arm of the X chromosome. These include the cytochrome b-245 beta polypeptide locus, Cybb located in the region XA (Mullins et al, 1990). The loci for clotting factors VIII and IX, cf-8 and cf-9

located in the region XB and XA6, respectively (Mullins et al, 1988). The locus for glucose-6-phosphate dehydrogenase, G6pd, located in the region XA6-A7 or XB (Mullins et al, 1988). The locus for phosphoglycerate kinase-1, Pgk-1 located in the XD region (Cavanna et al, 1988) and the alpha-galactosidase A locus, Ags located in the XF region (Paigen, 1979; Mullins et al, 1988); Gf-1 also named Gata1 (Chapman et al, 1991) involved in regulation of transcription of erythroid cells.

An homologous murine locus (mdx) has been noted for the Duchenne, Becker and Emery-Dreifuss dystrophies (Searle et al, 1987; Cavanna et al, 1988). The murine model for muscular dystrophy resulted from a spontaneous mutation (mdx) in C57BL/10 mice (Bulfield et al, 1984). Unlike human dystrophies, mdx is characterised by limb and cardiac muscle lesions without obvious dysfunction (Emery, 1980; Dangain and Vrbova, 1984; Bridges, 1986).

Mdx has been mapped to the region homologous to the DMD locus on the mouse X chromosome (Brockdorff et al, 1987; Chamberlain et al, 1987; Heilig et al, 1987), between the loci Hprt (hypoxanthine phosphoribosyltransferase) and pgk-1, in the mouse (Chapman et al, 1985; Peters et al, 1988). Evans et al, 1990 showed that the mouse dystrophin locus is positioned in the XC band of the X chromosome. Three alleles of mdx (mdx^{2cv}, mdx^{3cv}, mdx^{4cv}) were identified in mice treated with the mutagen N-ethylnitrosourea and it was also shown that reduced dystrophin levels are

a consequence of mutations at the dystrophin locus (Chapman et al, 1989). A point mutation in the dystrophin gene of mdx mice which results in the premature termination of translation of the dystrophin polypeptide has been identified (Sicinski et al, 1989).

It has also been shown that intracellular Ca^{2+} is elevated in mdx muscle and it is suggested that ion channels in mdx myotubes provide Ca^{2+}-leakage into the cell (Franco and Lansman, 1990).

Characterisation of X-linked Defects

Techniques to Evaluate X-chromosome Inactivation Patterns

X-Chromosome Inactivation

During female embryogenesis one of the two X chromosomes is inactivated resulting in dosage equivalence between females who have two X chromosomes and males who have one X chromosome (Lyon, 1961; Russell, 1961) and the inactive status is inherited by descendents within a somatic cell lineage (Chapman, 1987). X-chromosome inactivation is generally random such that tissues are composed of a mosaic mixture of equal numbers of cells expressing the maternal (X^M) and paternal (X^P) chromosome (West, 1982; Gartler and Riggs, 1983; Chapman, 1987).

Non-random X-chromosome inactivation or selection for cells expressing either the maternal or paternal X chromosome results in unequal numbers of cells and

greater expression of one kind of X chromosome. In studies on murine extraembryonic membranes, the X^P has been shown to be preferentially inactivated (Takagi and Sasaki, 1975; West et al, 1977). It is suggested that this differential expression is due to an imprinting process, probably by DNA modification such as methylation, prior to X inactivation, making X^M and X^P intrinsically different (Mohandas et al, 1981; Chapman et al, 1982; Monk, 1986). The fact that normally hypomethylated CpG-rich islands become methylated on the inactive X chromosome suggests a role for methylation in the maintenance of the inactive state (Bird, 1986; Norris et al, 1991).

Both human and murine X-chromosome inactivation is thought to proceed from an inactivation centre (XIC/Xic) Russell, 1963; Cattanach, 1975). Therman, 1974 hypothesised the existence of an X-inactivation centre in relation to a Barr body condensation centre.

The human XIC was initially located between Xq11.2 and Xq21.1 (Mattei, 1981). A visible bend at Xq13.3-q21.1 was suggested as being representative of the condensation process (Flejter et al, 1984) and was also expressed in other primates at a band homologous to human Xq13-q21 (Flejter et al, 1986). Brown and Willard, 1989 located the human XIC to Xq13. The gene XIST (X-inactivation specific transcript) from the region of the human XIC was found to be expressed on the inactive chromosome and was

suggested as being involved or influenced by the inactivation process (Brown et al, 1991a). XIST was further localised to XIC at Xq13 (Brown et al, 1991b). XIST RNA was shown to be localised within the nucleus at the position of the X-inactivation associated Barr body (Brown et al, 1992).

The mouse Xic was mapped to band XD (Rastan, 1983; Rastan and Robertson, 1985). Similarly to humans, murine Xist was shown to map to the region of Xic (Borsani et al, 1991), was shown to be exclusively expressed by the X chromosome and was suggested as being involved in X-chromosome inactivation (Brockdorff et al, 1991).

X-chromosome controlling elements have been shown to produce non-random inactivation of the X chromosome (Cattanach and Williams, 1972; West and Chapman, 1978). The murine locus (Xce) was mapped to the XD region, between mdx and Pgk-1 (Cattanach, 1970; Cattanach et al, 1970). Allelic variants of this locus influence X-chromosome inactivation such that an X chromosome expressing the Xce^{a} allele has a greater chance of being inactivated than an X chromosome carrying the Xce^{b} allele which, in turn, is more likely to be inactivated than an X chromosome carrying the Xce^{c} allele (Cattanach, 1972; Johnston and Cattanach, 1981). It has also been shown that the parental source of the Xce allele determines which X chromosome is inactivated (Rastan and Cattanach, 1983). For example, in females heterozygous for Xce (b/c),

the X^{M} chromosome bearing the <u>Xce</u>^{c} allele is less likely to be inactivated than the X^{p} chromosome carrying the same allele (Forrester and Ansell, 1985). Further evidence of parental effects on expression of the X chromosome, in addition to the possible existence of a fourth <u>Xce</u> allele have been demonstrated (Fowlis, 1988; Fowlis <u>et al</u>, 1991).

Analysis of Electrophoretic Alloenzymes

In addition to X-chromosome controlling elements, X-linked mutations may also result in non-random expression of the X chromosome. Detailed examples of these are given above in the overview of X-linked defects. In the heterozygote, the relative expression of the maternal or paternal X chromosome can be quantified by measuring the relative amounts of allelic X-linked enzyme which is used as a cell marker and can be separated by electrophoresis. Electrophoretic variants for enzymes HPRT, PGK-1 and G6PD are examples of such a marker. For example, female carriers of WAS who are heterozygous both for the WAS locus and for the G6PD locus which encodes for the enzyme G6PD with A/B polymorphism have expression of the B alloenzyme in T cells and platelets and equal expression of A and B alloenzymes in other cell types. Hence, non-random expression of the X chromosome demonstrates selection against those cells affected by the WAS defect. (Gealy <u>et al</u>, 1980). Also, female carriers of XLA who are heterozygous both for the XLA locus and for

the G6PD locus have expression of the A alloenzyme in B cells and equal expression of both alloenzymes in other cell types. Hence, non-random expression of the X chromosome demonstrates selection against those cells affected by XLA (Conley et al, 1986).

Similarly, in mice heterozygous for the Pgk-1 locus where cells express either the normal (PGK-1B) or the variant (PGK-1A) form of the enzyme, cells carrying the maternal or paternal X chromosome can be traced and quantified. For example, females heterozygous for both the xid mutation and the Pgk-1 locus demonstrate non-random expression of the X chromosome for B cells (Nahm et al, 1983; Forrester et al, 1987).

Analysis of Methylation Patterns

This method of analysis of X-chromosome inactivation patterns takes advantage of differences in methylation of specific cytosine bases in the DNA of active versus inactive X chromosomes (reviewed by Winkelston and Fearon, 1990).

Two X-chromosome encoded genes that vary in their pattern of methylation on the active and inactive X chromosome, HPRT and PGK, are also the sites of restriction fragment length polymorphisms (RFLPs) with restriction sites that bracket sequences that are variably methylated. To use the methylation technique (Vogelstein et al, 1987), DNA from females heterozygous for one of the RFLPs is sub-

jected to double digestion with restriction enzymes such that one enzyme reveals the RFLP allele and the other, a methylation-sensitive enzyme cuts the DNA derived from the non-methylated chromosome.

The inactive X chromosome is methylated at the PGK locus while the active X chromosome is methylated at the HPRT locus. Using a Southern blot, the DNA is analysed using a labelled probe from a sequence that flanks the PGK or HPRT gene.

If the cells examined have normal random X-chromosome inactivation, the DNA digested with both enzymes will show diminished intensity of both the bands seen in the lane containing DNA digested only with the RFLP-revealing enzyme because some of the DNA in each of the two bands has been digested by the methylation-sensitive enzyme. However, when DNA is derived from cells with non-random X-chromosome inactivation, then one of the bands will disappear in the lane digested with both enzymes as the DNA from that allele is nonmethylated.

Somatic Cell Hybrid Technique

A subsequent method to determine the X-chromosome inactivation pattern in cell lineages from women is based on forming hybrids between the human lymphocytes of interest and a hamster cell line deficient in the X-encoded enzyme, HPRT (Puck et al, 1987). Hybrids grown in selective media retain the human active X chromosome

resulting in HPRT activity. This X chromosome is identifiable using analysis for any X-linked polymorphic marker for which the donor woman was heterozygous. A normal random X-chromosome pattern demonstrates that some of the hybrids have retained the paternally-derived allele and some have retained the maternally-derived allele. A non-random X-chromosome inactivation pattern shows the hybrids have retained the same X chromosome.

Hence, analysis of electrophoretic alloenzymes, methylation patterns and somatic cell hybrids have been used to assess the X-chromosome inactivation patterns in female carriers of XLA (Conley et al, 1986; Fearon et al, 1987; Conley and Puck, 1988; Hendriks et al, 1989); XSCID (Puck and Conley 1987; Conley et al, 1988; Goodship et al, 1991; Conley, 1992; Hendriks et al, 1992; Puck, 1993; Wengler et al, 1993) and WAS (Fearon et al, 1988; Greer et al, 1989; Notarangelo et al, 1991). Defective cell lineages include B cells in XLA, B and T cells and in some cases granulocytes and natural killer cells in XSCID and B and T cells, monocytes and platelets in WAS.

Interpreting results of X-chromosome inactivation studies requires taking into account the degree to which random X-chromosome inactivation can result in variation from the expected mean of 50% of cells with the maternally-derived X chromosome active and 50% of cells with the paternally-derived X chromosome active. X-chromosome inactivation studies using the X-linked enzyme G6PD as a

cell marker and studies using the hybrid technique have shown that, in approximately 10% of normal females, random X-chromosome inactivation results in the expression of one X chromosome as the active X chromosome in 80% of the cells with the remaining 20% of cells expressing the other X chromosome (Fialkow, 1973; Stewart et al, 1989). This indicates a wide variation in normal random patterns of X-chromosome inactivation. Hence, the importance of evaluating the X-chromosome inactivation pattern in a cell lineage unaffected by an X-linked mutation from an individual in which there are cell lineages that are affected by the mutation. In normal women, the pattern of X-chromosome inactivation in all tissues tends to be similar (Fialkow, 1973).

Thus, studies of X-chromosome inactivation patterns can be used to identify specific cell types affected by an X-linked defect or mutation. In males, hemizygous for X-linked genes, cell types expressing the mutation are not detectable using the enzyme marker system since there is no selection against those cells.

Studies of X-chromosome inactivation patterns can also be used to indicate the stage in differentiation at which the cell type is affected by the the X-linked mutation. For example, in obligate carriers of XSCID, surface IgM^- B cells, committed to IgG- and IgA-expression, exhibited preferential use of the non-mutant X chromosome whereas balanced X-chromosome expression was seen for

less mature surface IgM^+ B cells. This indicated that the defective gene product of XSCID is expressed throughout B cell differentiation and its activity results in a relative rather than an absolute block in differentiation with progressively more skewing of X-chromosome inactivation patterns as B cells proliferate and differentiate (Conley et al, 1988).

Studies of X-chromosome inactivation patterns have been used in cell-lineage analyses and to determine contributing cell numbers of developing tissues by estimating the proportions of the genetically distinct cell populations. For example, variants of the X-linked enzyme G6PD have been used to study clonality of tumours to determine founder cell numbers of different tissues in man (Fialkow, 1973, 1983).

Mapping of Genetic Defects

A new marker for a locus can be located using a genetic or linkage map which contains the order and approximate recombinational distance between genetic markers on a chromosome and a physical map which describes the physical features of a chromosome.

Linkage mapping to map loci in relation to each other utilises the process of recombination which is the rearrangement of genetic material at crossover points or chiasmata on nonsister chromatids of homologous

chromosomes during meiosis (Morgan, 1910; Tease and Jones, 1978). This results in the formation of different arrangements of genes in the gametes such that the original arrangements of alleles on the two chromosomes are called the parental combinations and two new combinations are called recombinants. Sturtevant, 1913 suggested that the percentage of recombinants is a quantitative index of the linear distance between two genes such that the greater the distance between genes on a chromosome the greater the chance that nonsister chromatids cross over in the region between the genes and hence the greater the proportion of recombinants that would be produced. Thus, analysis of the meiotic recombinational frequency can determine the map distance between genes on a chromosome such that 1 map unit or centimorgan (cM) is defined as a recombinant frequency of 1%.

The greater the interval between genes on a chromosome the greater the probability that multiple crossovers occur. Therefore, to take account of multiple crossovers between loci, the mapping function (Haldane, 1919) is used to relate distance along a chromosome and recombinational frequency.

Linkage analysis maps loci for genetic defects by determining which region of the chromosome is inherited with the locus for the defect. For the mouse, it is possible to establish the relative levels of error

associated with intergenic distances by altering the number of mice analysed in a cross. Gene orders can be determined by minimising the number of multiple crossover events for a given set of loci (Mullins et al, 1990).

Physical mapping can be used to trace loci of genetic defects by analysing the region of the chromosome containing such loci. Closely linked polymorphic loci are used as markers of loci for genetic defects. For example, the loci for Duchenne (Murray et al, 1982) and Becker (Kingston et al, 1983) muscular dystrophies have been linked with DNA markers. DNA sequence polymorphisms can be detected using restriction enzymes (Botstein et al, 1980). Restriction fragment length polymorphisms (RFLPs) can be detected using Southern transfers (Owerbach et al, 1980; Naylor et al, 1984). RFLPs can result from an insertion, a deletion or a difference in the number of repeats of a short sequence between two restriction enzyme cleavage sites. For example, genetic studies of CYBB the gene whose deficiency causes X-linked CGD, have detected RFLPs in the DNA of carriers (Francke et al, 1990).

The polymerase chain reaction (PCR) (Saiki et al, 1985) can be used to map genetic markers whereby PCR amplifies a region of DNA containing variable numbers of inserts. The size of a fragment synthesised by PCR identifies the size of the insert. Microsatellite markers can be analysed by PCR (Luty et al, 1990).

Fluorescence *in situ* hybridisation can be used to map regions of the chromosome by hybridising a radiolabelled RNA or DNA probe to fixed metaphase chromosomes. For example, the human gene encoding the CD40 ligand was biotin-labelled and shown to hybridise to the X chromosome at band Xq26 (Aruffo *et al*, 1993).

The development of yeast artificial chromosomes(YACs) as cloning vectors for large segments of DNA has made cloning of chromosome regions possible and permits the analysis of candidate genes in a targeted region of the chromosome. For example, using this method the region Xq24-Xq28 containing the XLP locus has been cloned (Schlessinger *et al*, 1991).

Flow Cytometric Analysis

The effect of an X-linked immunological defect in leukocytes can be studied by flow cytometric analysis in which leukocyte subpopulations are identified by analysing the relative size of cells (Loken and Stall, 1982), the integral structure and granularity of cells (Ritchie *et al*, 1983) and analysis of the relative proportion of leukocyte subpopulations by quantifying fluorescence-labelled cells to assess the distribution of surface antigen. Defined subpopulations of cells can be isolated (Herzenberg *et al*, 1976) for further study, namely electrophoretic alloenzyme analysis in the present study to evaluate the X-chromosome inactivation patterns

within a subpopulation of cells.

Aims Of This Study

Animals with genetic mutations can be used to study the factors controlling normal development and function and as models for the study of genetic disease in man. Comparison of human and murine genetic maps can indicate the chromosome regions where the genetic order is conserved and can identify mouse genetic loci which may be homologous to human mutations causing disease. Genetic defects can be mapped to the chromosome using linkage and physical mapping. In humans and mice, loci controlling the haematopoietic and muscular systems have been mapped to the X chromosome (see the comparative human and mouse X-chromosome map, Fig.1).

The aim of this study was to characterise the phenotypic effect of a novel X-linked mutation in offspring of male mice treated with the mutagen N-ethylnitrosourea (ENU) which has been shown to transmit mutations via the germ line at a frequency of 1 mutation per 700 loci tested (Johnson and Lewis, 1981; Russell et al, 1982a, 1982b; Justice and Bode, 1986). It has been demonstrated that treatment with ENU can produce X-linked mutations affecting the haematopoietic and muscular systems (Chapman et al, 1988, 1989).

Studies of X-chromosome inactivation patterns in heterozygous females using electrophoretic alloenzyme

analysis were used to characterise the phenotypic effect of the X-linked ENU mutation by delineating the cell lineages affected by the mutation, the stage at which the mutation acts and the penetrance of the mutation in the affected cells.

In the heterozygote, the relative expression of the maternal or paternal X chromosome was quantified by measuring the relative amounts of allelic X-linked alloenzyme (PGK-1) which is used as a cell marker and is separated by electrophoresis. Non-random expression of of the X chromosome in cells demonstrates selection against the cells affected by the mutation.

The phenotypic effect of the ENU mutation was studied by analysis of cells of brain, haematopoietic and muscular systems from female carriers of ENU that were heterozygous for the ENU mutation (ENU/+) such that the presence of cells carrying the + locus on the X chromosome was detected by visualisation of the alloenzyme, PGK-1B and the presence of cells carrying the ENU mutation was detected by visualisation of the PGK-1A alloenzyme.

Attempts to relate the ENU mutation to a candidate gene were made by assessing whether the ENU mutation was an allele of <u>xid</u> by assessing the X-chromosome inactivation patterns in cells of females which were heterozygous for the ENU mutation and <u>xid</u> (ENU/<u>xid</u>) such that the presence of cells carrying the <u>xid</u> locus on the X chromosome was

detected by the marker PGK-1B alloenzyme.

Studies of X-chromosome inactivation patterns in normal cells of heterozygous females using alloenzyme electrophoretic analysis were also used to assess possible factors influencing X-chromosome inactivation in various strains, including the parental strain from which ENU-mutant mice were derived. Genetic factors such as the mouse X-chromosome controlling element, Xce have been shown to affect X-chromosome inactivation (Cattanach and Williams, 1972; West and Chapman, 1978) and allelic variants of the Xce can influence X-chromosome inactivation patterns (Cattanach, 1972; Johnston and Cattanach, 1981). Parental factors such as the parental source of the Xce allele can also determine which X chromosome is inactivated (Rastan and Cattanach 1983; Forrester and Ansell, 1985; Fowlis, 1988; Fowlis et al, 1991).

Various heterozygous females were produced from crosses between different strains and evaluated to examine variation in X-chromosome inactivation patterns. Studies were made of various normal cell types, that is cells unaffected by either the ENU or the xid mutation. Results were related to possible genetic and parental factors in the various strains studied that might influence X-chromosome inactivation.

Flow cytometric analysis was used to study the phenotypic

effect of the ENU mutation on leukocyte subpopulations to assess whether the mutation affects relative leukocyte numbers in haematopoietic tissues of ENU-mutant males and their hybrids which were produced from crosses with either normal or xid mice. Leukocyte subpopulations were classified according to various cell properties such as relative size, integral structure and granularity. The relative proportion of leukocyte subpopulations was analysed by quantifying fluorescence-labelled cells to assess the distribution of cell surface antigen.

Furthermore, defined subpopulations of cells were isolated and the X-chromosome inactivation patterns of the defined cell subpopulations from the heterozygous females were evaluated to characterise which leukocyte subpopulations were affected by the ENU mutation.

A preliminary study to characterise the effects of the ENU mutation on the immune system in functional terms was made. Leukocyte responses in ENU-mutant mice and their hybrids which were produced from crosses with either normal or xid mice were assessed following sensitisation to the skin-sensitising agent oxazolone. Leukocyte responses were assessed using flow cytometric analysis and by making cell counts. Electrophoretic alloenzyme analysis of leukocytes from heterozygous females was used to study X-chromosome inactivation patterns following sensitisation to oxazolone.

Finally, it is discussed whether the phenotypic effects of the novel X-linked mutation, in ENU-mutant mice might be compared to known disorders in humans or mice. Studies of the X-linked mutation in ENU-mutant mice may prove to be useful as a model for human X-linked disorders of the haematopoietic and muscular systems, and in assessing the genetic factors that control cell development and function.

Fig.1/

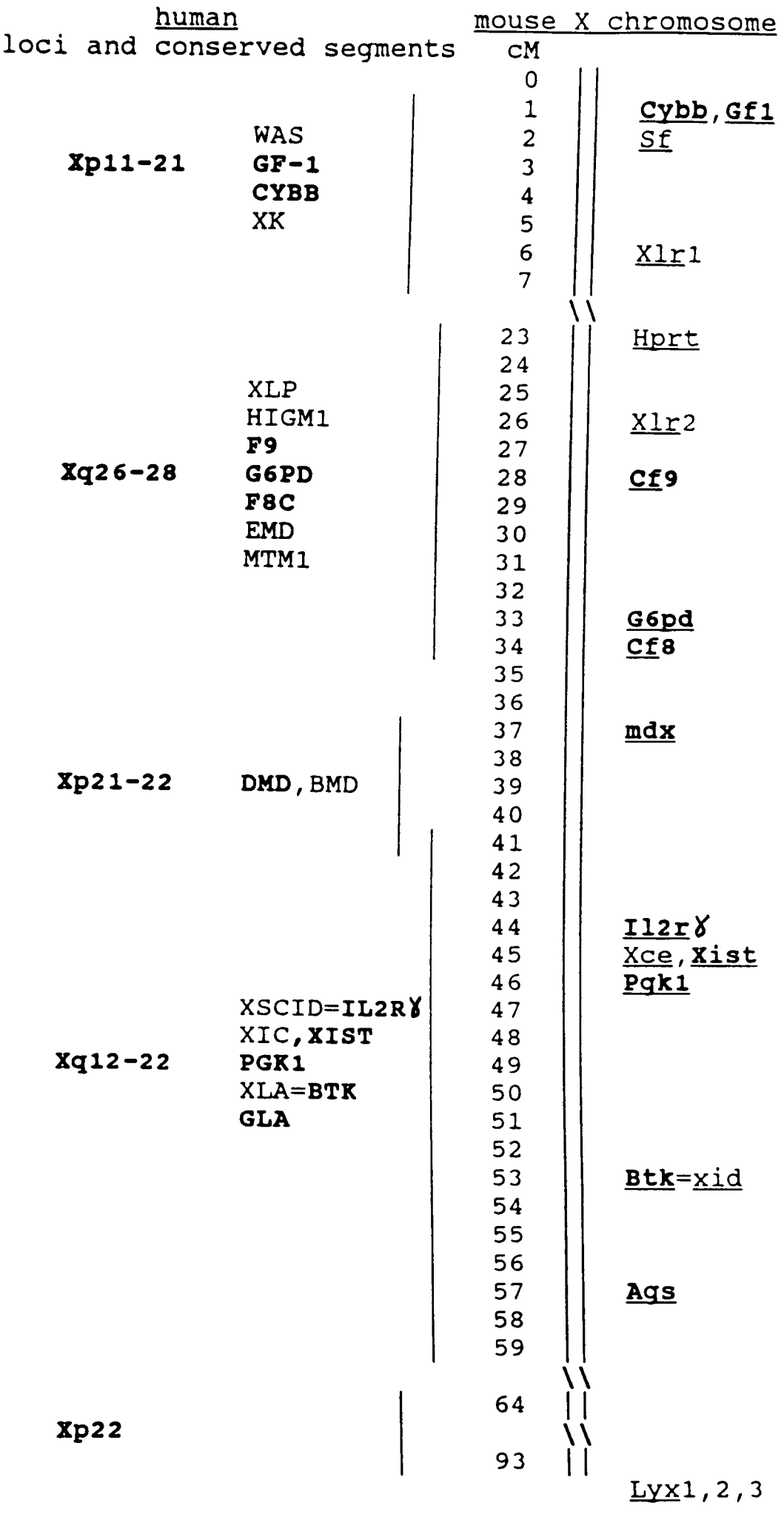


Fig.1 Comparative X-chromosome Map showing the location of murine loci given in the text in terms of recombinational distance in centimorgans from the centromere and the known homologies to human chromosomes given as loci in bold and lines for conserved segments on the left; slanted lines on the X chromosome represent excluded regions that are not relevant to this study, adapted from Lyon and Kirby in Mouse Genome (1994).

CHAPTER 2

Materials And Methods

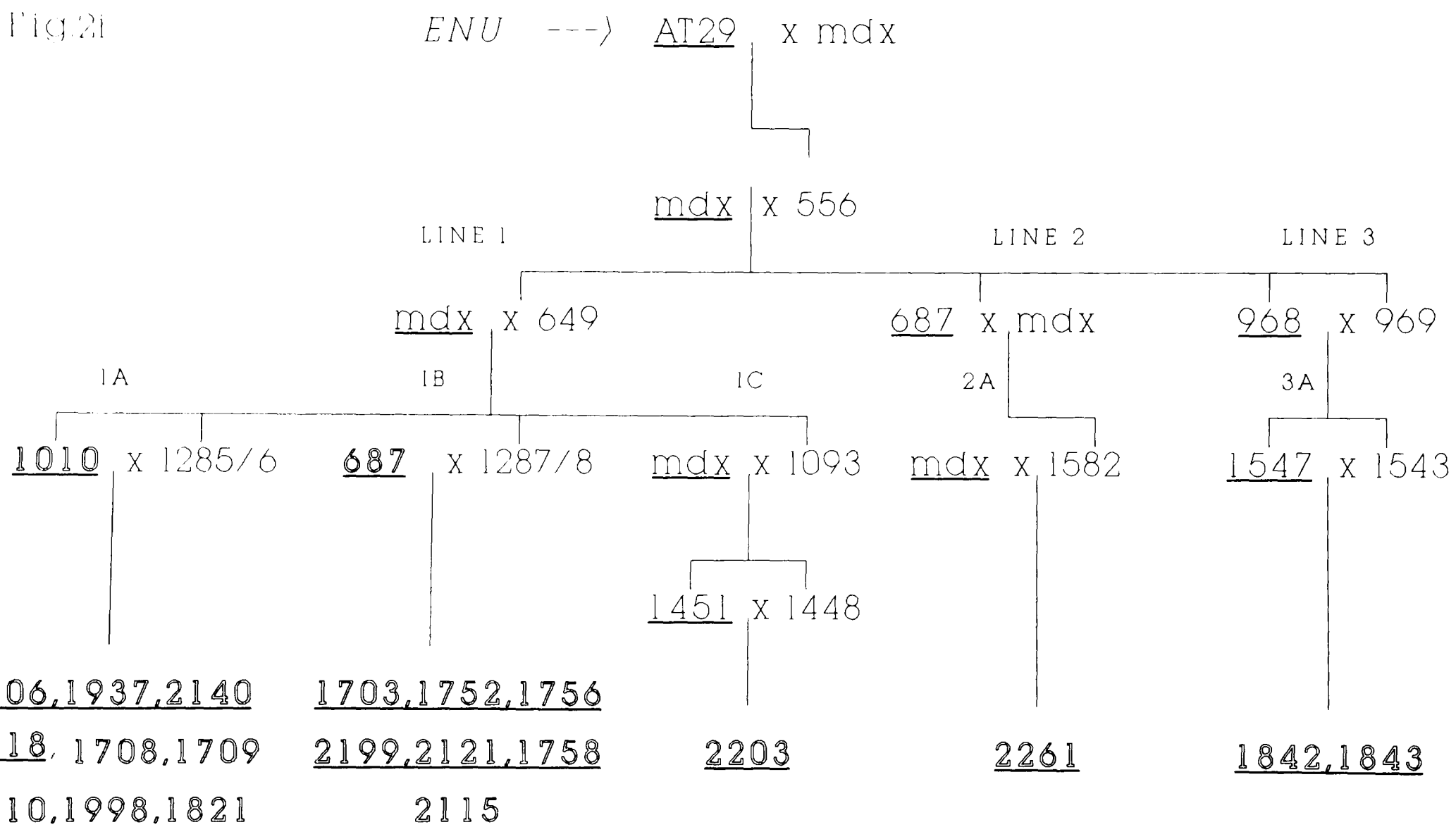
Mice

Strains of mice, with abbreviations in brackets, used in this study were CBA/Ca (CBA), CBA/Ca-Pgk-1a (CBA-Pgk-1a), CBA/N, C3H x C57BL/6 sublines (AT29, see below for genotype) and AT29xC57BL/10.mdx (female 556-derived) (AT29-ENU.556). AT29 and AT29-ENU.556 mice were obtained from V.M Chapman, Molecular and Cellular Biology Department, Roswell Park Memorial Institute, Buffalo, N.Y. 14263.

Phosphoglycerate kinase-1 (PGK-1) is an X-chromosome encoded enzyme marker present in all cells. Two Pgk-1 alleles exist in mice: CBA, CBA/N and C57BL/10.mdx are homozygous for the Pgk-1b allele and CBA/Pgk-1a, AT29 and AT29-ENU.556 are homozygous for the Pgk-1a allele (Fig. 2i). Alloenzymes of HPRT and PGK-1 (see below) can be separated and quantified by electrophoresis such that the relative amounts of the alloenzyme in tissue homogenates represents a relative measure of cells expressing either the maternal or paternal X chromosome and can be used to screen for mutations that result in unbalanced expression of the X chromosome, as discussed in the Introduction.

The techniques of mutagenesis and screening for X-linked mutations of AT29-ENU.556 mice were performed by Chapman

Fig 2i



AT29-ENU.556 PEDIGREE

MICE USED IN PRESENT STUDY IN BOLD

MALE MICE ARE UNDERLINED

et al, 1988).

(i) formation of the AT29-ENU.556 line

The AT29 (allelic transfer stock 29) line was produced by transferring a recombinant X chromosome carrying both the M. musculus Pgk-1a allele from the congenic strain C3H/HeHa Hprta, Pgk-1a and the M. castaneus Hprta allele to the C57BL/6JRos inbred strain.

The AT29-ENU.556 line was produced by treating male AT29 mice with ethyl nitrosourea (ENU/NEU, 200-250 μg/gm) and, following their return to fertility, mating treated mice to females of the C57BL/10.mdx which were homozygous for alleles Hprtb and Pgk-1b to produce the F1 heterozygous female, 556 which had unbalanced expression of both markers in tissues (see screening, below). AT29-ENU.556 mice were selected for being hemi/homozygous for alleles Hprta and Pgk-1a and were produced by mating siblings derived from the original C57BL/10.mdx x 556 cross (Fig. 2i).

(ii) screening for X-linked mutations

1762 F1 heterozygous females were tested for deviations from balanced mosaic expression of HPRT and PGK-1 in blood and tail tissue. By measuring both PGK-1 and HPRT levels, mutations specific to the indicator loci were distinguished from less specific mutations which affect normal balanced mosaicism.

9 of these F1 females had an unbalanced pattern of expression in blood for both markers such that only the maternally-derived alloenzyme was expressed. Female 556, also had unbalanced expression of both markers in tissues of tail and skeletal muscle. 7 female progeny of the cross between female 556 and male C57BL/10.mdx (Fig. 2i) were heterozygous for both indicator loci and 3 of these females had unbalanced mosaicism in blood. This shows that the mutation present on the Pgk-1a, Hprta X chromosome of the ENU-treated male alters the mosaic expression of the X chromosome and is heritable to heterozygous female offspring. Variation of non-mosaic expression of the X chromosome in blood of heterozygous females suggests that either the mutation has variable penetrance or its map position is distant from the Pgk-1a and Hprta marker loci. Furthermore, the sex ratio of mice receiving the X chromosome bearing the mutation did not differ from the expected 1:1 ratio indicating that the mutation is not a cell autonomous lethal condition.

Descendants of the cross between female 556 and male C57BL/10.mdx (Fig. 2i) were selected for the presence of the ENU mutation and used in the present study: females, homozygous for Pgk-1a, Hprta were mice: 1708, 1709, 1710, 1998, 1821, 2115 and males, hemizygous for Pgk-1a and Hprta were mice: 687, 1010, 1706, 1937, 2140, 1818, 2203, 2261, 1843, 1842, 1703, 1752, 1756, 1758, 2199, 2121. In the present study, an AT29-ENU.556 homozygous line and

hybrids between this line and CBA and CBA/N mice and CBA and CBA-Pgk-1a were formed. The male parent in any hybrid is written first. For example, in AT29-ENU.556 x CBA mice, the father is AT29-ENU.556 and the mother, CBA.

The mice in this study were maintained on a diet of Rat and Mouse No. 1 stock breeding pellets (Special Diet Services, Essex, England) and chlorinated water ad libitum. Offspring were transferred to stock cages after being weaned 21 days post partum.

Flow cytometry

(i) preparation of cell samples

Mice were sacrificed by ether anaesthesia and cells were suspended in FACS medium (Appendix 1) for both FACScan analysis and FACSIV sorting (Chapter 6, 7).

cell suspensions

Sub-cutaneous lymph nodes (2 brachial, 2 axillary, 2 inguinal), thymus, spleen and neonatal liver were placed in a petri dish containing FACS medium and tissues were dissociated by squeezing gently between two ground glass slides. Cell suspensions were produced by sieving (0.11mm mesh) the material into a test tube on ice.

Bone marrow cell suspensions were produced by flushing femora with FACS medium in a petri dish using a 25g needle and syringe and dissociating the marrow plug by

aspirating through the needle several times in a test tube on ice.

10 drops of blood were removed from the retro-orbital sinus of etherised mice using a heparinised capillary tube (Hawksley, Sussex, England). The blood was collected into a test tube on ice which contained FACS medium, and 100 units of Heparin solution (25,000 units/ml, diluted 1:25 in 0.9% saline, Evans Medical Ltd., Middlesex, England). The supernatant was removed following centrifugation at 550g for 10 minutes and 9ml of distilled water was added for 12 seconds to lyse the erythrocytes during resuspension of the pellet (Chandler et al, 1979). The distilled water shock was terminated by the addition of 1ml 10x concentrated Minimum Essential Medium-Eagle (Gibco) and, following centrifugation at 200g for 7 minutes and removal of the supernatant, the final pellet of white blood cells was resuspended in 0.3ml FACS medium.

(ii) cell counts

10µl of acridine orange/ethidium bromide solution (Appendix 1) was added to 10µl of each cell suspension. Cell counts were made using a fluorescent microscope on the basis that live cells appear green due to the uptake of acridine orange. Viability was routinely >90%. Cells from each tissue were washed and resuspended in FACS medium at a concentration of 1×10^7 cells/ml.

Table 2A: Antibodies used for FACS analysis of surface antigens

ANTIGEN	ANTIBODY (monoclonal and raised in rat unless stated)	REFERENCE	SOURCE	VOLUME 5 /(2x10 cells)
B220	RA36B2	Coffman and Weissman, 1981a,b	1a	20µl
Thy1.2	30-H12	Reif and Allen, 1964 Micklem et al, 1980 Abehsira et al, 1981	1a 1b	20µl 4µl
Ly1	53-7.3	Cantor and Boyse, 1975 Ledbetter and Herzenberg, 1979	1a 1b	20µl 4µl
Lyt2	53-6.7	Boyse et al,1968 Ledbetter and Seaman, 1982	1a 1b	20µl 4µl
L3T4	GK 1.5	Dialynas et al,1983	1a 1b	20µl 4µl
mIgM	PE conjugated, Goat-anti-mouse-IgM polycl.	Vitetta et al,1971,1972	2	0.28µl
mIgD	FITC conjugated, Sheep-anti-mouse-IgD polycl.		3	0.28µl
second step FITC conjugated	Goat-anti-rat-IgG (H+L chain specificity mouse adsorbed) polycl.		4	0.3µl

PE = Phycoerythrin
FITC = Fluorescein Isothiocynate
1a: made in author's laboratory
1b: Sera-Lab. Ltd. Crawley Down, Sussex, England (1/100 dilution)
2 : Caltag Labs., S.O., San Francisco, C.A.
3 : Nordic Immunology, Tilburg, The Netherlands
4 : TAGO Tissue Culture Services, England

(iii) staining

For analysis of cell surface antigens, 2×10^5 viable cells suspended in FACS medium were placed in the wells (20µl/well) of a round-bottomed microtitre plate (Sterilin, Middlesex, England) and the appropriate amount of antibody added (Table 2A). Wells adjacent to filled wells were left empty to minimise the risk of contamination by splashing. The plate was shaken on a micro-shaker (Dynatech, England) and incubated on melting ice for 40 minutes. FACS medium was then added to each well and the plate was centrifuged at 350g for 15 seconds. The supernatant was discarded and the pellets resuspended by shaking after refilling the wells with FACS medium. The cells were then centrifuged and resuspended again. If an indirect staining procedure was adopted, the primary antibody was revealed by incubating the washed cells for a further 40 minutes with a FITC-conjugated goat anti-rat IgG antibody (Tago Inc., CA, USA), used at 0.3µl per 2×10^5 cells. If the primary antibody had a conjugated fluorescent tag (direct staining), no further staining was necessary. All cells were again washed twice, and resuspended in FACS medium.

There were both positive and negative controls for analysis. Negative controls were unstained cells, while positive controls were stained with second step antibody alone. This provided measures of autofluorescence and

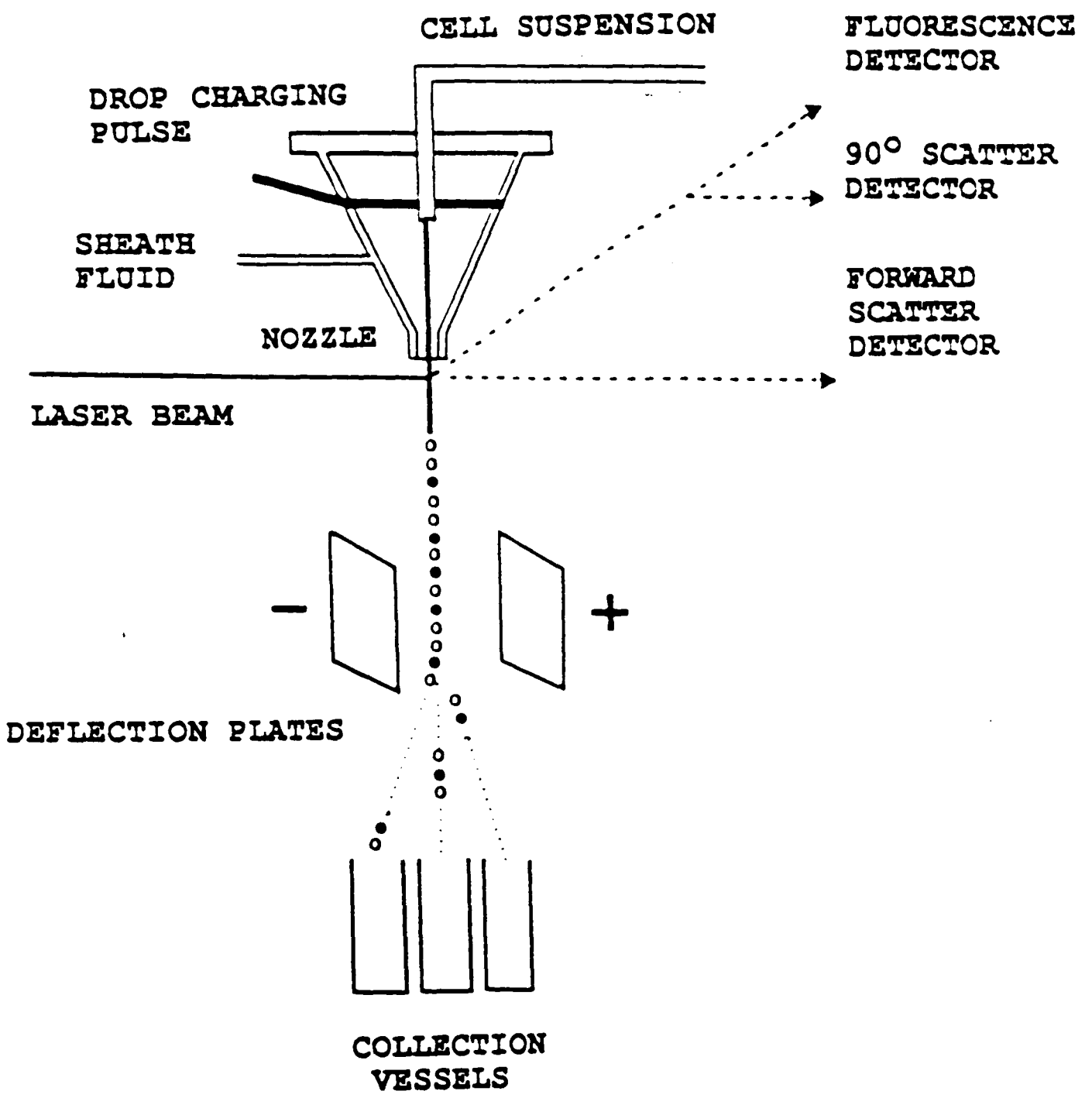


Fig. 2ii

Schematic illustration of a FACS machine in the process of sorting.

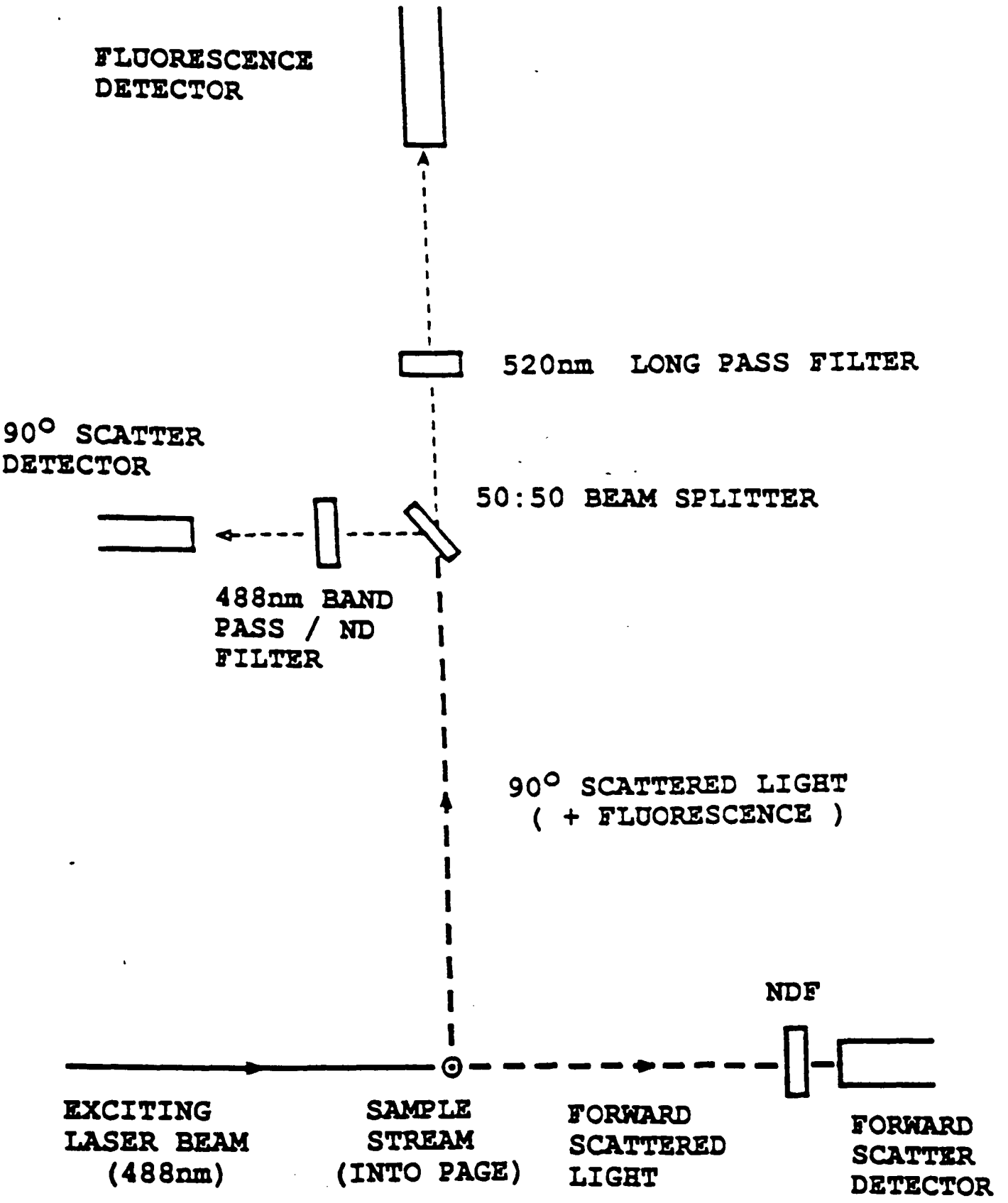


Fig. 2iii

Shematic diagram of FACS optics. NDF = neutral density filter

non-specific staining, respectively.

For cell sorting, 5×10^6 cells were stained with an appropriately scaled up volume of antibody (the volume of primary and secondary antibody was increased by a factor of 25, i.e. $5 \times 10^6 / 2 \times 10^5$). The staining procedure was similar to that above, but in FACS tubes (Falcon, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and cells were centrifuged at 400g for 7 minutes.

Analysis was performed on the FACScan and cell sorting was carried out using the FACSIV (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). (See Fig. 2ii for a general illustration of a FACS machine).

(iii) analysis

The FACS machine was fitted with an Argon laser (excitation wavelength 488nm) and logarithmic amplifiers (four decades per 255 channels) for the fluorescence channels.

Information resulting from analysis was stored in Consort 30 list mode data files. Analysis of data was carried out on hardware from Hewlett Packard, Belgium using the Lysys software package (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA); data was displayed using dotplots, histograms and contour plots.

For FACS analysis (Fig. 2iii), the suspension of cells was

conveyed through a sensing region by a fluid flow system. Cells were directed through the nozzle assembly and were centred in a cell-free sheath which formed a liquid jet that intersects the laser beam. As cells passed through the laser beam, light was scattered and fluorescent molecules (fluorescein isothiocyanate, FITC was mainly used in this study) became excited and fluoresced.

Excitation of fluorescent molecules on the cell surface resulted in emission of light of different wavelengths which was divided into different ranges by reflector filters and directed to a fluorescent detector for measurement. The emission spectra for the dyes FITC and PE overlap; this was compensated for electronically.

Light scatter gives a relative determination of cell-sized particles and describes the granularity and size of cells passing through the laser beam. Light is collected by detectors and converted into electronic signals, the magnitude of which is proportional to the light intensity.

Light scattered forwards (FSC) is collected at small angles of 3-12° (Parks et al, 1986). Forward light scatter measurements are used to initiate analysis, to permit gating out of dead cells or erythrocytes by demonstrating their low scatter profile (Loken and Herzenberg, 1975) and to determine relative cell sizes in mixed populations (Loken and Stall, 1982).

Light scattered at right angles or side scatter (SSC)

is collected within the range 70-110° (Parks et al, 1986). Side scatter measurements reflect integral structure and granularity of cells and thus permits identification or gating out of granulocytes which have granular cytoplasm (Ritchie et al, 1983). For example, in mixed populations of leukocytes, lymphocytes can be distinguished from cells with a relatively higher right angle scatter being structurally more complex. Such high scattering cells are a heterogeneous population that include monocytes and granulocytes. Forrester, 1986 defined a cell population with a relatively higher right angle scatter than lymphocytes as granulocytes for peripheral blood and bone marrow.

The ability to analyse forward and side gated populations means that fluorescence distributions can be obtained for a specific subpopulation of cells. Similarly, using two fluorescence parameters, gated analysis can distinguish cells which bear different levels of one surface antigen among a subpopulation of cells bearing a second antigen (Herzenberg et al, 1976).

Cell subpopulations were defined by quantifying fluorescence-labelled cells to assess the distribution of cell surface antigen (Table 2A). These included B ($B220^+$) cells and their subsets, population I with high IgD, low IgM; population II with high IgD, high IgM and population III with low IgD, high IgM which were classified according

to the density of membrane immunoglobulin isotype and also, T ($Thy1.2^+$) cells and the T cell and thymocyte subsets, $CD4^+$ ($L3T4^+$) helper cells, $CD8^+$ ($Lyt2^+$) cytolytic cells, $CD5^+$ ($Ly1^+$) cells, discussed in Chapter 3, 6 and 7.

Hence, cells in mixed cell populations were categorised according to a combination of cell properties such as the relative size of cells from forward light scatter measurements, the integral structure and granularity of cells from right angle scatter measurements and the expression of surface antigen quantified by fluorescence which is a measure of the density of surface antigen per cell.

(iv) cell sorting

For cell sorting, any combination of the above properties can be employed. During sorting, the nozzle assembly was vibrated vertically at 37,000 cycles per second which resulted in the stream of cells dividing into droplets at a fixed point after intersection with the laser beam. The conditions were set such that a droplet containing more than one cell is discarded. The fluorescent properties of the cell within the droplet were recorded as each droplet passed through the laser beam. Fluorescence and scatter criteria were established using a dotplot before formation of the droplet. Cells which fulfilled the requirement for sorting were charged before droplet formation and, upon passing between two charged deflection plates, negatively

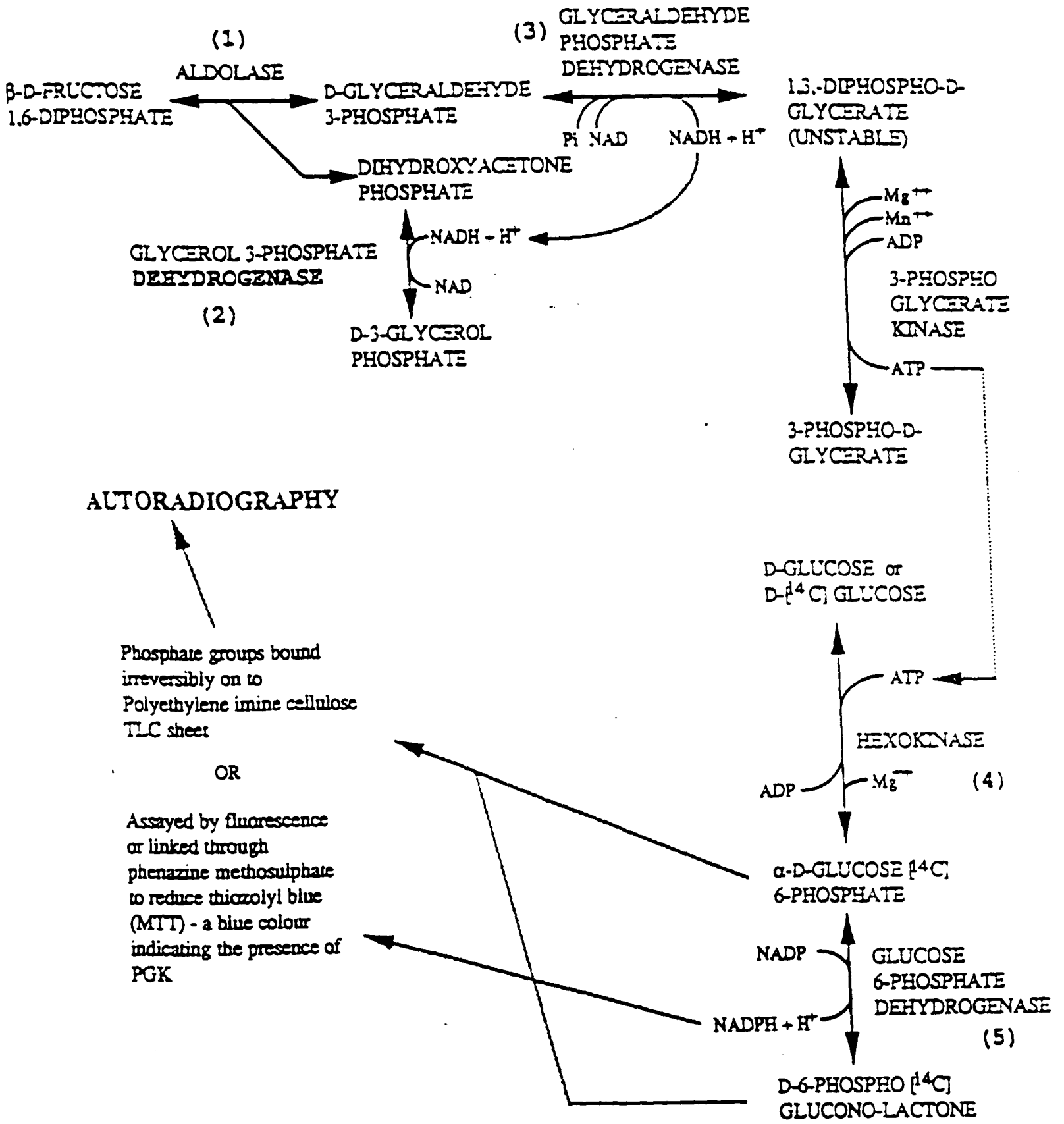


Fig. 2iv

The biochemical basis for the Phosphoglycerate Kinase assay.
(adapted from Ansell and Micklem, 1986).

charged droplets were collected separately from positively charged droplets, and both separated from the uncharged jet containing cells which did not meet the given criteria and were discarded.

Hence, defined subpopulations of cells were isolated for further study, namely electrophoretic alloenzyme analysis, in this study, to determine cell origin, discussed in Chapter 3.

Alloenzyme Analysis

Phosphoglycerate kinase-1 (PGK-1) is an enzyme found in all tissues and existing in two forms, the alloenzymes PGK-1A and PGK-1B which are separable by electrophoresis and can be measured relative to one another. The locus for Pgk-1 is on the X chromosome and the strains of mice used in this project were homozygous for either alloenzyme. The overall levels of each alloenzyme in any tissue of mice heterozygous for Pgk-1 depends on genetic factors and on X-chromosome inactivation patterns in the tissue progenitors which is discussed in the Chapter 1 (McLaren, 1972; West, 1975; Fialkow, 1973; Pappaioannou et al, 1981; McMahon et al, 1983).

PGK-1 is an enzyme of the glycolytic pathway, catalysing the conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid with the production of ATP. The enzyme assay for PGK-1 is illustrated in Fig. 2iv. Electrophoresis is carried out on cellulose acetate

membranes and PGK can be visualised either by autoradiography following incorporation of a ^{14}C-labelled substrate or by using MTT staining following reduction of a tetrazolium salt to formazan. The substrate for PGK-1, 1,3-diphospoglycerate, is unstable and is generated *in situ* on the electrophoresis membrane. Following the production of ATP, resulting from PGK-1 action, ATP is used in the production of glucose 6.phosphate and 6-phosphogluconolactone which is catalysed by hexokinase and glucose 6-phosphate dehydrogenase along with NADPH production. The system described was based on the work by Bucher *et al*, 1980 and modified by Ansell & Micklem, 1986.

(i) preparation of cell samples for electrophoresis

Sorted cells and homogenised pieces of the following tissues were frozen in sample buffer after washing in FACS medium (Appendix 1) soon after sacrifice: spleen, lymph node, thymus, brain, bone marrow, a drop of whole blood, neonatal liver and skeletal muscle of the leg.

Analysis of the leukocytes of spleen and blood required removal of erythrocytes by lysis using distilled water shock (see above) and analysis of blood platelets required removal of erythrocytes and other leukocytes: 6 drops of blood were collected in a non-heparinised tube containing 5ul of 38% sodium citrate (38g per 100ml distilled water). The tube was inverted and centrifuged at 300g for 5

minutes. The platelet-rich plasma was removed and centrifuged at 800g for 20 minutes. After the supernatant was discarded, the pellet was resuspended, washed in FACS medium, centrifiged at 300g for a further 5 minutes before being frozen in sample buffer.

Samples to be analysed were frozen at -70^{o} then thawed in sample buffer (Appendix 1) the volume of which was dependent on the cell number and the staining system used: a drop of blood (20µl) required 80µl buffer; samples for MTT-type electrophoresis required 2×10^{8} cells/ml buffer and sorted samples using autoradiography required 2×10^{7} cells/ml buffer. The buffer lyses cells and keeps enzymes stable during storage.

(ii) electrophoresis

Titan III cellulose acetate membranes (Helena Laboratories, Cat. No. 3024, Beamont, Texas, USA) were blotted to remove excess buffer after soaking in electrophoresis buffer for 20 minutes (Appendix 1). 0.25µl aliquots of sample were loaded at the cathodal end of cellulose acetate membranes using a "Super Z" applicator (Helena Labs). The membranes were positioned sample side down on a Helena electrophoresis tank containing 12.5 mg adenosine 5' monophosphate (AMP) (Sigma) in 50 ml electrophoresis buffer in both anodal and cathodal chambers. Filter paper wicks (Helena Labs, Cat.No.5081), soaked in the AMP/buffer solution, were applied over the

bridge sides, maintaining contact with the solution in the two chambers. A 10gm weight, positioned on top of each membrane ensured contact between the membranes and the wicks. Electrophoresis ran for 45 minutes at 15mA per membrane, at 4 °C.

PGK-1A, being more negatively charged, migrates closer to the anode than PGK-1B.

(iii) staining methods

Staining solutions for MTT and ^{14}C indicator systems were prepared 10 minutes before the end of electrophoresis.

(a) tetrazolium indicator system (MTT)

NADPH, produced from the reaction catalysed by G6PDH, is linked through phenazine methosulphate (PMS) to reduce dimethyl thiazolyl-diphenyl tetrazolium bromide (MTT). A blue colour indicates the presence of PGK-1.

75µl sample buffer, 400µl assay stock (see Appendix) , 100µl indicator stock (see Appendix), 10µl aldolase (6.5µ/ml) (Boehringer, W. Germany), 5µl GDH (2000µ/ml) (Sigma), 7.5µl GAPDH (10mg/ml) (Boehringer), 5µl hexo-kinase (6000µ/ml) (Sigma) and 10µl G6PDH (312µ/ml (Sigma) were added to 800µl electrophoresis buffer.

A mixture of 500µl MTT (10mg/ml distilled water) (Sigma), 500µl PMS (2.5mg/ml distilled water) (Sigma) and 2500µl 1.2% agar noble (Difco, Michigan, USA)

was boiled and retained at 56°C seconds before adding to the staining mixture. The final mixture was poured evenly over the membranes, allowed to set in darkness as the dyes are light-sensitive prior to incubation at 37°C until alloenzyme bands appeared. Membranes or gels were then fixed in an aqueous solution of 15% glycerol and 3% acetic acid.

(b) autoradiographic indicator system (^{14}C)

Addition of ^{14}C-labelled glucose as a substrate for the reaction catalysed by hexokinase results in the incorporation of ^{14}C into the indicator system. The ^{14}C-labelled products of the hexokinase and G6PDH reactions (glucose-6-phosphate and 6-phosphoglucono-lactone, respectively) bind covalently via their phosphate groups to the polyethylene imine (PEI) cellulose chromatography sheets (Bakerflex, Linton Products, Hysol, England). Any unconverted ^{14}C is washed out and PGK-1 visualised by autoradiography.

PEI sheets were soaked for 20 minutes in distilled water at 4°C, then blotted. The staining mixture was similar to that of the MTT system with reduced quantities of electrophoresis buffer (500µl) and sample buffer (30µl) and the glucose content of indicator stock is reduced to 1/10th (Appendix 1) such that the relative proportion of labelled glucose converted into phosphate products was increased. MTT, PMS and agar were ommitted from this system and

instead, 15µl D-(µ-^{14}C) glucose (1mCi/ml) (Amersham) was added to the stain prior to pouring the final mixture evenly over the PEI sheet. The electrophorised gel was placed on top, ensuring even contact and excluding air bubbles at the interface. Following a 15 minute incubation at 37°C, the PEI sheet was rinsed in 8mM Tris (Sigma) and washed with stirring for 4 hours in 8mM Tris at room temperature. After drying, PEI sheets were exposed to X-ray film (Kodak X-OMATS) for at least 3 days, or longer if the alloenzyme bands appeared faint. Films were developed for 2.5 minutes in a 1:4 solution of Kodak LX24 developer, washed in 2% acetic acid and fixed for 2.5 minutes in 1:4 solution of Kodak FX40 Fixer. Finally, the film was washed in distilled water for 20 minutes.

(iv) densitometry of alloenzyme bands

For both MTT and ^{14}C gels, the proportions of the two PGK-1 alloenzymes were assessed by a scanning densitometer (Chromoscan 3, Joyce Loebl, Vickers Instruments, Gateshead, England) using transmitted light for autoradiographic gels and reflected light for MTT-gels. Data were analysed and stored on a Sirius microcomputer. The programme calculated the area under each curve by integrating peaks produced by scanning.

(v) technical errors

Linearity experiments, with repeats have been carried out

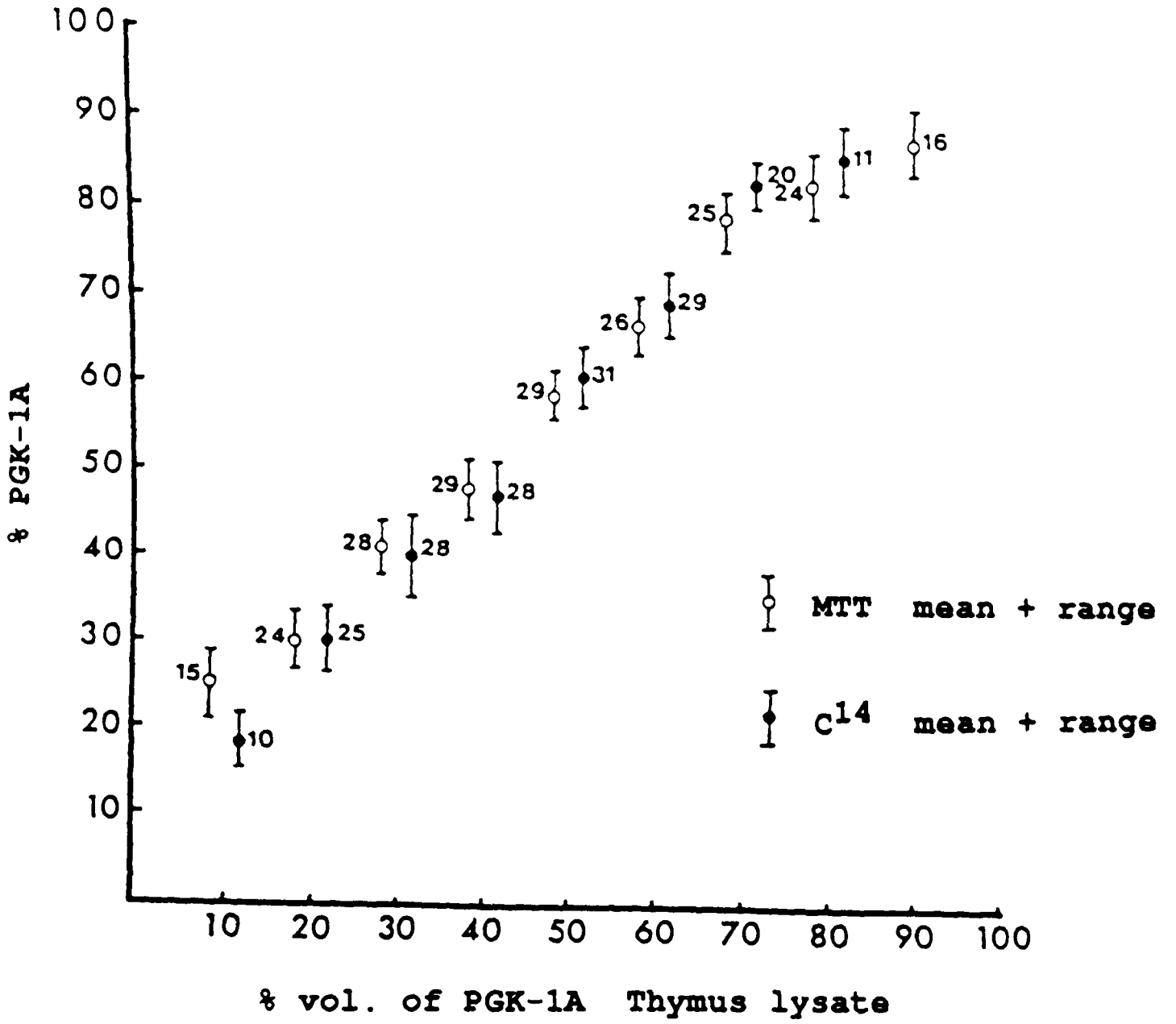


Fig. 2v

Linearity in the electrophoretic measurement of PGK-1A and PGK-1B mixtures. Figures shown on graph are number of samples per point (adapted from Ansell and Micklem, 1986).

in the author's laboratory (Ansell & Micklem, 1986; H. Taylor, pers. comm.) whereby, in artificial mixtures of PGK-1A and PGK-1B cell lysates, there is a linear relationship between the electrophoretic and volumetric measurement of %PGK-1A over the range 20-80%. Minor components tend to be overestimated outside this range (Fig. 2v). In practice, separations were discarded in which the total enzyme activity was too low, too high or were faulty for example, trailing, distorted, smearing, poorly separated or having lumpy agar. There was not much difference in the variation due to technical error between the MTT and ^{14}C system, although the latter was more sensitive (Ansell & Micklem, 1986).

Functional Analysis

Preliminary tests were carried out (Chapter 7) to examine the effect of 4-ethoxymethylene-2-phenyl oxazolone (BDH chemicals, Ltd.) hereafter named, oxazolone on the cellularity of the spleen and lymph nodes of homozygous and heterozygous mice which were aged 12 weeks, approximately.

(i) sensitisation

10 mg of oxazolone dissolved in 0.1 ml absolute ethanol at 60°C was applied to a shaved area of the skin, approximately 2 cm in diameter, on the right side of the thorax. Control animals received only 0.1ml of absolute ethanol at 60°C and were caged separately from treated

mice for 3 days.

(ii) assessment of sensitisation

The brachial lymph nodes of experimental and control mice were examined by eye for signs of swelling 3 days after treatment.

Cell suspensions were prepared for FACS analysis in the case of homozygotes or FACS sorting and PGK-1 alloenzyme analysis in the case of heterozygotes, as described above. Leukocyte counts were made using a Coulter Counter (Coulter Electronics Ltd., Luton, England) by gating out erythrocytes.

Data Analysis

In this study, a test of significance is applied when comparing the means of two different samples to see if they differ. When samples contain less than 30 members, the sample standard deviation becomes unreliable as an estimate of the standard deviation of the population. Hence, in significance testing, Student's t-test can be used. The t-distribution is similar to the normal distribution in being symmetrical about a mean but its dispersion varies according to the size of the sample. The bigger the sample, the closer the t-distribution approaches the normal distribution. The smaller the sample size, the greater is the difference between the means demanded by the t-test for a significant result.

There is a limit on the number of t-comparisons that can be made in a data set and that is 1 less than the number of mean values in the data set.

In this study, for comparisons tests between the means of two samples with low sample number, the distribution of the sample values was compared with the t-distribution, discussed above and Student's t-test was used such that a significant difference between the means of two samples was taken as $P<0.05$ when analysed.

In Chapter 3 correlation analysis was performed using the Minitab computer system to assess the effect of the ENU mutation indicated by the level of PGK-1A expression with age for selected tissues. Correlation concerns the strength association between the values of two variables which, in this case, were the mean %PGK-1A values and age in weeks. The mean %PGK-1A values were plotted against the age in weeks to produce the correlation coefficient, r and a scatter diagram which showed the amount of scatter or the strength of the linear association between the two variables and the direction of the linear association such that positive values implied a direct association and negative values implied an indirect association between the two variables. A correlation coefficient of +1 or -1 implied a perfect linear association and the closer r approached +1 or -1, the stronger was the association between the mean %PGK-1A values and age. A correlation coefficient of 0 implied no linear association and when r

approached 0 the weaker was the association between the mean %PGK-1A values and age. It should be noted that correlation analysis only picks up the linear association and it may miss more complicated associations where the variables are related in a nonlinear fashion (Groeneveld, 1988; Hogg and Ledolter, 1992)

CHAPTER 3

The Phenotypic Analysis Of An X-linked Mutation By X-inactivation Studies

X-linked mutations can result in unbalanced expression of the X chromosome. If a mutation at an X-linked locus directly affects a cell population by being lethal at a particular developmental stage or by affecting the ability of cells to proliferate there will be unbalanced expression of the X chromosome in the cells of females heterozygous for the mutation. Studies of X-chromosome inactivation patterns can be used to identify specific cell types affected by an X-linked mutation and the degree of imbalance of X-chromosome expression may reflect the extent of the mutation.

Analysis of X-chromosome inactivation patterns involves assessing the relative expression of the maternal or paternal X chromosome in heterozygotes by quantifying the relative amounts of allelic X-linked enzyme which is used as a cell marker and can be separated by electrophoresis.

For example, female carriers of XLA who are heterozygous both for the XLA and the G6PD locus encoding for the enzyme G6PD with A/B polymorphism have balanced expression of both alloenzymes in all cell types except B cells which are affected by XLA and demonstrate unbalanced expression of the A alloenzyme (Conley et al, 1986). Similarly, in mice heterozygous both for the xid

mutation and the Pgk-1 locus where cells express variant forms of the enzyme PGK-1, unbalanced expression of the X chromosome has been demonstrated for B cells (Nahm et al, 1983; Forrester et al, 1987).

The aim of this study was to characterise the phenotypic effect of a novel X-linked mutation in various tissues of offspring (AT29-ENU.556) of male mice (AT29) treated with the mutagen N-ethylnitrosourea (ENU). It has been shown that treatment with ENU can produce X-linked mutations affecting the haematopoietic and muscular systems (Chapman et al, 1988, 1989).

Studies of X-chromosome inactivation patterns in mice heterozygous for an ENU mutation using electrophoretic alloenzyme analysis were used to characterise the phenotypic effect of an X-linked ENU mutation by delineating the cell lineages affected by the mutation, the differential effect of the ENU mutation in various cell types and the effect of the mutation with age.

In this study, electrophoretic variants of the X-chromosome linked enzyme PGK-1 were used as markers to determine the expression of the X chromosome in mice heterozygous for the ENU mutation (ENU/+) such that the presence of cells carrying the + locus on the X chromosome was detected by visualisation of the allo-enzyme, PGK-1B and the presence of cells carrying the

ENU mutation was detected by the alloenzyme, PGK-1A. Hence, unbalanced expression of PGK-1A in a cell lineage delineates the lineage affected by the ENU mutation.

Interpreting the results of X-chromosome inactivation studies requires taking into account the degree to which random X-chromosome inactivation can result in variation from the expected mean of 50% of cells with the maternally-derived X chromosome active and 50% of cells with the paternally-derived X chromosome active. As a wide variation in normal random patterns of X-chromosome inactivation exists it is important to evaluate the X-chromosome inactivation patterns in cell lineages of heterozygous female controls that are unaffected by an X-linked mutation and also in cell lineages unaffected by an X-linked mutation from an individual in which there are cell lineages that are affected by an X-linked mutation.

Attempts to relate the ENU mutation to a candidate gene were made by assessing whether the ENU mutation was an allele of xid by assessing the X-chromosome inactivation patterns in cells of females which were heterozygous for the ENU mutation and xid (ENU/xid) such that the presence of cells carrying the xid locus on the X chromosome was detected by the PGK-1B alloenzyme.

The phenotypic effect of the ENU mutation was studied by analysis of X-chromosome inactivation patterns in tissues of females heterozygous for the ENU mutation and results

were compared with the X-chromosome inactivation patterns in tissues of control heterozygous females.

Hence, X-chromosome inactivation patterns were studied in heterozygous females produced from the following crosses:

(1) AT29-ENU.556 x CBA (ENU/+)

(2) AT29-ENU.556 x CBA/N (ENU/xid)

and PGK-1AB controls:

(3) AT29 x CBA

(4) AT29 x CBA/N

(5) CBA x Pgk-1a

As all mice referred to in this chapter are heterozygous females, to simplify the text, the term mice is used to denote heterozygous females. Studies were also made of mice derived from the reciprocal crosses of (1) and (3), above.

The tissues studied included the non-haematopoietic tissues brain, which was a control tissue as it was unaffected by the mutation, and skeletal muscle and the haematopoietic tissues blood, spleen, thymus, lymph node, bone marrow and neonatal liver. X-chromosome inactivation studies were extended to defined subpopulations of haemat opoietic cells to characterise the leukocyte sub-populations affected by the ENU mutation. The leukocyte subpopulations studied included platelets isolated from peripheral blood, white blood cells isolated from peripheral blood and spleen and cell subpopulations

leukocytes that were separated from lymphocytes by virtue of their relatively high right angle scatter being structurally more complex than lymphocytes. In the case of neonatal liver, lymphocytes were defined by their relatively low right angle scatter profile and not according to staining for antigen expression.

In the case of thymus, a combination of forward light scatter measurements defining relative cell size and intensity of fluorescence measuring the density of the antigen Thy1.2 permitted differentiation of thymocyte subsets of the inner cortex, outer cortex and medulla.

In the analysis of the effect of the ENU mutation with age mice were divided into: adults of >24 days, defined by sexual maturity; pre-adults of 15-24 days and young of <15 days.

phenotypic variation of AT29-ENU.556 heterozygous females

PGK-1 analysis of mice from (1) AT29-ENU.556 x CBA and (2) AT29-ENU.556 x CBA/N crosses revealed two distinct phenotypic groups for each cross, namely PI, PII and PIII, PIV respectively, the genetic basis of which is discussed in Chapter 4.

One phenotypic group from each of (1) and (2), namely PI and PIII, respectively had skewed patterns of PGK-1 expression, in favour of non-ENU-mutant (PGK-1B) cells. This was the case for all cell types studied, except

brain, high scattering leukocytes, possibly platelets and, depending on age, B cells of CBA/N mice (PIII).

The other phenotypic group from each of (1) and (2), named PII and PIV, respectively had PGK-1 patterns which reflected random expression of either chromosome. Hence, the assumption that PII and PIV mice did not carry the ENU-mutant X chromosome. These patterns were similar to those observed in normal (CBA-PGK-1AB) mice in all cell lineages, of all age groups, except in B cell populations in PIV mice where B lymphocytes carrying the xid mutation were selected against. The data for PIV mice were similar to published data (Forrester et al, 1987).

SECTION I.

Characterisation Of The Cell Lineages Affected By The ENU Mutation

%PGK-1A values were determined in adult (>24 day) mice of the nine groups discussed above. Results are presented in summary tables. The mean %PGK-1A values for whole tissue extracts of PI-PIV mice and AT29 control mice were compared using Student's t-test and the results are presented in the comparisons tables which follow an account of the results. Non-AT29 control mice (CBAxCBA-Pgk-1a) are included in summary tables but not used in t-tests.

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% PGK-1A : BRAIN

strain	mean	n	SD	age
(AT29 x CBA)	69	4	9.78	)24d
(CBA x AT29)	55	4	6.98	)24d
(CBA x CBA-Pgk-1a)	69	7	6.78	)24d
(AT29-ENU.556 x CBA) PII	67	8	11.81	)24d
(AT29-ENU.556 x CBA) PI	51	15	10.81	(24d
	63	34	12.90	)24d
(CBA x AT29-ENU.556) PI	64	5	6.02	)24d
(AT29 x CBA/N)	60	4	9.00	)24d
(AT29-ENU.556 x CBA/N) PIV	63	14	12.97	)24d
(AT29-ENU.556 x CBA/N) PIII	54	10	14.23	(24d
	64	14	12.35	)24d

Table 3.1

Non-haematopoietic tissues

Brain

With reference to the summary table 3.1 and the comparisons table 3.2, there was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 or AT29-ENU.556 crosses. Hence, the mean %PGK-1A values for each group were pooled for comparison with other crosses. It should be noted that for the AT29 control mice, the sample number was low which may have produced a false result. There was no significant difference between the mean %PGK-1A values for PI and AT29 control mice; for PI and PII mice; and for PIII and AT29 control mice and also for PIII and PIV mice.

Table 3.2 BRAIN : F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	~control	PII
#PI	>0.05 63 v 62	>0.05 63 v 67

	(AT29xCBA/N)	PIV
PIII	>0.05 64 v 60	>0.05 64 v 63

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values
#=(AT29-ENU.556xCBA)/(CBAxAT29-ENU.556)PI pooled mean %PGK-1A values

Skeletal muscle

With reference to summary table 3.3 and comparisons table 3.4, the ranges of %PGK-1A values for skeletal muscle

BIMODAL DISTRIBUTION OF %PGK-1A VALUES FOR SKELETAL MUSCLE OF PI* FEMALES

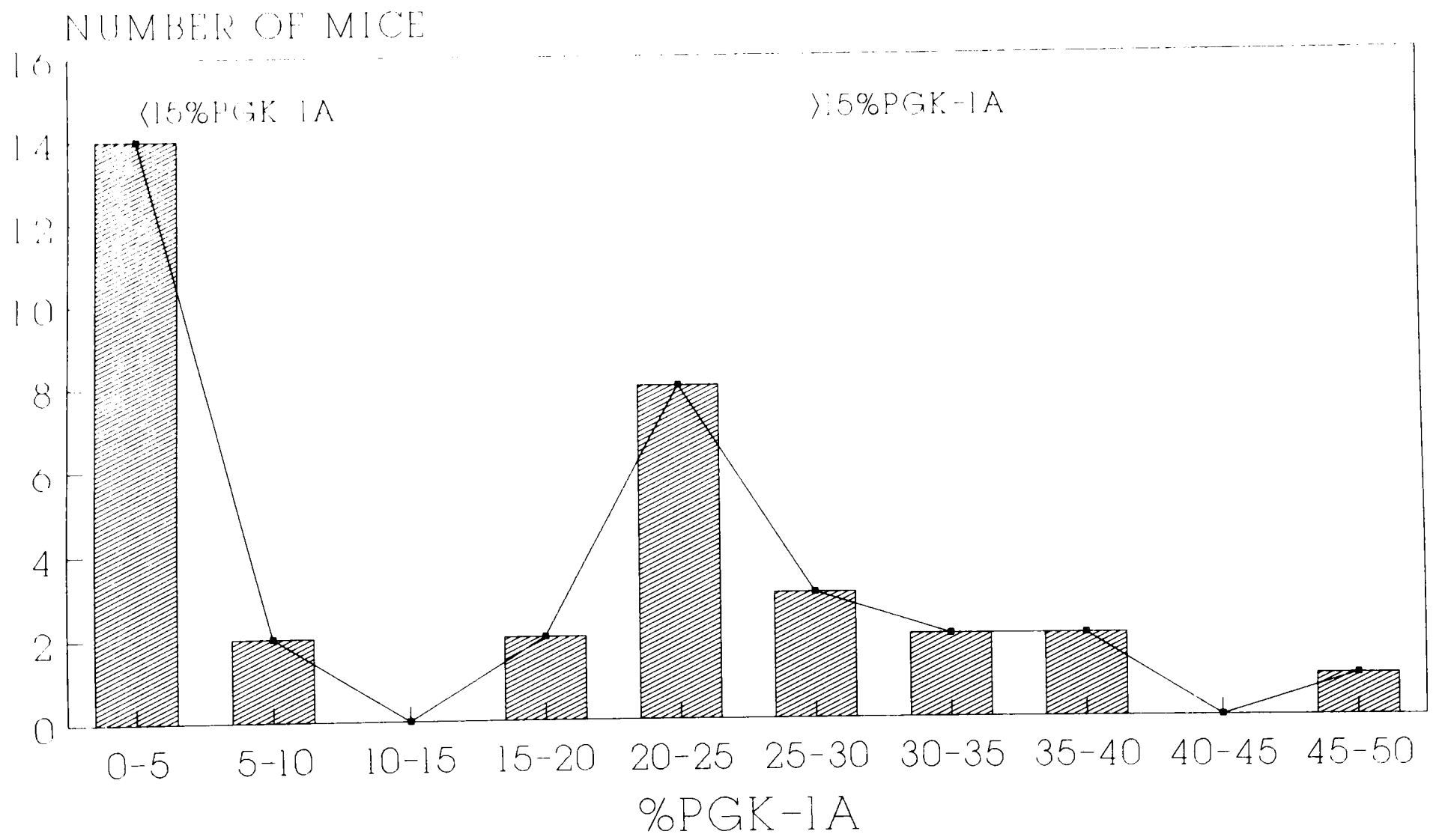


Fig.3.1 where * represents bimodal distribution of %PGK-1A values for PI females

were much broader than for most other tissues. For PI, PII and PIII mice the %PGK-1A values did not fit the t-distribution. An example of this is shown in Fig.3.1 where the distribution of %PGK-1A values is bimodal for (AT29-ENU.556xCBA) PI mice, denoted by PI*.

There was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 crosses. Hence, the data were pooled for comparison with other crosses. As PI* females had bimodal distribution of %PGK-1A values the group was divided into <15% and >15% PGK-1A sample groups, with equal numbers of mice in each and the >15% PGK-1A sample group was compared separately with other crosses (see Fig.3.1). Similarly, PII mice had bimodal distribution of %PGK-1A values so the sample was divided into <65% and >65% PGK-1A sample groups and the former group compared with other crosses.

The >15% PGK-1A sample group of PI* mice with a mean value of 26% PGK-1A was significantly lower than the mean value for PII mice with 56% PGK-1A and for the AT29 control mice with 53% PGK-1A and was significantly higher than the mean value for the PI mice of the reciprocal cross with 13% PGK-1A. However, if the PI* sample group was not split the overall mean of (AT29-ENU.556xCBA) PI mice was 14% PGK-1A compared with 13% PGK-1A for (CBAxAT29-ENU.556) PI mice of the reciprocal cross. It should be noted that there were only 3 PI mice in the reciprocal cross sample and it is possible that like PI*, PII and PIII mice, the

% PGK-1A : SKELETAL MUSCLE

strain	mean	n	SD	age
(AT29 x CBA)	61	4	12.36	)24d
(CBA x AT29)	46	4	6.61	)24d
(CBA x CBA-Pgk-1a)	64	7	5.86	)24d
(AT29-ENU.556 x CBA) PII	68	8	10.28	)24d
	33	8	10.45	(15d
(AT29-ENU.556 x CBA) PI	19	4	7.89	15-24d
	14	34	14.06	)24d
(CBA x AT29-ENU.556) PI	13	3	11.15	)24d
(AT29 x CBA/N)	56	4	7.41	)24d
(AT29-ENU.556 x CBA/N) PIV	63	14	17.55	)24d
	41	8	10.91	(15d
(AT29-ENU.556 x CBA/N) PIII	24	2	2.12	15-24d
	16	14	11.18	)24d

Table 3.3

distribution of %PGK-1A values for PI mice of the reciprocal cross might have also been bimodal had a larger sample been analysed.

Skeletal muscle of PIII mice also had bimodal distribution of %PGK-1A values. Hence, the sample was divided into <15% and >15% PGK-1A sample groups and the >15% PGK-1A sample group was compared separately with other crosses. The mean value for PIII mice with 24% PGK-1A was significantly lower than the mean value for PIV mice with 63% PGK-1A and for the AT29 control mice mice with 56% PGK-1A.

Table 3.4 SKELETAL MUSCLE : F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	(CBAx AT29-ENU.556) PI	~control	PII <65% PGK-1A
(AT29-ENU.556 xCBA) PI* >15% PGK-1A	<0.05 26v13	<0.01 26v53	<0.05 26v56

	(AT29xCBA/N)	PIV
PIII >15% PGK-1A	<0.01 24v56	<0.01 24v63

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values
* denotes bimodal distribution of PI females in Fig.3.1.

Haematopoietic tissues

Blood

Unsorted material

With reference to summary table 3.5 and the comparisons table 3.6, there was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 crosses, although the low sample number may have produced a false result. Hence, for simplicity the data were pooled for comparison with other crosses. The mean value for (AT29-ENU.556xCBA) PI mice with 26% PGK-1A was significantly lower than that for PI mice of the reciprocal cross with 47% PGK-1A and than that for PII mice with 72% PGK-1A and also than the mean value of the AT29 control mice with 58% PGK-1A.

The mean value for PIII mice with 26% PGK-1A was significantly lower than that for PIV mice with 70% PGK-1A and than that for the AT29 control mice with 59% PGK-1A.

Table 3.6 BLOOD/

% PGK-1A : WHOLE BLOOD

strain	mean	n	SD	age
(AT29 x CBA)	64	4	2.06	>24d
(CBA x AT29)	52	3	7.00	>24d
(CBA x CBA-Pgk-1a)	71	7	12.84	>24d
(AT29-ENU.556 x CBA) PII	72	8	6.28	>24d
	55	9	10.33	<15d
(AT29-ENU.556 x CBA) PI	42	4	6.45	15-24d
	26	35	12.64	>24d
(CBA x AT29-ENU.556) PI	47	5	9.98	>24d
(AT29 x CBA/N)	59	4	5.50	>24d
(AT29-ENU.556 x CBA/N) PIV	70	14	9.34	>24d
	59	5	6.76	<15d
(AT29-ENU.556 x CBA/N) PIII	44	2	2.83	15-24d
	26	14	13.39	>24d

Table 3.5

Table 3.6 BLOOD : F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	(CBAx AT29-ENU.556) PI	~control	PII
(AT29-ENU.556 xCBA) PI	<0.05 26 v 47	<0.01 26 v 58	<0.01 26 v 72

	(AT29xCBA/N)	PIV
PIII	<0.01 26 v 59	<0.01 26 v 70

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values

Blood Leukocytes

With reference to table 3.19 and 3.20 at the end of Chapter 3 in >24 day mice, for white blood cells, the mean value for PI mice was 17% PGK-1A which was much lower than that for PII mice with 74% PGK-1A and the AT29 control mice with 38% PGK-1A. Similarly, the mean value for white blood cells of PIII mice was 20% PGK-1A which was lower than that for PIV mice with 79% PGK-1A and the AT29 control mice with 57% PGK-1A. The mean %PGK-1A value for white blood cells of PII and PIV mice was higher than that for the AT29 control mice, given above.

For blood platelets of PI mice, the mean was 33% PGK-1A which was lower than that for PII mice with a mean value of 70% PGK-1A. PIII mice had a mean value of 24% PGK-1A which was lower than that for PIV mice with a mean value of 79% PGK-1A.

Spleen

% PGK-1A : WHOLE SPLEEN

strain	mean	n	SD	age
(AT29 x CBA)	60	4	3.42	)24d
(CBA x AT29)	54	3	5.69	)24d
(CBA x CBA-Pgk-1a)	72	7	10.39	)24d
(AT29-ENU.556 x CBA) PII	73	7	8.12	)24d
(AT29-ENU.556 x CBA) PI	36	8	9.21	(24d
	27	27	8.11	)24d
(CBA x AT29-ENU.556) PI	44	3	7.21	)24d
(AT29 x CBA/N)	62	4	6.24	)24d
(AT29-ENU.556 x CBA/N) PIV	80	9	5.59	)24d
(AT29-ENU.556 x CBA/N) PIII	51	5	6.48	(24d
	34	8	9.66	)24d

Table 3.7

Spleen

Unsorted material

With reference to summary table 3.7 and the comparisons table 3.8, there was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 crosses. Hence, the data were pooled for comparison with other crosses. The mean value for (AT29-ENU.556xCBA) PI mice with 27% PGK-1A was significantly lower than that for PI mice of the reciprocal cross with 44% PGK-1A and that for PII mice with 73% PGK-1A and the AT29 control mice with 57% PGK-1A.

Similarly, for CBA/N F1s, the mean value for PIII mice with 34% PGK-1A was significantly lower than that for PIV mice with 80% PGK-1A and that for the AT29 control mice with 62% PGK-1A.

Table 3.8 SPLEEN: F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	(CBAx AT29-ENU.556) PI	~control	PII
(AT29-ENU.556 xCBA) PI	<0.01 27 v 44	<0.01 27 v 57	<0.01 27 v 73

	(AT29xCBA/N)	PIV
PIII	<0.01 34 v 62	<0.01 34 v 80

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values

Splenic Leukocytes

With reference to tables 3.21-3.29 at the end of Chapter 3 for >24 day mice, the mean value for splenic white blood cells of PI mice was 24% PGK-1A which was lower than that of PII mice with 74% PGK-1A. The mean value for splenic white blood cells of PIII mice was 37% PGK-1A which was lower than that of PIV mice with 84% PGK-1A.

Sorted material

Without carrying out comparison tests, there were obvious differences between the mean %PGK-1A values for sorted splenic cell populations of ENU-mutant and control mice. Note that the mean %PGK-1A values for either T or B cells were obtained by pooling %PGK-1A values of their subsets. For example, the mean %PGK-1A value for splenic B cells was derived by pooling %PGK-1A values of $B220^{+}$ and $Thy1.2^{-}$ cells sorted by flow cytometry (Table 3.21, for >24 day mice).

The mean value for splenic B cells of PI mice was 2% PGK-1A which was lower than that of the AT29 control mice with 51% PGK-1A and than that of PII mice with 68% PGK-1A. Similarly, the mean value for splenic T cells of PI mice was 2% PGK-1A which was lower than that of the AT29 control mice with 48% PGK-1A and than that of PII mice with 69% PGK-1A. However, high scattering cells of PI females had a mean value of 43% PGK-1A.

The mean value for splenic B cells of PIV mice was 92% PGK-1A and that of the AT29 control mice was 78% PGK-1A which were both greater than that of PIII mice with 50% PGK-1A. Similarly, for splenic T cells, the mean value of PIV mice was 72% PGK-1A and that of the AT29 control mice was 65% PGK-1A which were both greater than the mean value of PIII mice with 21% PGK-1A. The mean value for high scattering cells of PIII mice was 48% PGK-1A.

The mean %PGK-1A values for B and T cells of PII and PIV mice were higher than those of the AT29 control mice which is consistent with the data for whole spleen, whole blood and blood leukocytes.

Thymus

Unsorted material

Refer to summary table 3.9 and the comparisons table 3.10. There was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 or AT29-ENU.556 crosses. Hence, the data for the respective groups were pooled for comparison with other crosses. The mean value for PI mice with 13% PGK-1A was significantly lower than that for PII mice with 68% PGK-1A and the AT29 control mice with 59% PGK-1A.

Similarly, the mean value for PIII mice with 14% PGK-1A was significantly lower than that for PIV mice with 70% PGK-1A and the AT29 control mice with 57% PGK-1A.

% PGK-1A : THYMUS

strain	mean	n	SD	age
(AT29 x CBA)	63	3	5.51	)24d
(CBA x AT29)	55	3	4.62	)24d
(CBA x CBA-Pgk-1a)	66	7	10.68	)24d
(AT29-ENU.556 x CBA) PII	68	7	12.92	)24d
(AT29-ENU.556 x CBA) PI	23	7	8.84	(24d
	13	29	11.11	)24d
(CBA x AT29-ENU.556) PI	9	3	15.01	)24d
(AT29 x CBA/N)	57	4	5.50	)24d
(AT29-ENU.556 x CBA/N) PIV	70	11	10.56	)24d
(AT29-ENU.556 x CBA/N) PIII	39	6	9.38	(24d
	14	8	8.48	)24d

Table3.9

Table 3.10 THYMUS : F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	~control	PII
(AT29-ENU.556 xCBA) #PI	<0.01 13 v 59	<0.01 13 v 68

	(AT29xCBA/N)	PIV
PIII	<0.01 14 v 57	<0.01 14 v 70

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values
#=(AT29-ENU.556xCBA)/(CBAxAT29-ENU.556)PI pooled mean %PGK-1A values

Sorted material

Refer to tables 3.30-3.38 at the end of Chapter 3, for >24 day mice. As for sorted splenic material, the mean %PGK-1A value for thymocytes was obtained by pooling the %PGK-1A values of the thymocyte subsets sorted by flow cytometry. There were obvious differences without comparison tests between the mean %PGK-1A values for thymocytes of ENU-mutant and control mice.

The mean value for thymocytes of the AT29 control mice was 53% PGK-1A and that of PII mice was 63% PGK-1A which were both greater than that of PI mice which was 4% PGK-1A. Similarly, the mean value for thymocytes of the AT29 control mice was 54% PGK-1A and that of PIV mice was 71% PGK-1A which were both higher than that of PIII mice which was 4% PGK-1A. The mean %PGK-1A values for thymocytes of PII and PIV mice were higher than that for the AT29 control mice which was also the case for T cells of lymph

node.

With reference to table 3.30 and 3.31, for >24 day mice, there was little variation in the PGK-1 expression between the various subsets of thymocytes studied for either PI or PIII mice.

Lymph node

Unsorted material

With reference to summary table 3.11 and the comparisons table 3.12, there was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 crosses. Hence, the data of the reciprocal crosses were pooled for comparison with other crosses. The mean value for (AT29-ENU.556xCBA) PI mice with 13% PGK-1A was significantly lower than that for PI mice of its reciprocal cross with 33% PGK-1A and than that for both PII mice with 64% PGK-1A and the AT29 control mice with 62% PGK-1A.

The mean value for PIII mice with 23% PGK-1A was significantly lower than that for PIV mice with 77% PGK-1A and the AT29 control mice mice with 62% PGK-1A.

Table 3.12 LYMPH NODE/

% PGK-1A : LYMPH NODE

strain	mean	n	SD	age
(AT29 x CBA)	68	3	8.74	)24d
(CBA x AT29)	55	3	4.16	)24d
(CBA x CBA-Pgk-1a)	69	7	9.80	)24d
(AT29-ENU.556 x CBA) PII	64	4	11.89	)24d
	21	3	8.08	15-24d
(AT29-ENU.556 x CBA) PI	13	26	10.38	)24d
(CBA x AT29-ENU.556) PI	33	2	1.41	)24d
(AT29 x CBA/N)	62	3	4.04	)24d
(AT29-ENU.556 x CBA/N) PIV	77	12	7.76	)24d
(AT29-ENU.556 x CBA/N) PIII	28	1	/	15-24d
	23	4	9.93	)24d

Table 3.11

Table 3.12 LYMPH NODE : F1 COMPARISONS (>24 day mice) showing values of P and the mean %PGK-1A values

	(CBAx AT29-ENU.556) PI	~control	PII
(AT29-ENU.556 xCBA) PI	<0.05 13 v 33	<0.01 13 v 62	<0.01 13 v 64

	(AT29xCBA/N)	PIV
PIII	<0.01 23 v 62	<0.01 23 v 77

each cell contains values of P and the mean %PGK-1A values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values

Sorted material

With reference to Tables 3.39-3.47 at the end of Chapter 3 for >24 day mice, the mean %PGK-1A value for either B or T cells was obtained by pooling the %PGK-1A values of their subsets sorted by flow cytometry, as discussed above. There were obvious differences between the mean %PGK-1A values for lymph node cell subsets of ENU-mutant and control mice, without comparisons tests.

The mean value for B cells of PI mice was 3% PGK-1A which was lower than that of PII mice with 60% PGK-1A and also that of the AT29 control mice with 52% PGK-1A. Similarly, the mean value for T cells of PI mice was 2% PGK-1A which was lower than that of PII mice with 62% PGK-1A and than that of the AT29 control mice with 50% PGK-1A.
Similarly, the mean value for B cells of PIII mice was 32% PGK-1A which was lower than that of PIV mice with 92% PGK-1A. The mean value for T cells of PIII mice was 11%

PGK-1A which was lower than that of PIV mice with 72% PGK-1A and also lower than that of the AT29 control mice with 63% PGK-1A. The mean %PGK-1A value for T cells of PIV mice and for B and T cells of PII mice were higher than the values of the AT29 control mice, given above.

Bone marrow

Unsorted material

With reference to summary table 3.13 and comparisons table 3.14, there was no significant difference between the mean %PGK-1A values for mice of the reciprocal AT29 and AT29-ENU.556 crosses. Hence, the respective data were pooled for comparison with other crosses. The mean value for PI mice with 40% PGK-1A was significantly lower than that for PII mice with 68% PGK-1A and than the AT29 control mice with 57% PGK-1A.

The mean value for PIII mice with 40% PGK-1A was significantly lower than that for PIV mice with 70% PGK-1A and than that for the AT29 control mice with 57% PGK-1A.

Table 3.14 BONE MARROW/

% PGK-1A : BONE MARROW

strain	mean	n	SD	age
(AT29 x CBA)	59	3	6.11	)24d
(CBA x AT29)	54	2	8.48	)24d
(CBA x CBA-Pgk-1a)	66	7	13.18	)24d
(AT29-ENU.556 x CBA) PII	68	8	6.76	)24d
(AT29-ENU.556 x CBA) PI	35	3	7.09	15-24d
	40	24	11.39	)24d
(CBA x AT29-ENU.556) PI	39	2	2.83	)24d
(AT29 x CBA/N)	57	2	9.90	)24d
(AT29-ENU.556 x CBA/N) PIV	70	11	9.66	)24d
(AT29-ENU.556 x CBA/N) PIII	42	1	/	15-24d
	40	7	8.06	)24d

Table 3.13

Table 3.14 BONE MARROW : F1 COMPARISONS (>24 day mice) showing values of P and the the mean %PGK-1A values

	~control	PII
#PI	<0.01 40 v 57	<0.01 40 v 68

	(AT29xCBA/N)	PIV
PIII	<0.05 40 v 57	<0.01 40 v 70

each cell contains the values of P and the mean values
~=(AT29xCBA)/(CBAxAT29) control pooled mean %PGK-1A values
#=(AT29-ENU.556xCBA)/(CBAxAT29-ENU.556) PI pooled mean %PGK-1A values

Sorted material

With reference to Tables 3.48-3.58 at the end of Chapter 3 in >24 day mice, there were obvious differences without comparisons tests between the mean %PGK-1A values for sorted bone marrow cells of ENU-mutant and control mice. For B cells the mean value of PI mice was 10% PGK-1A which was lower than that of the AT29 control mice with a value of 47% PGK-1A and than that of PII mice with a value of 68% PGK-1A. For non-B ($B220^-$) cells, the mean value for PI mice was 18% PGK-1A which was lower than that for the AT29 control mice with 50% PGK-1A and than that for PII mice with 50% PGK-1A. However, for high scattering cells of PI mice the mean value was 52% PGK-1A, that of the AT29 control mice was 59% PGK-1A and that of PII mice was 50% PGK-1A.

The mean value for bone marrow B cells of PIII mice was 22% PGK-1A which was lower than that of the AT29 control

mice with 62% PGK-1A and than PIV mice with 70% PGK-1A. The mean value for non-B ($B220^-$) cells of PIII mice was 13% PGK-1A which was lower than that of the AT29 control mice with 56% PGK-1A and than PIV mice with 57% PGK-1A. However, for high scattering cells of PIII mice the mean value was 39% PGK-1A and that of the AT29 control mice was 52% PGK-1A and that of PIV mice was 73% PGK-1A. The mean %PGK-1A value for B cells of PII and PIV mice was higher than that for the AT29 control mice which is consistent with the data for B cells of other tissues.

The following table 3.15 summarises data presented in Section I. It gives the %PGK-1A modal range (mean $\pm$ standard deviation) and the mean %PGK-1A values for unsorted tissues of the mice of the nine crosses. Values underlined refer to tissues affected by the ENU mutation. It shows the variation of PGK-1A expression between tissues and between individual mice within a cross and the PGK-1A variation between mice of the nine crosses. (CBAxCBA-Pgk-1a) mice are included as non-AT29 controls to demonstrate variation of PGK-1A expression between normal PGK-1AB heterozygotes.

Table 3.15/

Table 3.15: summary table for Section I showing the %PGK-1A modal ranges and the mean %PGK-1A values for>24 day mice

	%PGK-1A						
	BRAIN	MUSCLE	BLOOD	SPLEEN	NODE	THYMUS	MARROW
(AT29xCBA)	59-79 69	51-79 61	62-66 64	57-63 60	59-77 68	57-69 63	53-65 59
(CBAxAT29)	48-62 55	39-53 46	45-59 52	48-60 54	51-59 55	50-60 55	46-62 54
PIa	50-76 63	0-28 14	13-39 26	19-35 27	3-23 13	2-25 13	29-51 40
PIb	58-70 64	2-24 13	37-57 47	37-51 44	32-34 33	0-24 9	36-42 39
PII	55-79 67	58-78 68	66-78 72	65-81 73	52-76 64	55-81 68	61-75 68
(AT29xCBA/N)	57-69 60	49-63 56	55-65 59	56-68 62	58-66 62	51-63 57	47-67 57
PIII	52-76 64	5-27 16	13-39 26	24-44 34	13-33 23	6-22 14	32-48 40
PIV	50-76 63	45-81 63	61-79 70	74-86 80	69-85 77	59-81 70	60-80 70
(CBAx CBA-Pgk-1a)	62-76 69	58-70 64	58-84 71	62-82 72	59-79 69	55-77 66	53-79 66

a=(AT29-ENU.556xCBA), b=(CBAxAT29-ENU.556)
tissues affected by the ENU mutation are underlined

Results of comparisons tests revealed no significant difference between the mean %PGK-1A values when the AT29 X chromosome was maternally-derived (CBAxAT29) than when the X chromosome was paternally-derived (AT29xCBA) for all tissues.

There was a significant difference between the mean %PGK-1A values for PI mice of the reciprocal crosses in which the mean %PGK-1A values were lower for blood, spleen

and lymph node of PIa mice. However, there was no significant difference between the mean %PGK-1A values for PI mice of the reciprocal crosses for brain, skeletal muscle, thymus and bone marrow. Hence, variation between PIa and PIb mice of the reciprocal crosses appeared to be tissue-specific.

Comparing tissues affected by the ENU-mutation, those with the lowest mean %PGK-1A values were skeletal muscle, lymph node and thymus of PIa, PIb and PIII mice.

SECTION II

Assessment Of The Effect Of The ENU Mutation With Age

The effect of the ENU mutation with age was assessed in various tissues and sorted cell populations by analysis of the X-chromosome inactivation patterns in mice derived from eight of the crosses discussed above, excluding mice of the CBA x CBA-Pgk-1a cross. The mean %PGK-1A values were plotted against age to produce the developmental profiles of the mice studied. Each point on the graph for whole tissues is a mean %PGK-1A value of mice of the same age. For sorted cell populations, individual %PGK-1A values were plotted against age. Profiles provide a crude indication of PGK-1 variation with age, complicated by individual variation. It should be noted that, for thymus and lymph node, the amount of material available for PGK-1 analysis decreases with age. Hence, as reflected in the data, priority was given to sorted material, rather than

to whole tissue extracts. Developmental profiles are presented at the end of Chapter 3 (Fig. 3.2-3.28).

With reference to the developmental profiles presented at the end of this chapter, the interpretation of the variation in the mean %PGK-1A values with age was difficult possibly due to variation between individuals within a cross. Hence, the effect of the ENU mutation with age was assessed in selected tissues of PI and PII mice by correlation analysis of PGK-1 expression with age, as discussed in Chapter 2.

For brain, skeletal muscle, blood and lymph node of PI and PII mice, the correlation coefficient, r is given which quantifies the degree of linear association between the mean %PGK-1A values and age. The correlation coefficient measures the direction and strength of a linear association. Positive values imply a direct association and negative values imply an indirect association. The value of the correlation coefficient gives a measure of the strength of the linear association. A correlation coefficient of +1 or -1 implies a perfect linear association and the closer r approaches +1 or -1, the stronger is the association between the mean %PGK-1A values and age. A correlation coefficient of 0 implies no linear association and when r approaches 0 the weaker is the association between the mean %PGK-1A values and age.

Non Haematopoietic Tissues

Brain

The mean %PGK-1A values for brain of all crosses was variable. Peaks and troughs of each profile were probably attributable to variation between individuals within a cross and not to differences in age as they occurred randomly, at different time points. Despite the variation in the mean %PGK-1A values, the level of expression of PGK-1A for PI and PIII mice was similar to that for PII, PIV and the AT29 control mice (Fig. 3.2 and 3.3).

The correlation between mean %PGK-1A values and age was assessed in PI and PII mice of age 0-100 weeks. For PII mice r=-0.233 with n=7 and for PI females r=0.051 with n=24. Although there was a difference in the correlation coefficients of PI and PII mice which may be attributable to the small sample size for PII mice, the correlation coefficients approached 0 which implies that the association between the mean %PGK-1A values and age for brain of PI and PII mice was relatively weak (see below). This suggests that the mean %PGK-1A values did not change with age, as suggested above (Fig 3.2).

Skeletal muscle

There was a decline in the mean %PGK-1A values with age for PI and PIII mice. The greatest decrease in these values appeared to occur during the first 12 weeks of age,

after which there appeared to be a relatively gradual decline in the mean %PGK-1A values. The variation in PGK-1A expression for PII, PIV and the AT29 control mice did not appear to alter with age and probably reflected individual variation (Fig. 3.4 and 3.5).

The correlation between the mean %PGK-1A values and age was assessed in PI and PII mice of age 0-100 weeks and also in PI mice of age 0-11 weeks and 14-100 weeks to assess whether the greatest decrease in the mean %PGK-1A values occurred during the first 12 weeks of age, as described above. For PII mice of age 0-100 weeks $r=0.032$ and for PI mice of age 0-100 weeks $r=-0.264$. For PI mice of age 0-11 weeks $r=-0.694$ and for PI mice of age 14 100 weeks $r=0.100$.

Comparing the correlation coefficients for skeletal muscle of PI and PII mice of age 0-100 weeks revealed a relatively strong indirect association between the mean %PGK-1A values and age for skeletal muscle of PI mice. There was also a relatively strong indirect association between the mean %PGK-1A values and age for skeletal muscle compared with brain of PI mice of the same age group. Hence, the results suggest that for skeletal muscle of PI mice the mean %PGK-1A values declined with age.

Comparison of the correlation coefficients for the two age groups for skeletal muscle of PI mice suggests a

stronger indirect association between the mean %PGK-1A values and age for mice of age 0-11 weeks than for mice of the 14-100 week age group. This suggests that the decline in the mean %PGK-1A values was greater during the period 0-11 weeks than during the 14-100 week period which relates to the information derived from the developmental profile, as discussed above (Fig 3.4).

Haematopoietic tissues

Blood

The pattern of PGK-1A expression with age for blood (Fig. 3.6 and 3.7) of PI and PIII mice was similar to that for skeletal muscle (Fig. 3.4 and 3.5) where the greatest decrease in the mean %PGK-1A values appeared to occur during the first 12 weeks of age, followed by a relatively gradual decrease with age. The variation in PGK-1A expression for PII, PIV and the AT29 control mice did not appear to alter with age and was probably due to individual variation.

The correlation between the mean %PGK-1A values and age was assessed in PI and PII mice of age 0-100 weeks and also in PI mice of age 0-11 weeks and 14-100 weeks to assess whether the decrease in the mean %PGK-1A values was greater during the first 12 weeks as described above. For PII mice of age 0-100 weeks $r=-0.384$ and for PI mice of age 0-100 weeks $r=-0.668$. For PI mice of age 0-11 weeks $r=-0.571$ and for PI mice of age 14-100 weeks $r=-0.574$.

Comparing the correlation coefficients for blood of PI and PII mice of age 0-100 weeks revealed that the decline in the mean %PGK-1A values with age for PI mice was relatively greater than that for PII mice. In fact, this decline in the mean %PGK-1A values with age for blood of PI mice was rather striking when compared to brain of PI mice of the same age group.

Comparing the correlation coefficients for blood of PI mice of age 0-11 weeks with PI mice of age 14-100 weeks showed that there was little difference in the degree of the decline in the mean %PGK-1A values with age. This differed from skeletal muscle in which there was a greater decline in the mean %PGK-1A values during the 0-11 week period.

Lymph node

Unsorted material

The profile for PI mice demonstrated a decline in the mean %PGK-1A values with age and that for PIII mice gave an inconclusive result. The variation in the mean %PGK-1A values for PII, PIV and AT29 control mice probably reflected individual variation. Note that lymph node was only collected in sufficient quantities for PGK-1 analysis from mice of age >15 days that is 2.57 weeks on the profiles (Fig 3.18 and 3.19).

The correlation between the mean %PGK-1A values and age

was assessed in lymph node of PI mice of age 2.57-100 weeks and also of age 2.57-11 weeks and age 14-100 weeks to assess whether the level of PGK-1A expression altered with age. There was no correlation analysis made for lymph node of PII mice as the sample number was low (n=4). For the 2.57-100 week period r=-0.403 and for the 2.57-11 week period r=0.059 and for the 14-100 week period r=-0.403. The results demonstrate a relatively strong indirect association between the mean %PGK-1A values and age for lymph node of PI mice compared to brain of PI mice of age 0-100 weeks, implying that the mean %PGK-1A values declined over the 100 week period.

Comparing the correlation coefficients of the period up to 11 weeks with that from 14-100 weeks suggests that the decline in the mean %PGK-1A values was more pronounced after 14 weeks of age. This differs from the correlation analysis for skeletal muscle which implied that the decline in the mean %PGK-1A values was more pronounced during the 0-11 week period.

Sorted material

The profile for lymph node B and T cells of PI mice demonstrated a decline in the %PGK-1A values during the 100 week period. For B and T cells there appeared to be a marked decline in the level of PGK-1A expression at around 12 weeks of age (Fig. 3.20 and 3.21).

For lymph node T cells of PIII mice the %PGK-1A values

appeared to remain at a constant level from age 12 weeks (Fig. 3.23 and 3.24). The profile for the B cell populations of PIII mice was more difficult to interpret (Fig. 3.22). The profile for $B220^{+}$ cells appeared to decrease at about 12 weeks of age and increased by 60 weeks of age. This result is consistent with the profile for splenic and bone marrow B cells (Fig. 3.12 and Fig 3.28). However, it appeared that the %PGK-1A values of other lymph node B cell populations of PIII mice were at a constant level from 12-60 weeks old.

Spleen

Unsorted material

The mean %PGK-1A values for PI mice appeared to decrease gradually with age. The profile for PIII mice demonstrated this pattern up to 12 weeks of age, after which there was an increase in the mean %PGK-1A values with age. The profiles for PII, PIV and the AT29 control mice did not demonstrate a decline in the mean %PGK-1A values with age but the profiles were variable which was probably due to individual variation (Fig. 3.8 and 3.9).

Sorted material

The profiles for splenic B and T cell populations of PI mice demonstrated a decline in the %PGK-1A values with age, particularly during the first 3 weeks and around 12 weeks of age (Fig. 3.10 and 3.11).

For splenic T cells of PIII mice there was unbalanced expression of PGK-1 (Fig. 3.13). However, the level of PGK-1A expression for splenic T cells of PI mice was generally higher than that for thymocytes (Fig. 3.17) and, unlike thymus but similar to lymph node (Fig. 3.23 and 3.24), appeared to have a constant level of PGK-1A expression with age, following a decrease during the first 3 weeks.

For splenic B cells of PIII mice there was an increase in the %PGK-1A values from 12-60 weeks of age (Fig 3.12). This is consistent with the profile for B cells of bone marrow (Fig. 3.28) and the profile for some B cells of lymph node of PIII mice (Fig. 3.22).

Thymus

Unsorted material

There appeared to be a relatively steep decline in the mean %PGK-1A values during the first 12 weeks followed by a relatively gradual decline after 12 weeks of age for PI and PIII mice but not for PII, PIV or the AT29 control mice where variation in PGK-1A expression appeared to reflect individual variation and not variation with age (Fig. 3.14 and 3.15).

Sorted material

The pattern for thymocyte populations of PI and PIII mice reflected the pattern seen for unsorted thymus, where

there was a relatively steep decrease in the %PGK-1A values during the first 12 weeks of age and a relatively gradual decrease after 12 weeks of age. There did not appear to be a difference in the level of PGK-1A expression between thymocyte subsets. All thymocytes of ENU-mutant crosses eventually expressed very low %PGK-1A values (Fig. 3.16 and 3.17).

Neonatal liver and Bone marrow

Unsorted material

Fig. 3.25 and 3.26 shows the developmental profile for both neonatal liver in PI and PIII mice up to 3 days old and for bone marrow in all mice after 15 days old as it was not possible to collect bone marrow material in sufficient quantities for PGK-1 analysis from mice younger than 15 days old. The mean %PGK-1A values for bone marrow of PI and PIII mice were lower than those for PII, PIV and the AT29 control mice and there appeared to be a slight decline in the level of PGK-1A expression with age.

Sorted material

Fig. 3.27 and 3.28 shows the developmental profile for neonatal liver lymphocytes from mice up to 3 days old and for bone marrow lymphocytes from mice of >15 days old. The profiles for the sorted cell populations for neonatal liver and bone marrow appeared to be consistent with the result for unsorted bone marrow. Taking the %PGK-1A values

for both neonatal liver lymphocytes and bone marrow B and non-B ($B220^-$) lymphocytes of PI mice, there appeared to be a relatively steep decline in the profile during the first 12 weeks after which there was a relatively gradual decline up to 100 weeks old (Fig. 3.27).

Taking the %PGK-1A values for both neonatal liver lymphocytes and bone marrow B and non-B ($B220^-$) lymphocytes of PIII mice there appeared to be a relatively steep decline in the profile during the first 12 weeks after which the %PGK-1A values appeared to increase up to 60 weeks of age (Fig. 3.28). This profile is consistent with that observed for splenic B cells (Fig. 3.12) and some lymph node B cells (Fig.3.22) of PIII mice.

<u>Comparisons Of The Effect Of The ENU Mutation In Various Age Groups</u>

In the previous section the effect of the ENU mutation with age in all tissues of mice was assessed using the developmental profiles given at the end of this chapter and correlation analysis for brain, skeletal muscle, blood and lymph node of PI and PII mice. This section examines these results more closely by making statistical comparisons of the effect of the ENU mutation in selected tissues from mice of post-natal age: <15 day, 15-24 day and >24 day. The level of PGK-1A expression was determined in all whole tissues except brain and in sorted cell populations of PI mice and also in blood, spleen, lymph

node and bone marrow and in sorted B cells of PIII mice. Differences between the mean %PGK-1A values of the various age groups were recognised as significant by Student's t-test. Only statistical results that were significant are presented in Tables 3.16 and 3.17.

Skeletal muscle

There was a significant difference between the mean values for <15 day PI mice with 33% PGK-1A and 15-24 day PI mice with 19% PGK-1A. Hence, only <15 day PI mice were compared with >24 day PI mice (Table 3.3 and 3.16).

In the >24 day group there was bimodal distribution of %PGK-1A values. Hence, the group was divided into PI mice with <15% PGK-1A which had a mean value of 1% PGK-1A and >15% PGK-1A which had a mean value of 26% PGK-1A and each sample compared separately with <15 day PI mice with a mean value of 33% PGK-1A. There was a significant difference between the mean value for <15 day PI mice and the mean value for each group of the >24 day PI mice, given above (Table 3.16).

Haematopoietic Tissues

Blood

Unsorted material

There was a significant difference between the mean values for <15 day PI mice with 55% PGK-1A and 15-24 day PI mice

with 42% PGK-1A. Similarly, there was a significant difference between the mean values for <15 day PIII mice with 59% PGK-1A and 15-24 day PIII mice with 44% PGK-1A. Hence, only <15 day PIII mice were compared with >24 day PIII mice. Both PI and PIII >24 day mice had mean values of 26% PGK-1A which were significantly lower than the mean values for PI and PIII <15 day mice, given above (Table 3.5 and 3.16).

Spleen

Unsorted material

There was no significant difference between the mean values for <15 day PI mice with 41% PGK-1A and 15-24 day PI mice with 30% PGK-1A. Similarly, there was no significant difference between the mean values for <15 day PIII mice with 52% PGK-1A and 15-24 day PIII mice with 45% PGK-1A. Hence, values of all <24 day PI or PIII mice were pooled and compared with >24 day PI or PIII mice. The mean values for PI and PIII >24 day mice of 27% and 34% PGK-1A, respectively were significantly lower than those for PI and PIII <24 day mice of 36% and 51% PGK-1A, respectively (Table 3.16, 3.21, 3.22).

Sorted material

For splenic high scattering cells of <24 day PI mice, there was a mean value of 46% PGK-1A compared to >24 day PI mice with a single value of 43% PGK-1A. There were

significant differences between the mean values for <24 day PI mice and >24 day PI mice for all splenic B and T cells where <24 day PI mice had the higher mean %PGK-1A values (Table 3.17 and 3.21).

For splenic B cells of PIII mice, there was a significant difference between the mean % PGK-1A values for <24 day and >24 day with the latter group having the higher value (Table 3.17 and 3.22).

Bone marrow

Unsorted material

It was not possible to successfully obtain marrow from <15 day mice in sufficient quantities for PGK-1 analysis therefore, 15-24 mice were compared with >24 day mice. There was no significant difference between the mean values for bone marrow of 15-24 day PI mice with 35% PGK-1A and >24 day PI mice with 40% PGK-1A. For bone marrow of PIII mice, there was a single value of 42% PGK-1A for 15-24 day mice compared with the mean value of 40% PGK-1A for >24 day mice (Table 3.48 and 3.50).

Sorted material

For high scattering cells of bone marrow of PI mice there was a single value of 62% PGK-1A for 15-24 day mice compared with the mean value of 44% PGK-1A for >24 day mice. For B cells of bone marrow of PI mice there was a single value for 15-24 day mice of 41% PGK-1A compared

with the mean value for >24 day mice of 10% PGK-1A. For non-B ($B200^-$) cells of bone marrow of PI mice there was a single value for 15-24 day mice of 48% PGK-1A compared with the mean value of 18% PGK-1A for >24 day mice (Table 3.48).

Lymph node

Unsorted material

It was not possible to successfully obtain lymph node material from <15 day mice in sufficient quantities for PGK-1 analysis therefore, 15-24 day mice were compared with >24 day mice. For PI mice, there was no significant difference between the mean values of 15-24 day mice with 21% PGK-1A and >24 day mice with 13% PGK-1A. For lymph node of PIII mice, there was a single value for 15-24 day mice of 28% PGK-1A compared with the mean value for >24 day mice of 23% PGK-1A (Table 3.39, 3.40).

Sorted material

The mean %PGK-1A value for lymph node T cells of PI mice aged 15-24 day was significantly higher than the mean %PGK-1A value for >24 day mice (Table 3.17 and 3.39).

Thymus

Unsorted material

For thymus there was no significant difference in the mean

values between <15 day PI mice with 29% PGK-1A and 15-24 day PI mice with 18% PGK-1A. Hence, all mean %PGK-1A values for <24 day PI mice were pooled to give a mean value of 23% PGK-1A and compared with >24 day PI mice. The mean value for <24 day PI mice was 23% PGK-1A which was significantly greater than the mean value for >24 day PI mice with 13% PGK-1A (Table 3.16 and 3.30).

Sorted material

The mean %PGK-1A value for thymocytes of <24 day PI mice was significantly greater than the mean %PGK-1A value of >24 day PI mice (Table 3.17 and 3.30).

Comparisons Of The Effect Of The ENU Mutation Between Neonatal Liver Lymphocytes and B and T Cells

The level of PGK-1A expression for 1-3 day neonatal liver lymphocytes (NLL) was compared with that for B and T cells of PI mice and B cells of PIII mice of age <24 day and >24 day to identify the stage at which the ENU mutation takes effect. Differences in the mean %PGK-1A values between NLL and B and T cells were recognised as significant by Student's t-test. Only statistical results that were significant are presented in Table 3.18.

Sorted material

Neonatal liver compared with spleen

Refer to table 3.18, 3.21 and 3.49. There was a

significant difference between the mean values for NLL with 44% PGK-1A and splenic T cells of <24 day PI mice with 33% PGK-1A and also >24 day PI mice with 2% PGK-1A.

There was a significant difference between the mean values for NLL with 44% PGK-1A and splenic B cells of >24 day PI mice with 2% PGK-1A but not between NLL and splenic B cells of <24 day PI mice with 35% PGK-1A.

There was no significant difference between the mean values for neonatal liver high scattering cells with 54% PGK-1A and splenic high scattering cells of <24 day PI mice with 46% PGK-1A and for splenic high scattering cells of >24 day PI mice there was a single value of 43% PGK-1A.

There was not a significant difference between the mean values for NLL with 44% PGK-1A and splenic B cells of <24 day PIII mice with 25% PGK-1A and also splenic B cells of >24 day PIII mice with 50% PGK-1A (Table 3.22 and 3.51).

Neonatal liver compared with bone marrow

Refer to table 3.18, 3.48 and 3.49. There was a significant difference between the mean values for NLL with 44% PGK-1A and bone marrow B cells of >24 day PI mice with 10% PGK-1A. For bone marrow B cells of <24 day PI mice there was a single value of 41% PGK-1A.

There was not a significant difference between the mean values for neonatal liver high scattering cells with 54%

PGK-1A and bone marrow high scattering cells of >24 day PI mice with 44% PGK-1A. For bone marrow high scattering cells of <24 day PI mice there was a single value of 62% PGK-1A (Table 3.18).

For PIII mice, there was a significant difference between the mean values for NLL with 44% PGK-1A and bone marrow B cells of >24 day mice with 22% PGK-1A (Table 3.18, 3.50, 3.51).

Neonatal liver compared with lymph node

The mean value of NLL with 44% PGK-1A was significantly higher than that for lymph node B cells of >24 day PI mice with 3% PGK-1A and than that for lymph node T cells of <24 day PI mice with 16% PGK-1A and also than that for lymph node T cells of >24 day PI mice with 2% PGK-1A (Table 3.18, 3.39, 3.49).

There was not a significant difference between the mean values for NLL with 44% PGK-1A and lymph node B cells of >24 day PIII mice with 32% PGK-1A (Table 3.40 and 3.51).

Neonatal liver compared with thymus

The mean value of NLL with 44% PGK-1A was significantly higher than that for thymocytes of <24 day PI mice with 33% PGK-1A and than that for thymocytes of >24 day PI mice with 4% PGK-1A (Table 3.18, 3.30, 3.49).

TABLE 3.16: The Effect Of The ENU Mutation With Age For Unsorted Tissues

	AGE COMPARISONS		
	<15day v 15-24day	<15day v >24day	<24day v >24day
PI MUSCLE	*(33 v 19)	<15%PGK-1A >15%PGK-1A ** (33 v 1)	* (33 v 26)	/
PI BLOOD	*(55 v 42)	** (55 v 26)	
PIII	*(59 v 44)	** (59 v 26)	/
PI SPLEEN	/	/	* (35 v 27)
PIII			** (51 v 34)
PI THYMUS	/	/	* (23 v 13)

The table gives for unsorted tissue the significance level *=P<0.05, **=P<0.01 and the mean %PGK-1A values for younger v older PI and PIII mice

Note there is bimodal distribution of %PGK-1A values for muscle of >24 day PI mice

TABLE 3.17: The Effect Of The ENU Mutation With Age For Sorted Cell Populations

			AGE COMPARISONS OF MICE PI		PIII
SPLEEN	T cells	B-	** (35 v 4)		/
		T+	** (27 v 0)		/
	B cells	B+	** (36 v 2)	B+	* (24 v 50)
		T-	** (35 v 2)		/
	IC	T+	** (33 v 2)		/
THYMUS	M	T+	* (26 v 3)		/
	all thymocytes	mean#	** (33 v 2)		/
L.NODE	T cells	mean#	** (16 v 1)		/

The table gives for sorted cells the significance level *=P<0.05, **=P<0.01 and the mean %PGK-1A values for younger v older PI and PIII mice

note the age comparisons for spleen, thymus is <24 day v >24 day and l.node is 15-24 day v >24 day

key to cell types (see tables 3.21,3.22,3.30,3.39)

staining for antigen: for B cells ($B220^+$) and ($Thy1.2^-$); for T cells ($Thy1.2^+$) and ($B220^-$);

mean# refers to pooled mean %PGK-1A values: for lymph node T cells or thymocytes ($Thy1.2^+$),($B220^-$),($L3T4^{+or-}$), ($Lyt2^{+or-}$)

For thymocytes: IC=inner cortex, M=medulla

TABLE 3.18: Comparison Of The Effect Of The ENU Mutation In Neonatal Liver Lymphocytes With B and T Cells

COMPARISONS BETWEEN NLL AND B,T CELLS

		NLL v 15-24 or <24day	NLL v >24day
PI SPLEEN	B cells	/	** (44 v 2)
	T cells	* (44 v 33)	** (44 v 2)
PI THYMUS	thymocytes	* (44 v 33)	** (44 v 4)
PI MARROW	B cells	/	** (44 v 10)
PIII	B cells	/	* (44 v 22)
PI L.NODE	B cells	/	** (44 v 3)
	T cells	** (44 v 16)	* (44 v 2)

The table gives the significance level *=P<0.05, **=P<0.01 and the mean %PGK-1A values for NLL v B and T cells. For B, T cells, mean %PGK-1A values are pooled values for cells defined below.

note the age comparisons are NLL v 15-24 day lymph node and NLL v <24 day spleen, thymus; and NLL v >24 day all tissues above

a significant result for PIII mice was only obtained for comparisons between NLL and B cells of bone marrow of >24 day mice

key to cell types for pooled mean %PGK-1A values, staining for antigen:

Spleen: B cells=$B220^+$, $Thy1.2^-$; T cells= $Thy1.2^+$, $B220^-$, $L3T4^+$ $Lyt2^+$, $Ly1^+$ (table 3.22)

thymocytes=$Thy1.2^+$, $L3T4^{+or-}$, $Lyt2^{+or-}$, inner, outer cortex, medulla (table 3.30)

marrow: B cells=$B220^+$ (table 3.48, 3.50)

l.node: B cells=$B220^+$, $Thy1.2^-$; T cells=$Thy1.2^+$, $B220^-$, $L3T4^+$ $Lyt2^+$ (table 3.39)

NLL=neonatal liver lymphocytes from 1-3 day PI or PIII mice, unstained for antigen (table 3.49, 3.51)

DISCUSSION

Studies of X-chromosome inactivation patterns in cells of AT29-ENU.556 mice that were heterozygous for the X-linked mutation ENU were used to characterise the phenotypic effect of the mutation by delineating the cell lineages affected by the mutation, the differential effect of the mutation in cell lineages and the effect of the mutation with age.

The presence of cells carrying the ENU mutation was detected by the expression of the alloenzyme PGK-1A such that unbalanced expression of PGK-1A in a cell lineage delineated the cells affected by the mutation.

X-chromosome inactivation patterns were studied in cell lineages of heterozygous female controls that were unaffected by the mutation as there is a wide variation in normal random patterns of X-chromosome inactivation (comparison tables 3.2, 3.4, 3.6, 3.8, 3.10, 3.12, 3.14) and X-chromosome inactivation patterns were also studied in brain tissue of AT29-ENU.556 heterozygous females which was unaffected by the mutation and was used as a control tissue (summary table 3.15).

As the patterns of PGK-1 expression change with age in AT29-ENU.556 heterozygous females where there are random patterns once inactivation has taken place, the success of cells or competition is favoured and not skewed inactivation as an explanation for the variations in PGK-1

expression.

cell lineages affected by the mutation in adult mice

Unbalanced expression of the X chromosome carrying the ENU mutation was seen for skeletal muscle and the haemato-poietic tissues, blood, spleen, thymus, lymph node and bone marrow (comparison tables and summary table 3.15) and for the cell subpopulations constituting these tissues namely, erythrocytes and B and T cells and possibly platelets (table 3.19, 3.20, 3.21, 3.39).

The cells of blood, spleen and bone marrow which were defined as high scattering and were distinguished from lymphocytes by their relatively high right angle scatter being structurally more complex, were not affected by the ENU mutation having balanced X-chromosome expression similar to that for brain in the same mice (summary table 3.15) and similar to that for the same cells in control mice (table 3.21, 3.48, 3.53, 3.56).

The differential effect of the ENU mutation in tissues and cell subpopulations was demonstrated. Skeletal muscle, thymus and lymph node were affected by the mutation to a greater extent than blood, spleen and bone marrow (summary table 3.15). This reflected the presence of cells in these tissues that were shown to be unaffected by the mutation such as high scattering cells in the spleen and bone marrow and also the presence of cells that were affected by the mutation to a lesser extent such as erythrocytes in

whole blood and whole spleen and bone marrow B cells, which were predominantly pre-B cells, which tended to shift the patterns of PGK-1 expression towards balanced ratios (table 3.21, 3.19, 3.48).

It was also demonstrated that the ENU mutation had differential effects on cell subpopulations within a cell lineage. For example, the patterns of PGK-1 expression for bone marrow B cells, which were predominantly pre-B cells, showed that the pre-B cells were affected by the mutation to a lesser extent than the circulating B cells of the spleen and lymph nodes. Unbalanced expression was also seen for bone marrow lymphocytes that stained negatively for B220 implying the mutation takes effect before the pre-B cell stage. These results suggest the mutation has subtle differential effects within the B cell lineage (Table 3.21, 3.39, 3.48).

The T cell subsets $L3T4^+$ helper, $Lyt2^+$ cytolytic, $Ly1^+$ and the thymocytes of the cortex and medulla were all similarly affected by the mutation (table 3.21, 3.30, 3.39).

<u>studies of mice heterozygous for the ENU and xid mutations</u>

Attempts to relate the ENU mutation to a candidate gene were made by assessing whether the ENU mutation was an allele of <u>xid</u> by studying the X-chromosome inactivation patterns in females heterozygous for ENU and <u>xid</u>.

Studies of X-chromosome inactivation patterns have revealed that the defect in xid is intrinsic to B cells and that T cells, pre-B cells, other haematopoietic cells and non-haematopoietic cells are unaffected by the xid mutation (Nahm et al, 1983; Conley et al, 1986; Forrester, 1986).

The extent of the mutation, relative to xid which affects mature B lymphocytes, was assessed by comparing (AT29-ENU.556 x CBA/N) PIII heterozygotes with (AT29 x CBA/N) and non-mutant (PIV) control heterozygotes. For PIII females, skeletal myocytes and unsorted haematopoietic tissues were affected by the mutation, to the same order as PI samples (summary table 3.15).

Examination of the constituent populations of the haematopoietic tissues revealed a more complex pattern of PGK-1 expression in PIII females. For splenic B cells there was balanced PGK-1 expression and for lymph node B cells there was unbalanced expression of PGK-1 in favour of cells carrying xid and, for B cells of bone marrow there was unbalanced expression of PGK-1 in favour of cells carrying xid (table 3.22, 3.40, 3.50).

If balanced X-chromosome expression depends on whether both mutations affect the same population of cells and the penetrance of each mutation, then tendency to balanced expression for splenic B cells reflects simultaneous effects and similar penetrance of both mutations in mature

B cells.

Discrete differences between lymph node and spleen in the degree of PGK-1 expression may reflect tissue differences in ratios of B and T cells and not differential effects of the mutations on B cells of these tissues because in PI females the ENU mutation had an equal effect on B lymphocytes of the spleen and lymph node. Unbalanced expression of PGK-1 in B cells of bone marrow was greater than that for B cells of the spleen and lymph node, which implies that the ENU mutation also acts in pre-B cells, unlike xid, as B cells of marrow are predominantly pre-B cells. These results are consistent with previous findings in +/xid heterozygotes which showed unbalanced PGK-1 expression in favour of normal B cells in the spleen and lymph node and slightly unbalanced PGK-1 expression in marrow as xid acts in circulating mature B cells and not in pre-B cells (Witkowski et al, 1985; Forrester et al, 1987).

For thymocytes, the level of PGK-1 expression was similar for PI and PIII females (table, 3.30, 3.31). However, in PIII mice, T cells of the lymph node and the spleen, in particular, demonstrated unbalanced PGK-1 expression but to a lesser degree than those of PI females (table 3.21, 3.22 and 3.39, 3.40). This is interesting since xid does not affect thymocytes and the degree of unbalanced PGK-1 expression was similar for PIII and PI females (see also Forrester, 1986). This suggests that ENU-mutant T cells

compete less well with normal T cells than they do with xid-bearing T cells which might suggest that xid-bearing T cells are subtly defective. These results may relate to previous findings which showed defective function of a T-cell helper subset in CBA/N mice (Bottomly and Mosier, 1979, Scher, 1982).

effect of the ENU mutation with age

All cells affected by the ENU mutation demonstrated variation in PGK-1 expression with age.

The effect of the ENU mutation with age was assessed using developmental profiles (Fig. 3.2-3.28) and correlation analysis which quantifies the degree of linear association between PGK-1A expression and age studied in skeletal muscle, blood and lymph node and compared with brain and control mice.

For skeletal muscle, blood and lymph node of PI mice, the effect of the mutation increased with age, particularly between 0-11 weeks for skeletal muscle and between 14-100 weeks for lymph node. There was little difference in the degree of the decline in PGK-1A expression in comparisons between the 0-11 week period and the 14-100 week period for blood.

T cells and thymocytes

There was a significant difference in the mean %PGK-1A values between neonatal liver lymphocytes and T cells of

the spleen, lymph node and thymocytes of PI mice (table 3.18). There was a significant difference in the mean %PGK-1A values between T cells of pre-adult and adult PI mice for spleen, lymph node and thymocytes (table 3.17).

Developmental profiles (Fig. 3.2-3.28) showed that the level of PGK-1A expression for thymocytes of PI and PIII mice and for T cells of the spleen and lymph node of PI mice decreased most noticeably during the first 3 weeks and after 12 weeks of age. For PIII mice the level of PGK-1A expression of T cells in the spleen and lymph node remained relatively constant from 12 weeks of age, after decreasing during the first 3 weeks.

Results show that the effects of the mutation in T cells were apparent in pre-adult mice and became more pronounced with age, except for lymph node and splenic T cells of PIII mice which supports the idea that balanced PGK-1 levels after 12 weeks of age was attributable to the presence of xid- and ENU-defective B cells in these tissues.

B cells

There was a significant difference in the mean %PGK-1A values between neonatal liver lymphocytes and B cells of the spleen, marrow and lymph node of PI adult mice and of the bone marrow of PIII adult mice (table 3.18)

There was a significant difference in the mean %PGK-1A

values between B cells of pre-adult and adult mice for the spleen of PI and PIII mice (table 3.17).

Developmental profiles showed that the level of PGK-1A expression for B cells of the spleen and lymph node in PI mice decreased most noticeably during the first 3 weeks and after 12 weeks of age. For PIII mice, the level of PGK-1A expression for B cells of the lymph node and the spleen, in particular, increased most noticeably after 12 weeks of age. The level of PGK-1A expression for marrow pre-B cells of PI and PIII mice decreased most noticeably during the first 3 weeks of age, after which levels were constant.

The results demonstrate a significant difference in the effect of the mutation between neonatal liver lymphocytes and B and T cells of PI mice. In PIII mice, there was a shift towards balanced PGK-1 expression after 12 weeks of age which suggests that the effects of xid became more pronounced during this period. This is consistent with previous findings in which xid B cells of adult mice are at a greater competitive disadvantage than those of mice of age 2-6 weeks (Forrester et al, 1987).

Balanced levels of PGK-1 expression depend on the X-chromosome inactivation patterns in the tissue progenitors (McLaren, 1972; West, 1975; Fialkow, 1973; Pappaioannou et al, 1981; McMahon et al, 1983) and on the nature of the X-chromosome controlling element (Xce). Allelic variants of

this locus and the parental source of the allele influence X inactivation such that an X chromosome carrying the $\underline{Xce}^{a}$ allele has a greater chance of being inactivated than an X chromosome carrying the $\underline{Xce}^{b}$ which, in turn, is more likely to be inactivated than an X chromosome carrying the $\underline{Xce}^{b}$ allele (Cattanach, 1972; Johnston and Cattanach, 1981). Moreover, in females heterozygous for Xce (b/c), a maternally-derived X chromosome bearing the $\underline{Xce}^{c}$ allele is less likely to be inactivated than a paternally-derived X chromosome carrying the same allele (Forrester and Ansell, 1985). Further evidence for parental effects on X chromosome expression and the possible existence of a fourth allele for AKR/J mice have been demonstrated following studies of their hybrids (Fowlis, 1988; Fowlis et al, 1991). In this study variation of PGK-1 expression may depend on parental factors where this effect may be specific to certain tissues and on the nature of the Xce allele which is discussed in Chapter 5.

Variation in PGK-1 expression between tissues within an individual may be due to the constituent cells arising from different progenitor pools such as haematopoietic cells compared with brain or skeletal muscle or tissues seeded from the same progenitor pool where sampling events concerning seeding of a tissue involved few cells, discussed in Chapter 5.

Individual variation within a strain may be reflected by bimodal distribution of values of %PGK-1A, such that a

strain can be divided into separate sample groups (see Fig. 3.1 for skeletal muscle) where there is a reasonable sample number for each group. Therefore, subtle differential effects of the mutation may be difficult to recognise due to variation of PGK-1 mosaicism.

Studies of X-chromosome inactivation patterns using alloenzyme analysis have delineated distinct cell lineages in which the ENU mutation acts, namely, B and pre-B and T cells, erythrocytes and possibly platelets and skeletal myocytes and also its effect on these lineages with age. Variation of PGK-1 expression between affected lineages may reflect the differential effects of the mutation and variation of PGK-1 expression within a lineage may reflect differential effects of the mutation on cells at different stages of maturation.

Loci controlling the affected lineages have been mapped to the X chromosome. Although several loci that control muscle development are X-chromosome specific, it is rather interesting to find skeletal muscle affected by the same mutation as haematopoietic cells. Two murine X-chromosome loci can be eliminated as possibilities. These are the muscular mutation mdx, since its effect cannot be detected by unbalanced expression of PGK-1 in skeletal muscle (Chapman, 1990, personal communication) and the xid mutation as, unlike the ENU mutation, it does not act in pre-B cells.

DEVELOPMENTAL PROFILE
TISSUE : BRAIN

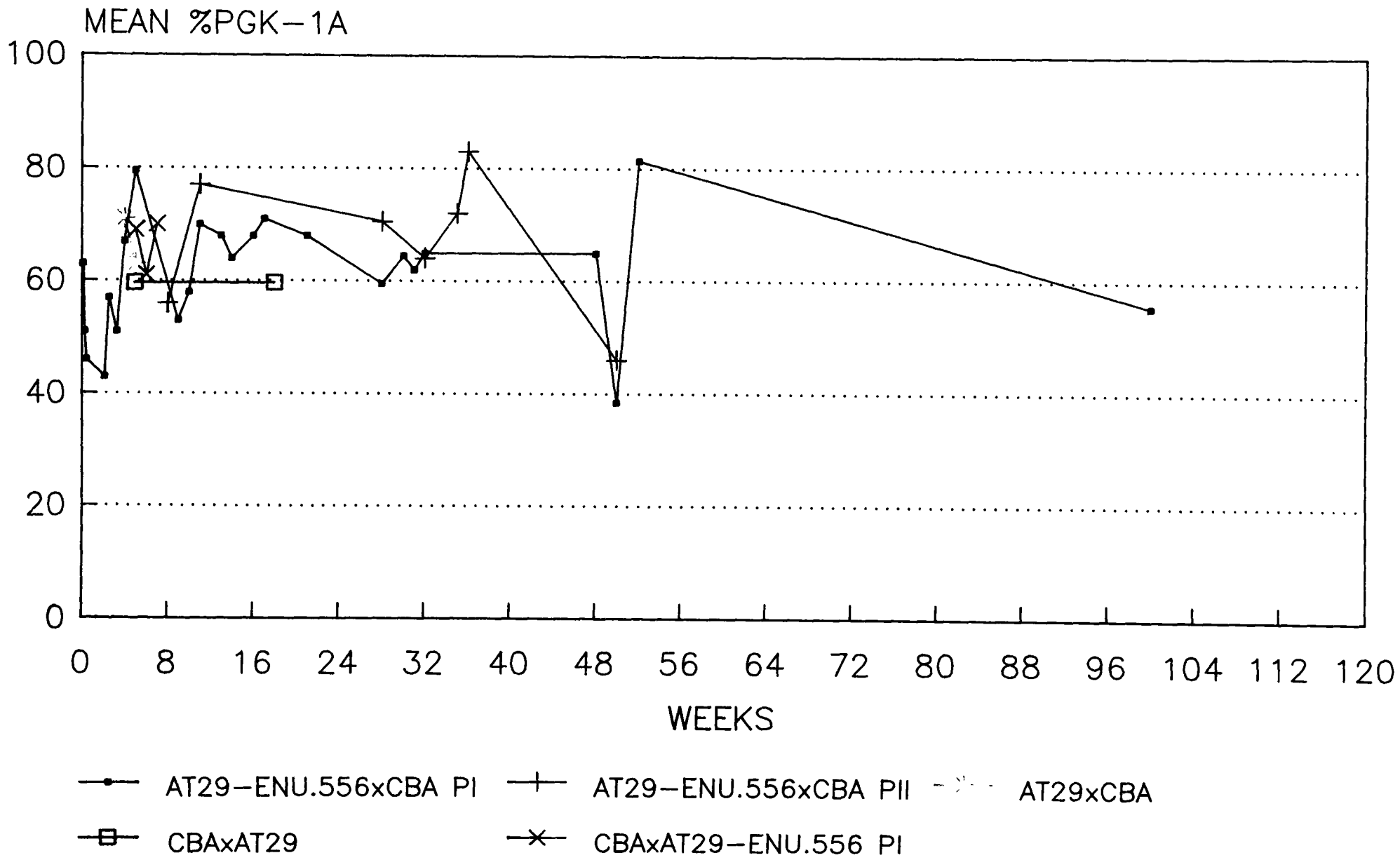


Fig. 3.2

DEVELOPMENTAL PROFILE
TISSUE : BRAIN

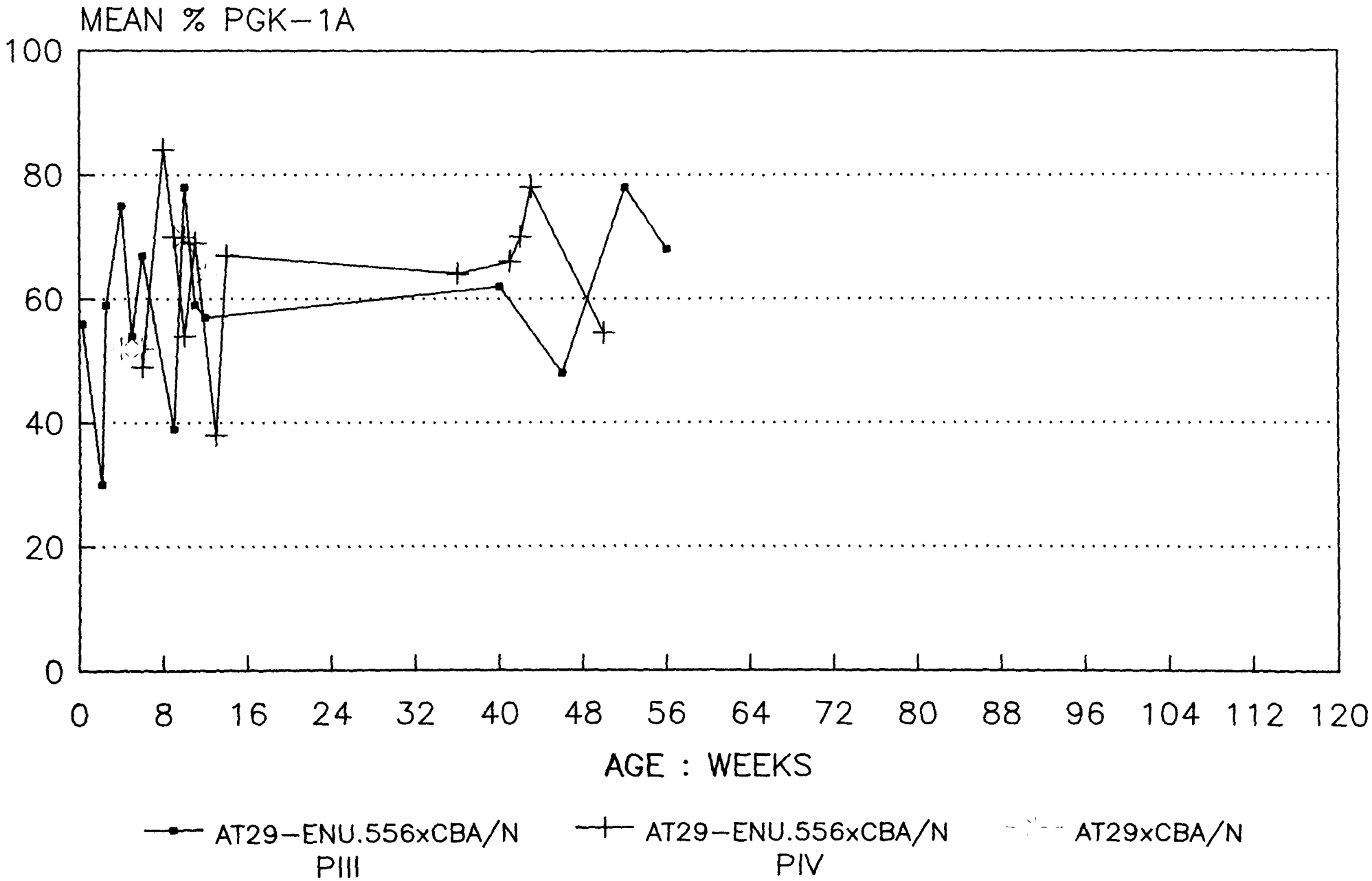


Fig. 3.3

DEVELOPMENTAL PROFILE
TISSUE : SKELETAL MUSCLE

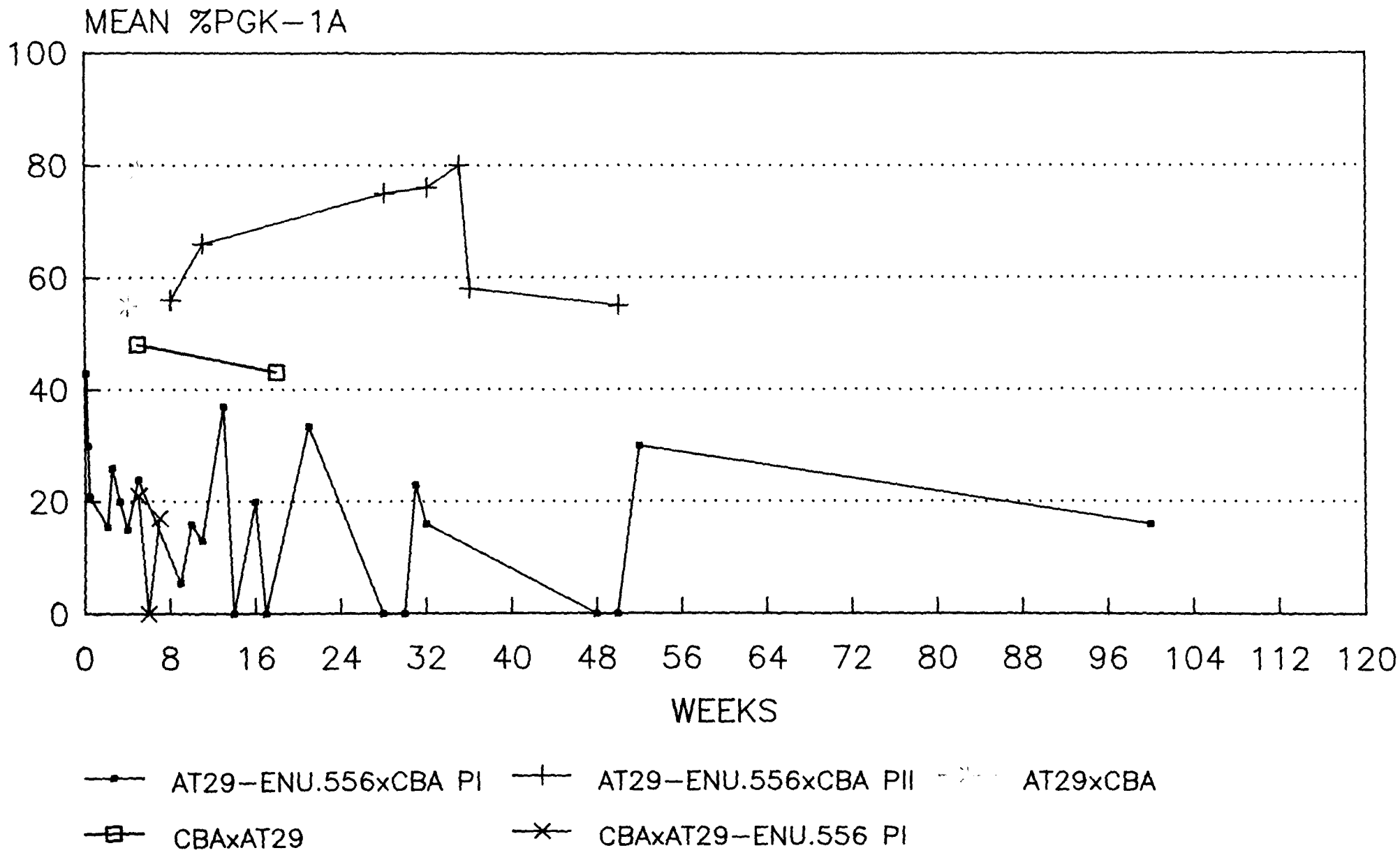


Fig. 3.4

DEVELOPMENTAL PROFILE
TISSUE : SKELETAL MUSCLE

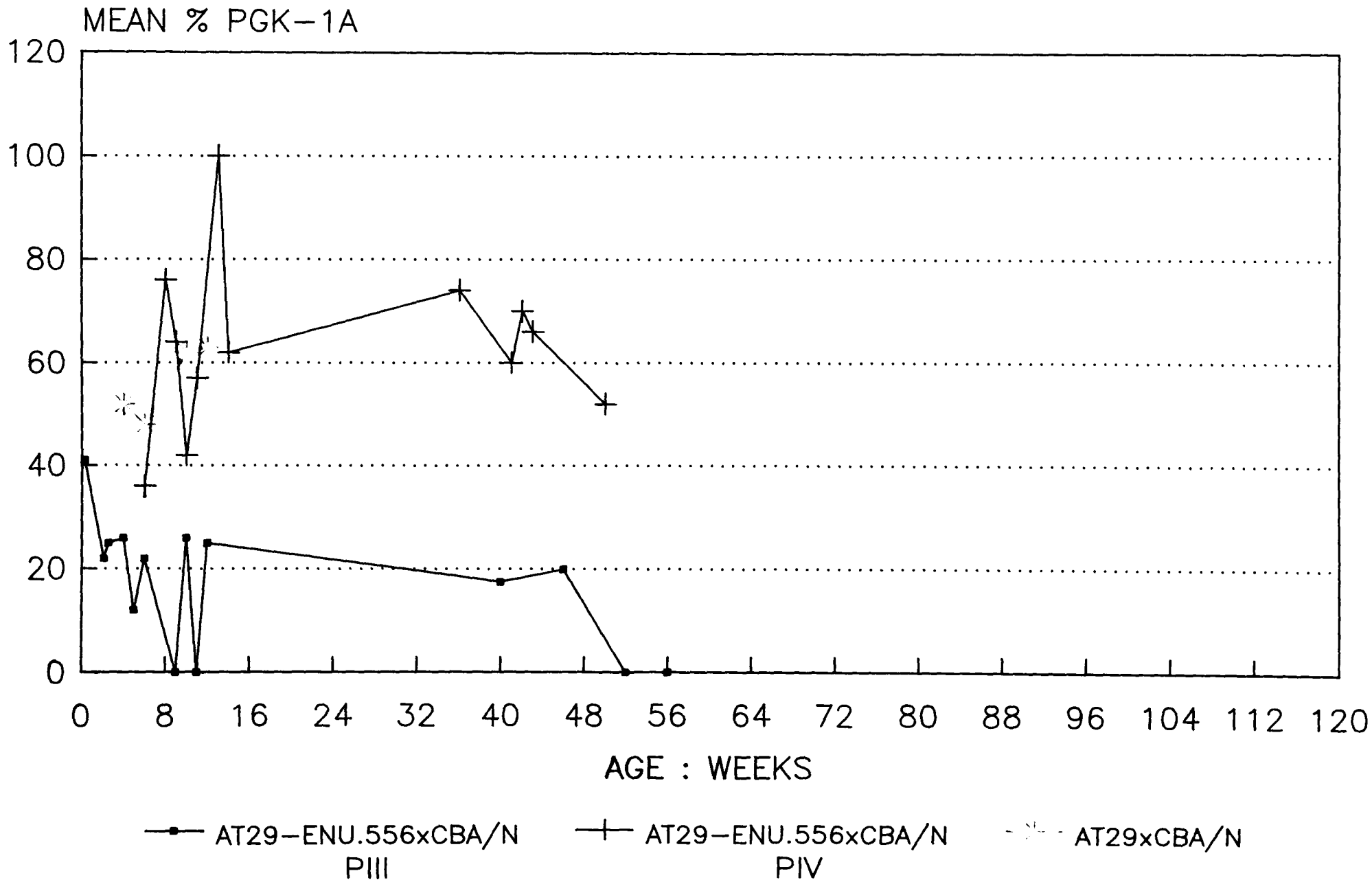


Fig. 3.5

%PGK-1A:BLOOD CELL POPULATIONS

cell type	mean	st deviation	n	age	
WHOLE	26	12.64	35	>24d	
WBC	17	14.19	11	>24d	PI
PLATELETS	33	10.73	12	>24d	
WHOLE	72	6.28	8	>24d	
WBC	74	/	1	>24d	PII
PLATELETS	70	4.00	3	>24d	
WHOLE	52	7.00	3	>24d	CBAxAT29
WBC	38	/	1	>24d	Table 3.19

%PGK-1A:BLOOD CELL POPULATIONS

cell type	mean	st deviation	n	age	
WHOLE	26	13.39	14	>24d	
WBC	20	3.54	2	>24d	PIII
PLATELETS	24	5.29	3	>24d	
WHOLE	70	9.34	14	>24d	
WBC	79	6.66	3	>24d	PIV
PLATELETS	79	7.94	6	>24d	
WHOLE	59	5.50	4	>24d	AT29xCBA/N
WBC	57	/	1	>24d	Table 3.20

Key to tables for blood:

WHOLE = unsorted blood
WBC = unsorted white blood cells

DEVELOPMENTAL PROFILE
TISSUE : WHOLE BLOOD

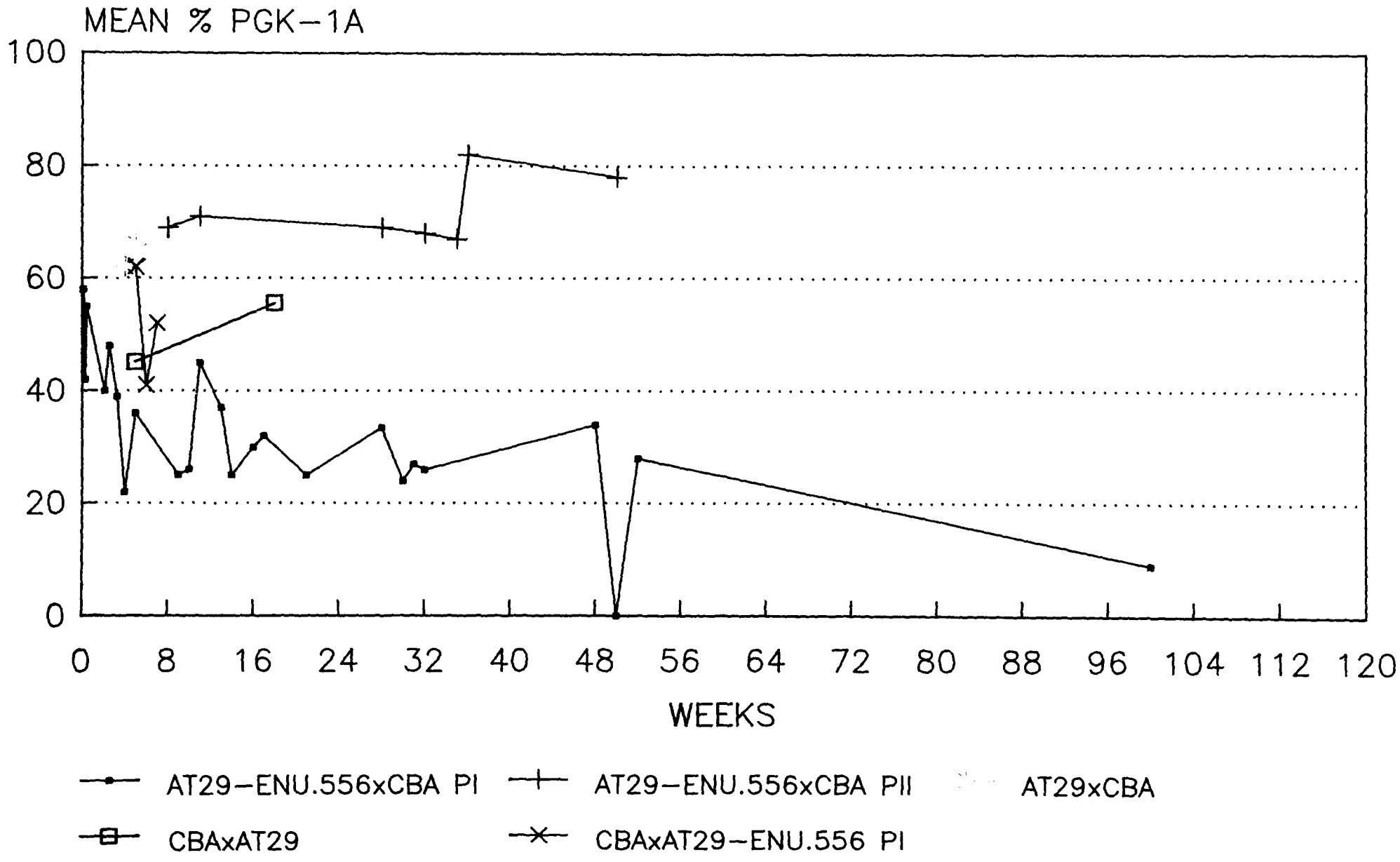


Fig. 3.6

DEVELOPMENTAL PROFILE
TISSUE : WHOLE BLOOD

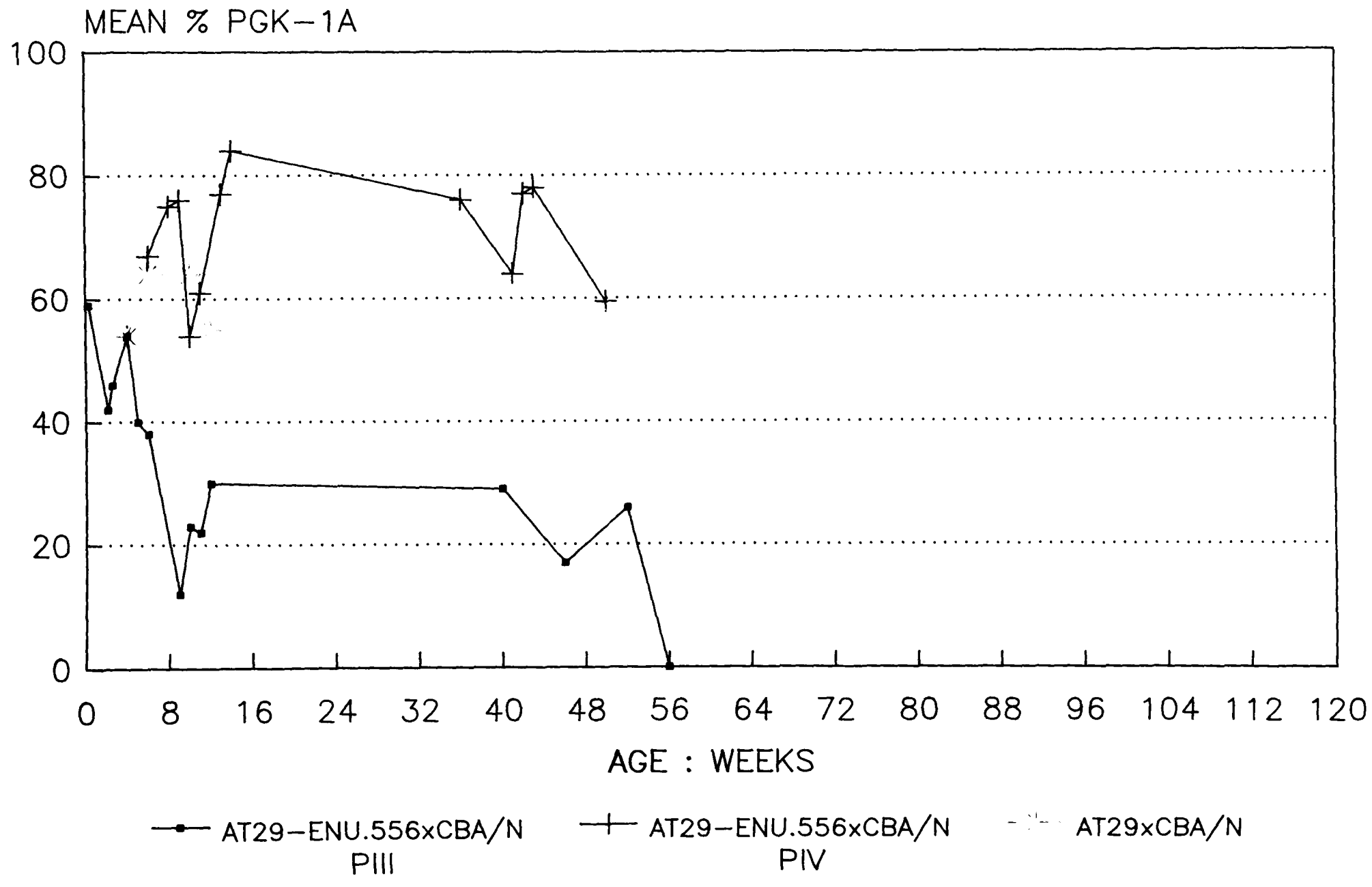


Fig. 3.7

%PGK-1A : (AT29-ENU.556 x CBA) PI

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	36	9.21	8	⟨24d
	27	8.11	27	⟩24d
WBC	24	7.08	16	⟩24d
B AND T				
(B/T)+	0	0.00	2	⟩24d
L4-	0	/	1	⟩24d
OTHER				
(B/T)-	11	19.63	3	⟩24d
high scatter	46	7.31	7	⟨24d
	43	/	1	⟩24d
T				
B-	35	7.62	5	⟨24d
	4	8.27	9	⟩24d
T+	0	0.00	7	⟩24d
L4+	0	/	1	⟩24d
T d	26	/	1	⟨24d
T b	27	/	1	⟨24d
B				
B+	36	10.65	6	⟨24d
	2	6.00	9	⟩24d
T-	35	7.07	2	⟨24d
	2	5.66	8	⟩24d

Table 3.21

Key to tables and figures for spleen:
WHOLE = unsorted spleen
WBC = unsorted white blood cells
HIGH SCATTER = cells with higher right angle scatter than lymphocytes
antibody staining: B = B220; T = Thy 1.2; L4 = L3T4; L2 = Lyt2;
d,b = dull or bright staining

%PGK-1A:(AT29-ENU.556 x CBA/N) PIII

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	51	6.48	5	(24d
	34	9.66	8	)24d
WBC	37	/	1	)24d
B AND T (B/T)+	35	/	1	)24d
L4-	43	9.90	2	)24d
L2-	34	/	1	)24d
L1-	41	/	1	)24d
OTHER high scatter	48	/	1	)24d
T B-	36	17.68	2	(24d
	20	10.76	7	)24d
T+	22	4.04	3	)24d
L4+	20	3.54	2	)24d
L2+	25	/	1	)24d
L1+	16	/	1	)24d
B B+	24	14.14	2	(24d
	50	8.39	3	)24d
T-	28	/	1	(24d
	50	6.08	3	)24d
B d	27	/	1	)24d
B b	53	18.38	2	)24d

Table 3.22

%PGK-1A : (AT29 x CBA)

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	60	3.42	4	)24d
T B-	38	/	1	)24d

Table 3.23

%PGK-1A : (CBA x AT29)

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	54	5.69	3	)24d
T B-	58	/	1	)24d
B B+	51	/	1	)24d

Table 3.24

%PGK-1A : (CBA x CBA-Pgk-1a)

SPLEEN: CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	72	10.39	7	)24d
T B-	87	/	1	)24d
T d	86	/	1	)24d
T b	84	/	1	)24d
B B+	85	/	1	)24d

Table 3.25

%PGK-1A : (CBA x AT29-ENU.556)PI

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	44	7.21	3	)24d
T B-	0	/	1	)24d
T+	0	/	1	)24d
B B+	0	0	2	)24d
T-	10	13.44	2	)24d

Table 3.26

%PGK-1A (AT29-ENU.55o x CBA) PII

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	73	3.12	7	)24d
WBC	74	3.10	4	)24d
B AND T				
(B/T)+	64	/	1	)24d
T				
B-	68	/	1	)24d
T-	70	/	1	)24d
B				
T-	68	/	1	)24d

Table 3.27

%PGK-1A (AT29 x CBA/N)

SPLEEN CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	62	6.24	4	)24d
T				
B-	65	/	1	)24d
B				
B+	81	/	1	)24d
T-	100	/	1	)24d
B d	65	/	1	)24d
B b	67	/	1	)24d

Table 3.28

%PGK-1A:(AT29-ENU.55o x CBA/N)PIV

SPLEEN : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	80	5.59	9	)24d
WBC	84	3.89	7	)24d
B AND T				
(B/T)+	86	11.73	6	)24d
OTHER				
(B/T)-	78	15.97	6	)24d
T				
B-	74	17.44	5	)24d
T+	70	7.85	4	)24d
B				
B+	91	8.59	5	)24d
T-	93	9.34	7	)24d

Table 3.29

DEVELOPMENTAL PROFILE
TISSUE : WHOLE SPLEEN

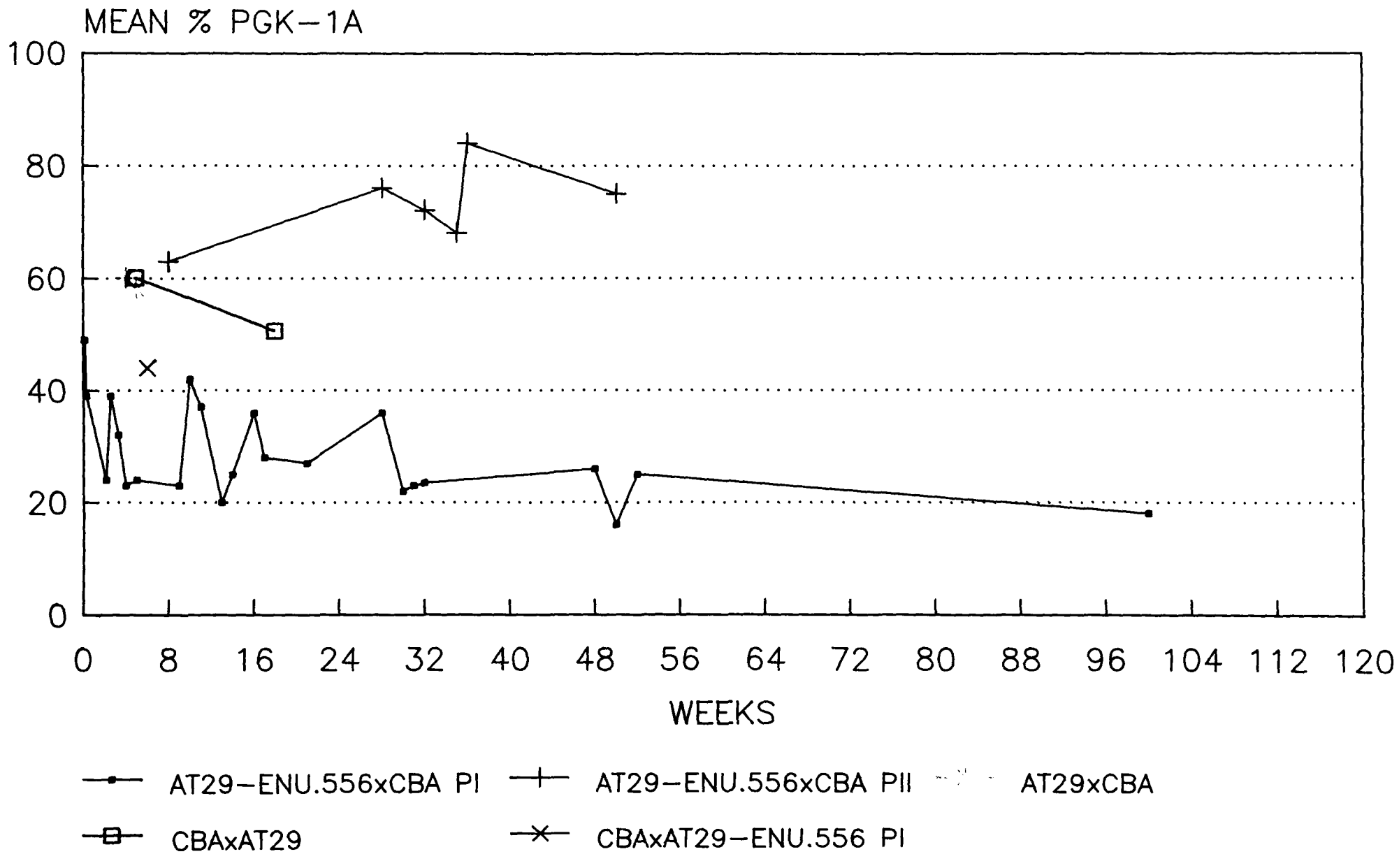


Fig. 3.8

DEVELOPMENTAL PROFILE
TISSUE : WHOLE SPLEEN

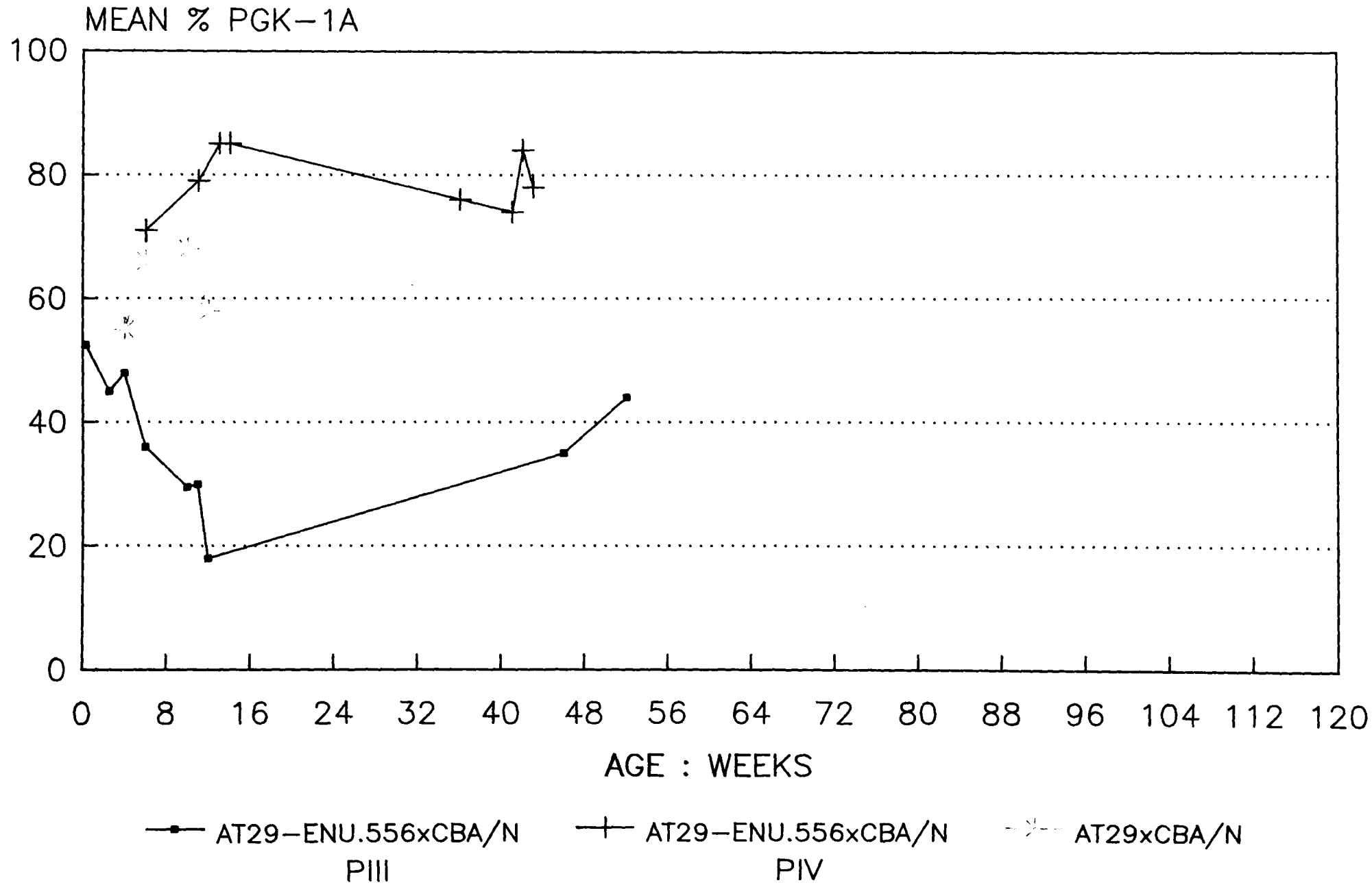
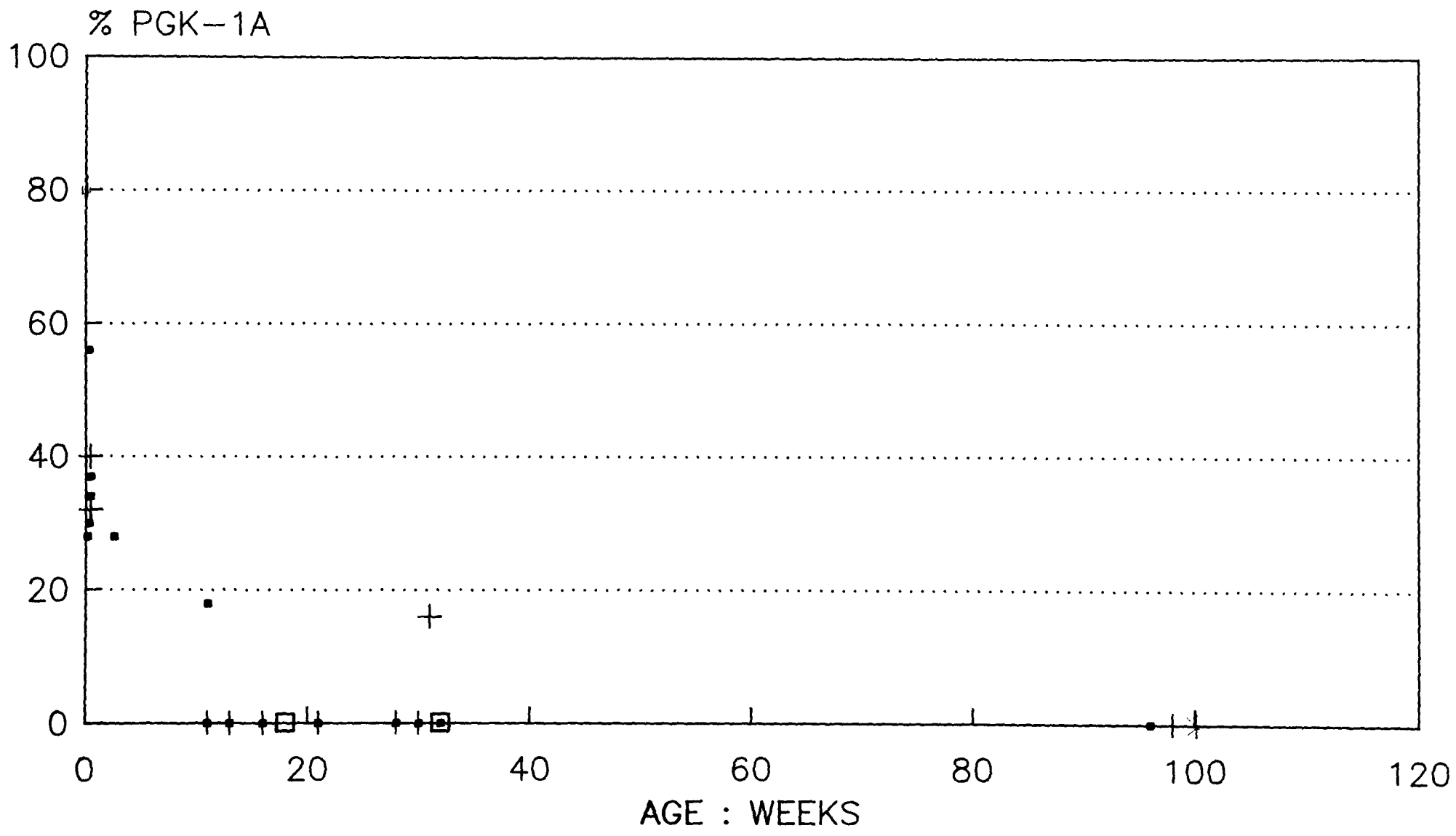


Fig. 3.9

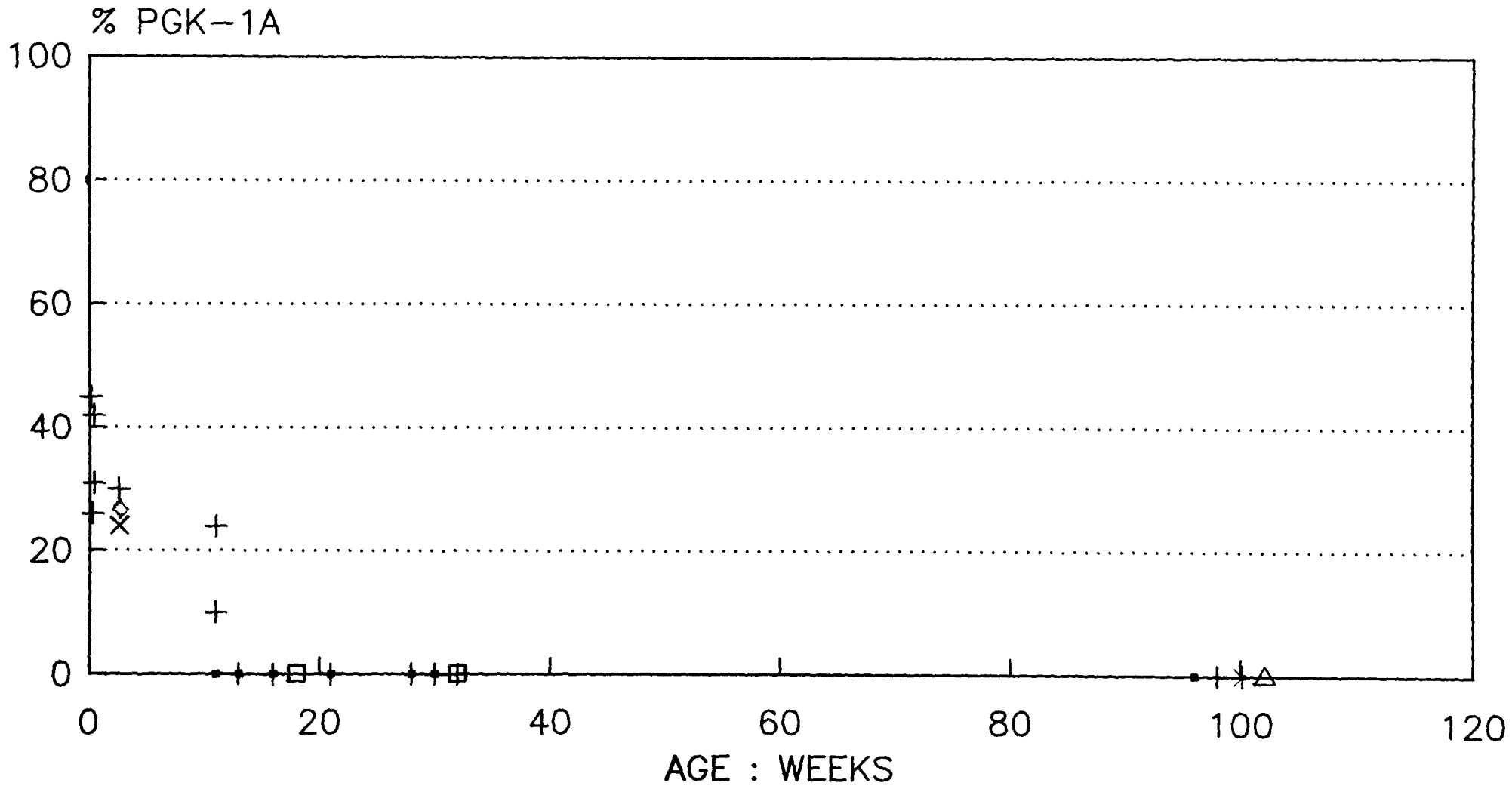
DEVELOPMENTAL PROFILE
(AT29-ENU.556xCBA)PI : SORTED SPLEEN



cell populations which include B cells

Fig. 3.10

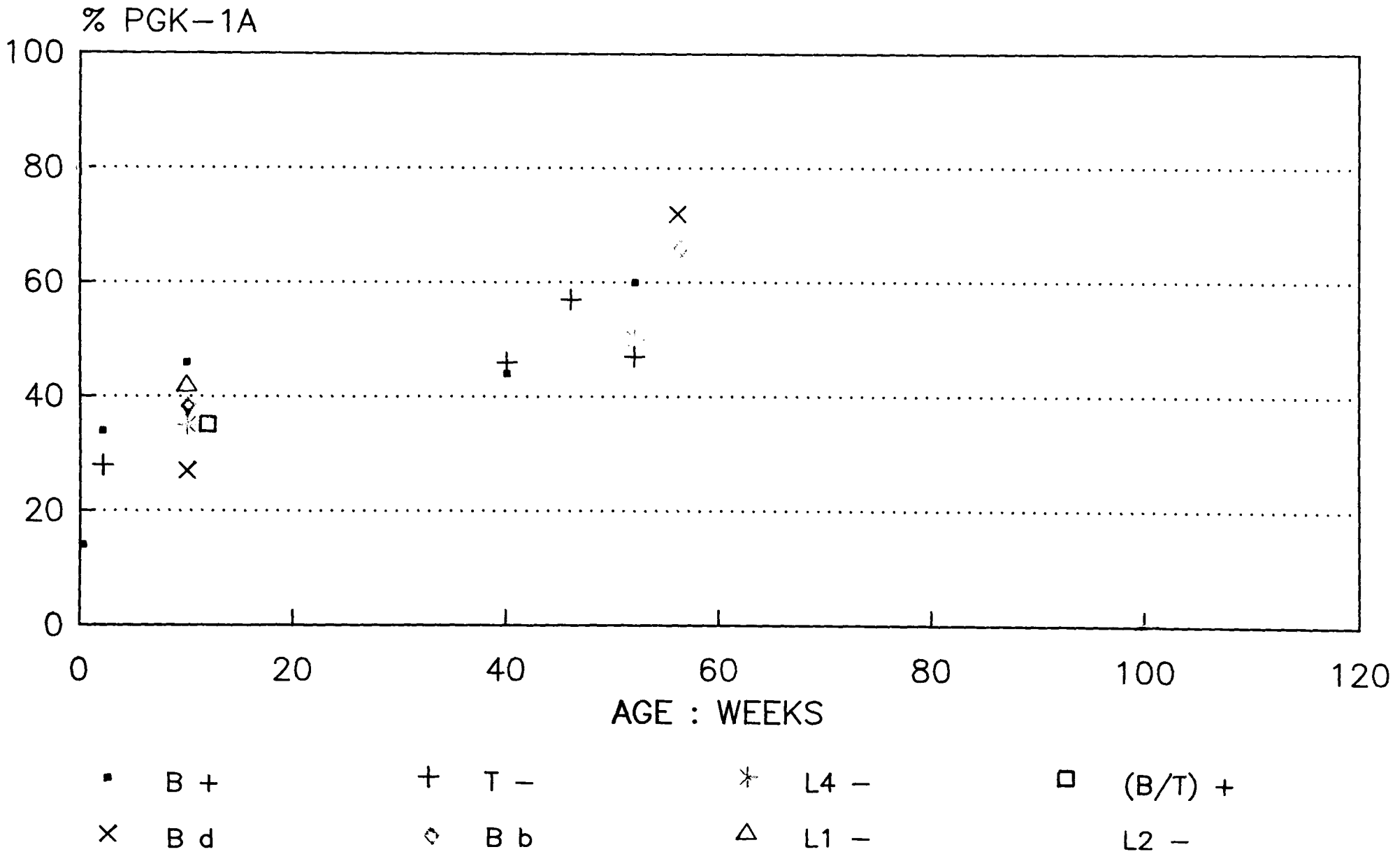
DEVELOPMENTAL PROFILE
(AT29-ENU.556xCBA)PI : SORTED SPLEEN



cell populations which include T cells

Fig. 3.11

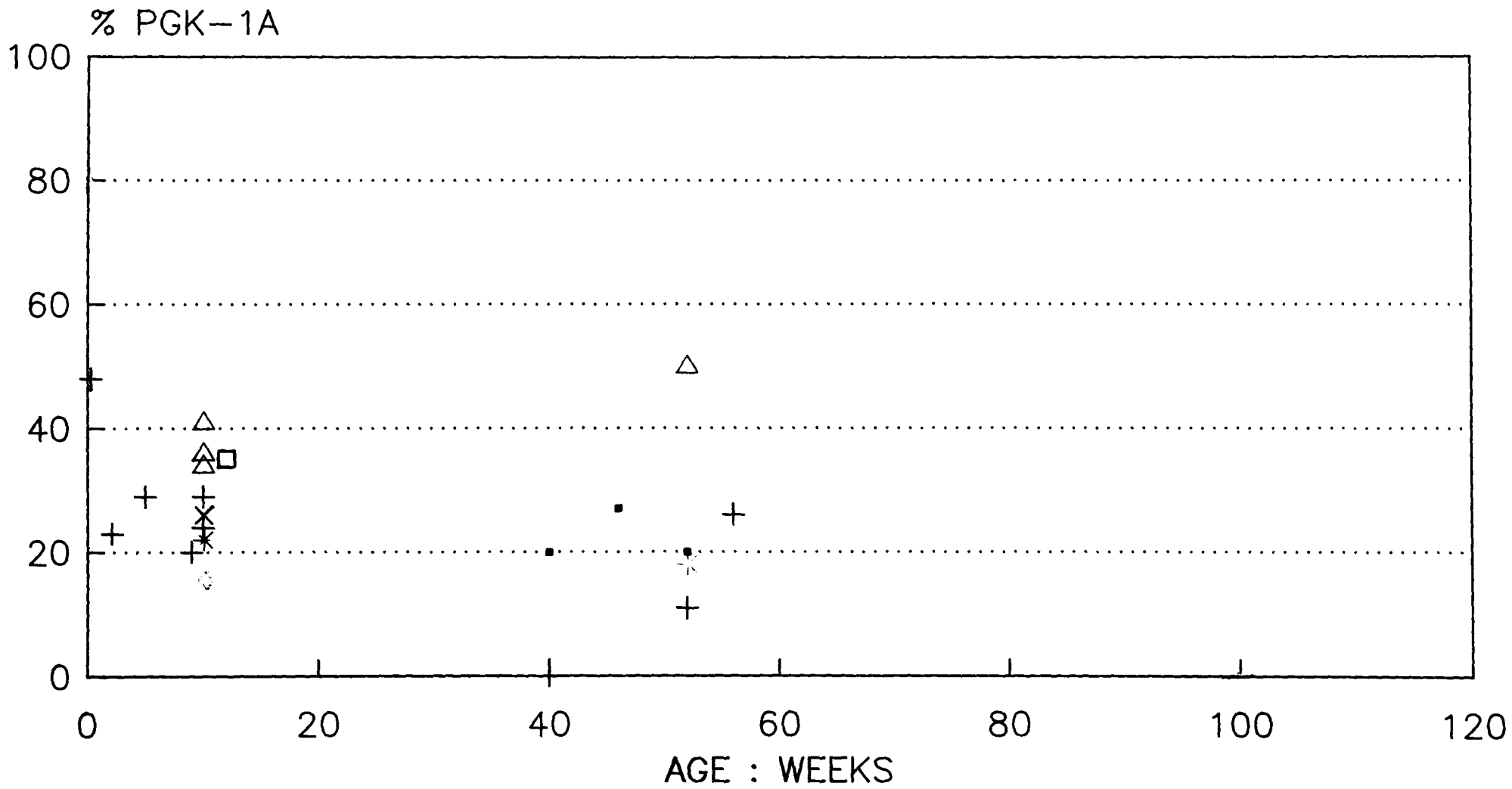
DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA/N)PIII : SORTED SPLEEN



cell populations which include B cells

Fig. 3.12

DEVELOPMENTAL PROFILE
(AT29-ENU.556xCBA/N)PIII : SORTED SPLEEN



cell populations which include T cells

Fig. 3.13

%PGK-1A : (AT29-ENU.556 x CBA) P1

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	23	8.84	7	⟨24d
	13	11.11	29	⟩24d
T L4+	28	7.21	3	⟨24d
	0	/	1	⟩24d
L4-	39	5.57	3	⟨24d
	0	/	1	⟩24d
L2+	30	0.71	2	⟨24d
L2-	40	9.19	2	⟨24d
I.CORTEX T+	33	11.06	3	⟨24d
	2	4.54	7	⟩24d
O.CORTEX T+	42	/	1	⟨24d
	3	6.20	9	⟩24d
MEDULLA T+	26	19.09	2	⟨24d
	3	7.56	7	⟩24d

Table 3.30

Key to tables and figures for THYMUS:

WHOLE = unsorted thymus
I.CORTEX, O.CORTEX, MEDULLA = thymocytes within thymic compartments
antibody staining: T = Thy1.2; L4 = L3T4; L2 = Lyt2; L1 = Ly1;
d,b = dull or bright staining

%PGK-1A:(AT29-ENU.556 x CBA/N) PIII

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	39	9.38	6	⟨24d
	14	8.48	8	⟩24d
T L4+	22	17.68	2	⟨24d
L4-	0	/	1	⟩24d
L4 d	7	/	1	⟩24d
L1+	0	/	1	⟩24d
L1-	10	/	1	⟩24d
T-	30	/	1	⟨24d
I.CORTEX T+	24	9.19	2	⟨24d
	5	5.83	4	⟩24d
O.CORTEX T+	28	9.19	2	⟨24d
	0	0.00	3	⟩24d
MEDULLA T+	32	11.31	2	⟨24d
	6	6.51	3	⟩24d

Table 3.31

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	63	5.51	3	)24d
T L4+	48	/	1	)24d
T L4-	62	/	1	)24d
MEDULLA T+	46	/	1	)24d

Table 3.32

%PGK-1A : (CBA x AT29)

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	55	4.62	3	)24d
I.CORTEX T+	52	7.78	2	)24d
O.CORTEX T+	57	1.41	2	)24d
MEDULLA T+	51	11.31	2	)24d

Table 3.33

%PGK-1A : (CBA x CBA-Pgk-1a)

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	66	10.68	7	)24d
I.CORTEX T+	90	/	1	)24d

Table 3.34

%PGK-1A : (CBA x AT29-ENU.556) PI

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	9	15.01	3	)24d
I.CORTEX T+	0	/	1	)24d
O.CORTEX T+	0	0	2	)24d
MEDULLA T+	0	0	2	)24d

Table 3.35

%PGK-1A : (AT29-ENU.556 x CBA) PII

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	68	12.92	7	)24d
I.CORTEX T+	70	/	1	)24d
O.CORTEX T+	60	6.36	2	)24d
MEDULLA T+	62	/	1	)24d

Table 3.36

%PGK-1A : (AT29 x CBA/N)

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	57	5.50	4	)24d
T L4+	56	/	1	)24d
I.CORTEX T+	54	/	1	)24d
O.CORTEX T+	56	/	1	)24d
MEDULLA T+	53	8.48	2	)24d

Table 3.37

%PGK-1A:(AT29-ENU.556 x CBA/N)PIV

THYMUS : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	70	10.56	11	)24d
I.CORTEX T+	67	14.74	8	)24d
O.CORTEX T+	72	9.94	9	)24d
MEDULLA T+	75	7.31	6	)24d

Table 3.38

DEVELOPMENTAL PROFILE
TISSUE : THYMUS

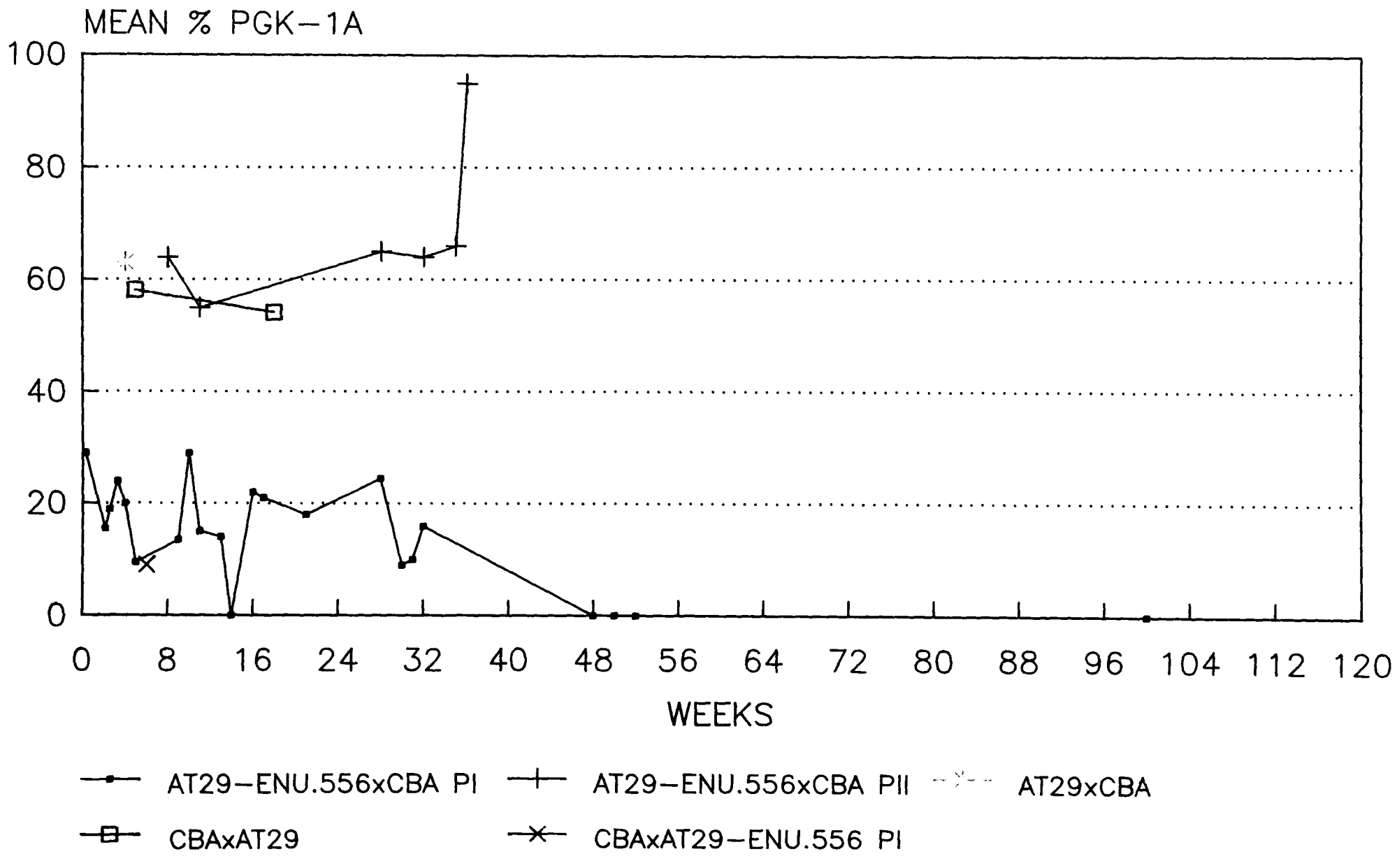


Fig. 3.14

DEVELOPMENTAL PROFILE
TISSUE : THYMUS

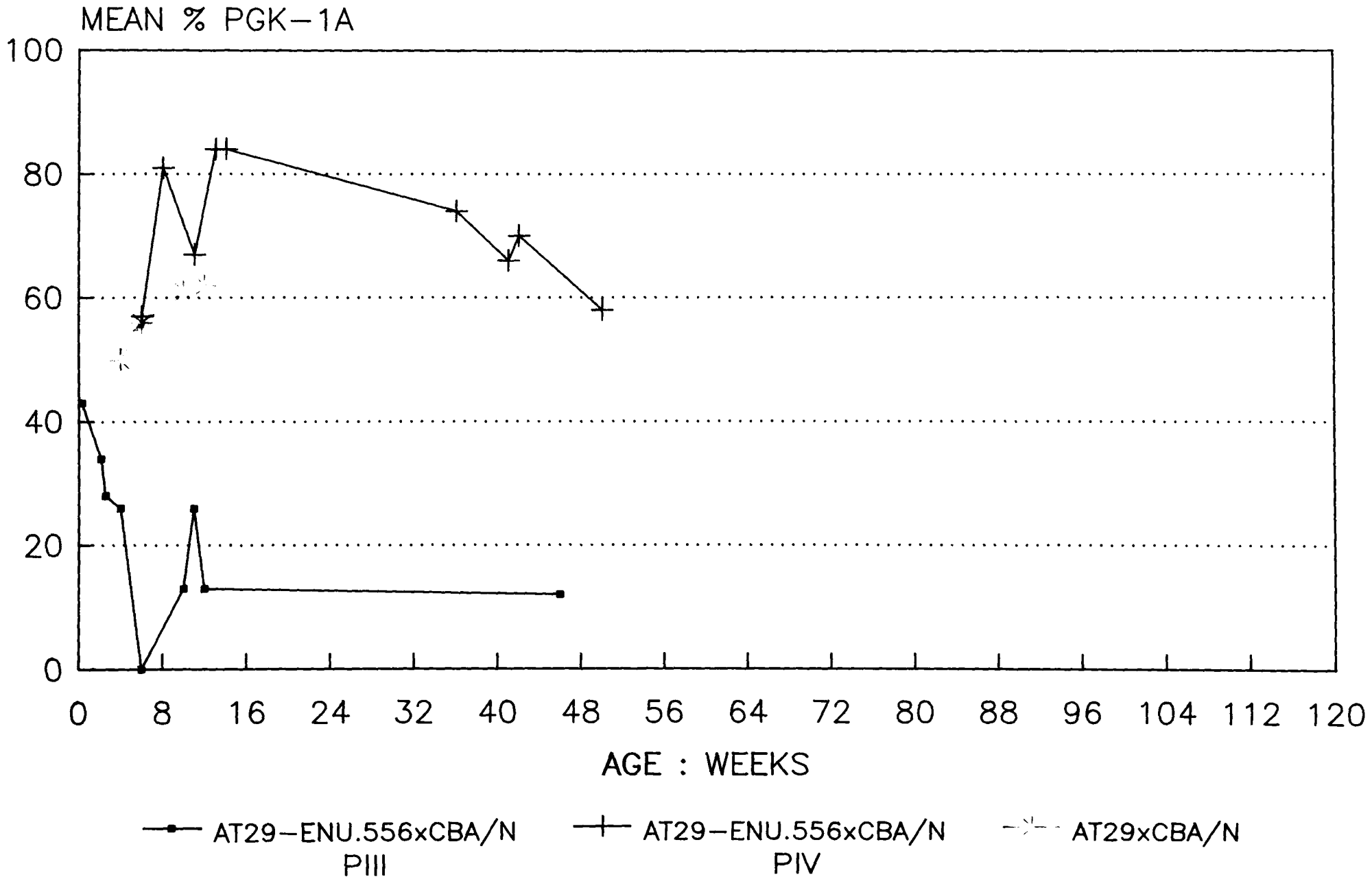


Fig. 3.15

DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA)PI : SORTED THYMUS

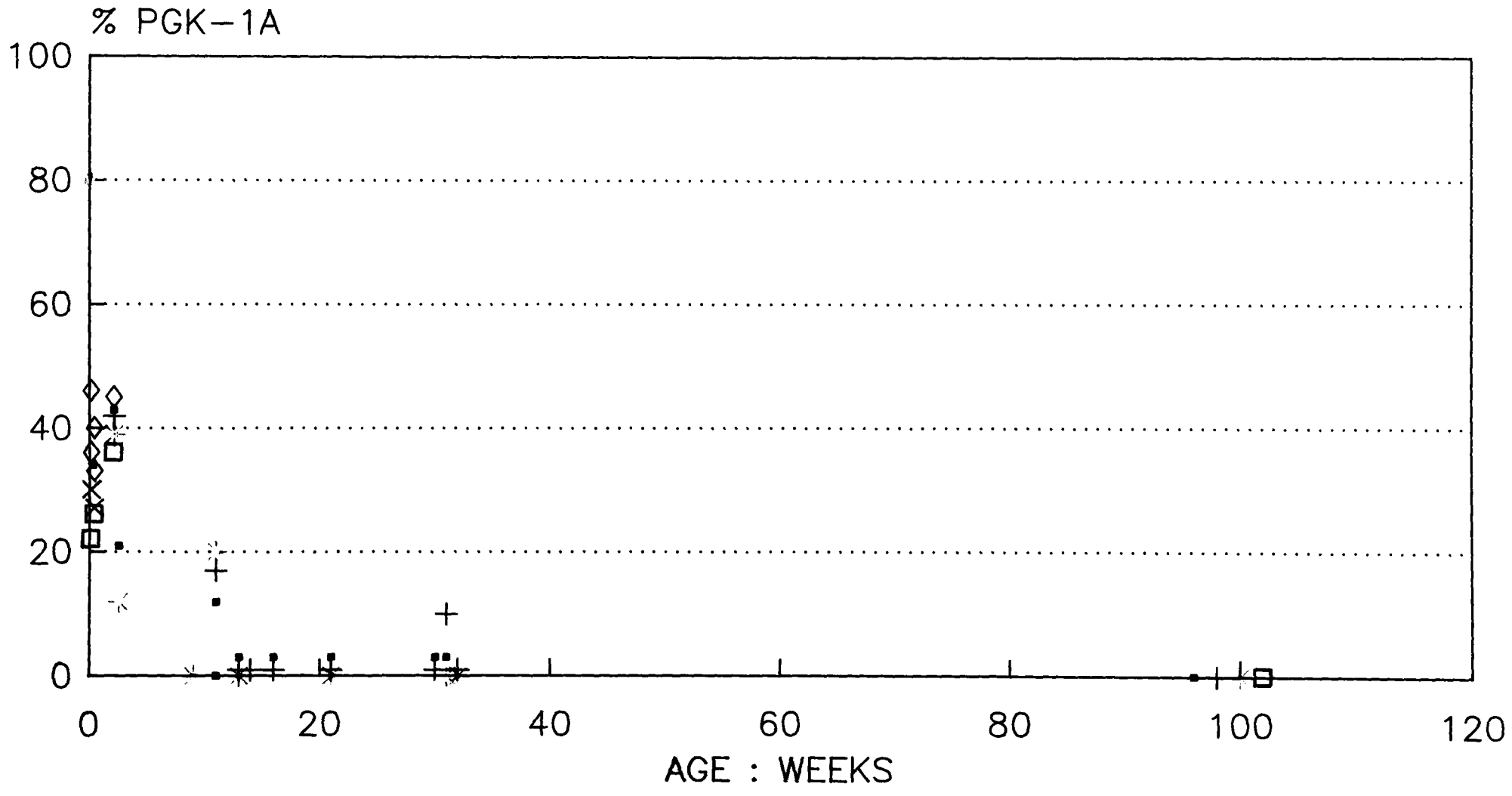


Fig. 3.16

DEVELOPMENTAL PROFILE
(AT29-ENU.556xCBA/N)PIII : SORTED THYMUS

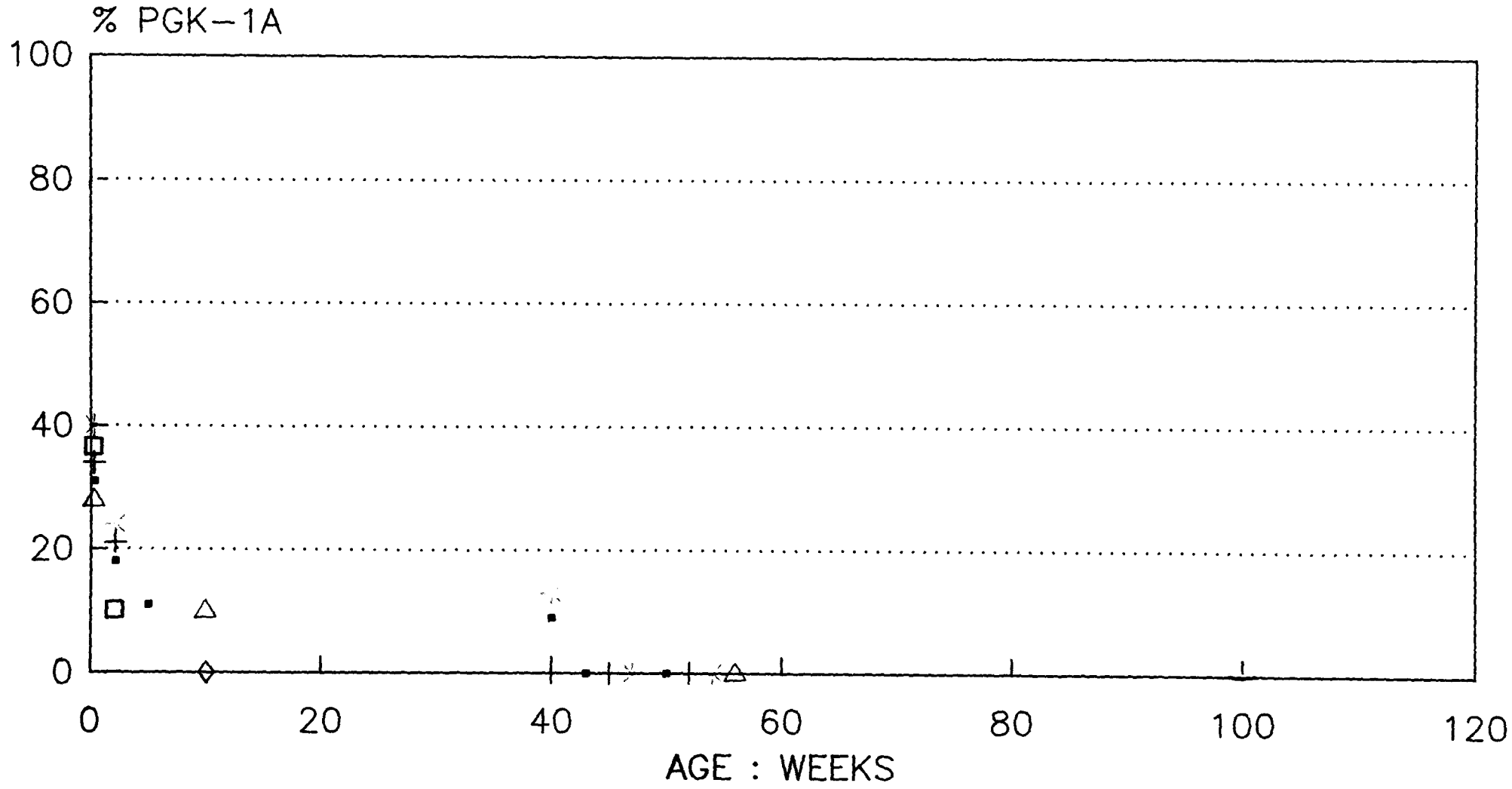


Fig. 3.17

%PGK-1A : (AT29-ENU.556 x CBA) P1

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	21	8.08	3	15-24d
	13	10.38	26	)24d
B AND T				
(B/T)+	0	/	1	)24d
L4-	1	1.73	3	)24d
L2-	5	7.07	2	)24d
OTHER				
(B/T)-	0	/	1	)24d
T				
B-	0	0.00	7	)24d
T+	0	0.00	6	)24d
L4+	3	5.20	3	)24d
L2+	6	7.78	2	)24d
T d	16	/	1	15-24d
T b	17	/	1	15-24d
B				
B+	2	4.95	8	)24d
T-	4	8.98	6	)24d

Table 3.39

Key to tables and figures for LYMPH NODE:

WHOLE = unsorted lymph node

antibody staining: B = B220; T = Thy1.2; L4 = L3T4; L2 = Lyt2; L1 = Ly1;
d,b = dull or bright staining

%PGK-1A:(AT29-ENU.556 x CBA/N) PIII

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	28	/	1	15-24d
	23	9.93	4	)24d
B AND T				
(B/T)+	20	10.61	2	)24d
L4-	10	8.96	3	)24d
L2-	11	3.00	3	)24d
L1-	26	5.66	2	)24d
T				
B-	0	/	1	15-24d
	13	12.52	5	)24d
T+	0	/	1	15-24d
	4	7.51	3	)24d
L4+	6	8.48	2	)24d
L2+	10	/	1	)24d
L1+	5	/	1	)24d
L4 d	18	0.71	2	)24d
L4 b	6	8.48	2	)24d
L2 d	17	1.41	2	)24d
L2 b	15	7.07	2	)24d
L1 d	17	/	1	)24d
L1 b	0	/	1	)24d
B				
B+	31	11.54	5	)24d
T-	12	4.65	4	)24d
B b	35	/	1	)24d

Table 3.40

%PGK-1A : (AT29 x CBA)

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	68	8.74	3	)24d
T				
B-	47	/	1	)24d
T+	55	/	1	)24d
L4+	55	/	1	)24d

Table 3.41

%PGK-1A : (CBA x AT29)

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	55	4.16	3	)24d
OTHER high scattering T+	53	/	1	)24d
T				
B-	49	/	1	)24d
T+	49	/	1	)24d
T d	47	/	1	)24d
T b	48	/	1	)24d
B				
B+	48	/	1	)24d
T-	56	/	1	)24d

Table 3.42

%PGK-1A : (CBA x CBA-Pgk-1a)

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	69	9.80	7	)24d
T				
B-	85	/	1	)24d
T d	88	/	1	)24d
T b	85	/	1	)24d
B				
B b	83	/	1	)24d

Table 3.43

%PGK-1A : (CBA x AT29-ENU.556)PI

LYMPH NODE: CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	33	1.41	2	)24d
T T+	0	0	2	)24d
B T-	0	0	2	)24d

Table 3.44

%PGK-1A : (AT29-ENU.556 x CBA) PII

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	64	11.89	4	)24d
T B-	64	/	1	)24d
T+	60	2.12	2	)24d
B B+	68	/	1	)24d
T-	52	/	1	)24d

Table 3.45

%PGK-1A : (AT29 x CBA/N)

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	62	4.04	3	)24d
B AND T				
L4-	70	/	1	)24d
L2-	64	/	1	)24d
T				
T+	54	/	1	)24d
L4 d	71	/	1	)24d
L4 b	58	/	1	)24d
L2 d	62	/	1	)24d
L2 b	62	/	1	)24d
Ll d	75	/	1	)24d
Ll b	54	/	1	)24d

Table 3.46

%PGK-1A:(AT29-ENU.556 x CBA/N)PIV

LYMPH NODE : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	77	7.76	11	)24d
B AND T				
(B/T)+	76	6.27	4	)24d
OTHER				
(B/T)-	64	/	1	)24d
T				
B-	75	7.28	7	)24d
T+	73	10.25	8	)24d
T b	78	/	1	)24d
B				
B+	90	9.50	3	)24d
T-	91	9.15	5	)24d

Table 3.47

DEVELOPMENTAL PROFILE
TISSUE : LYMPH NODE

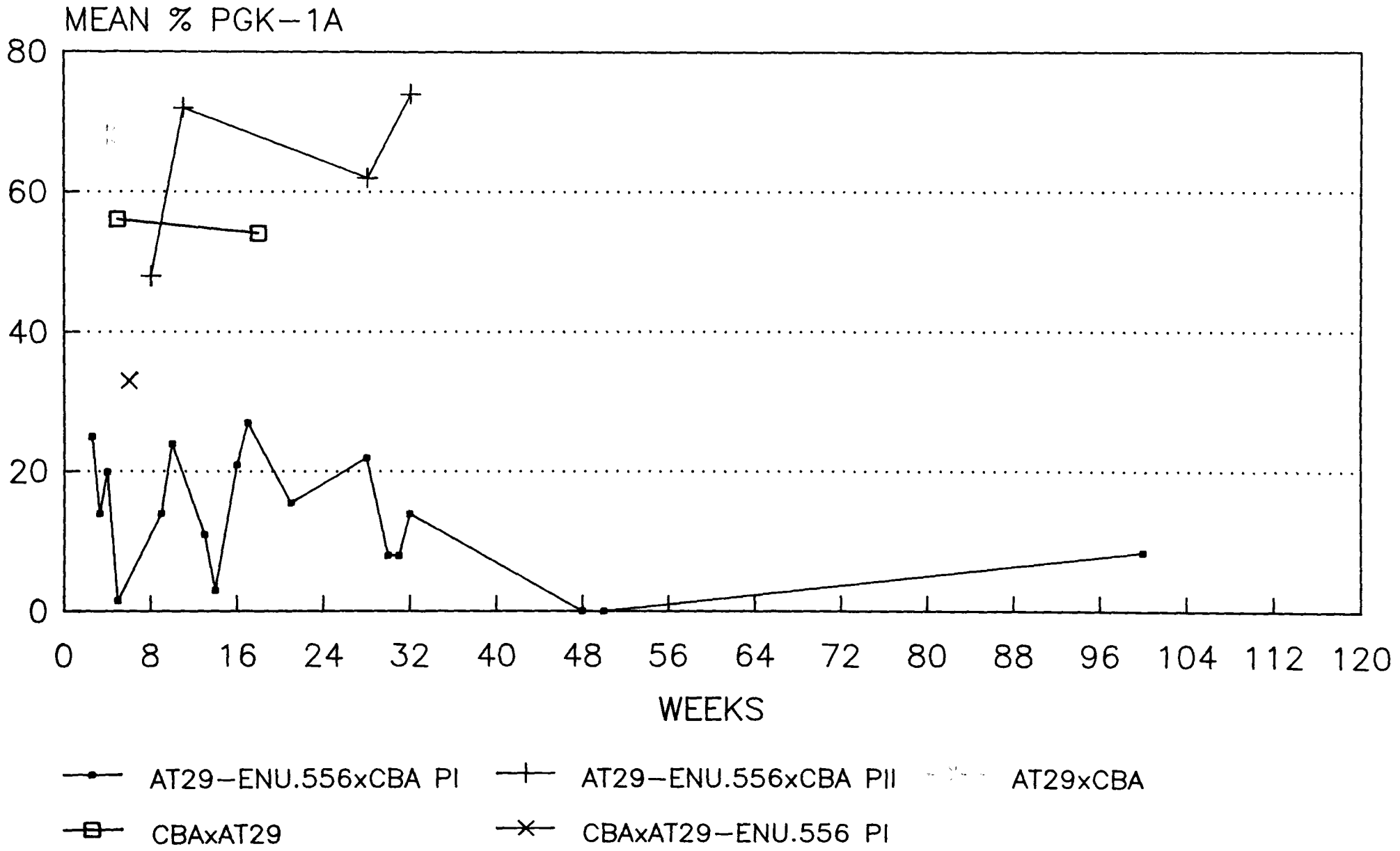


Fig. 3.18

DEVELOPMENTAL PROFILE
TISSUE : LYMPH NODE

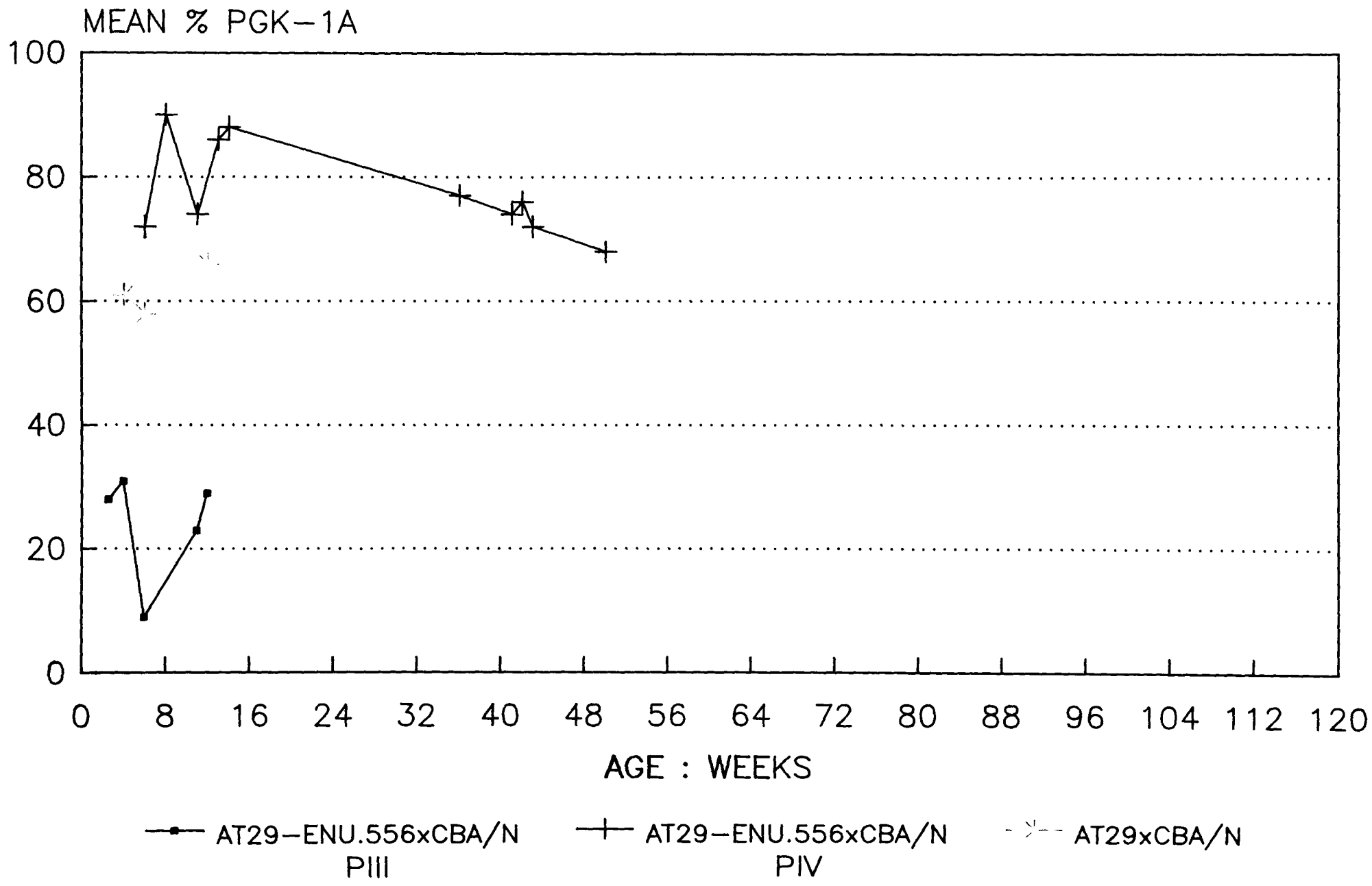
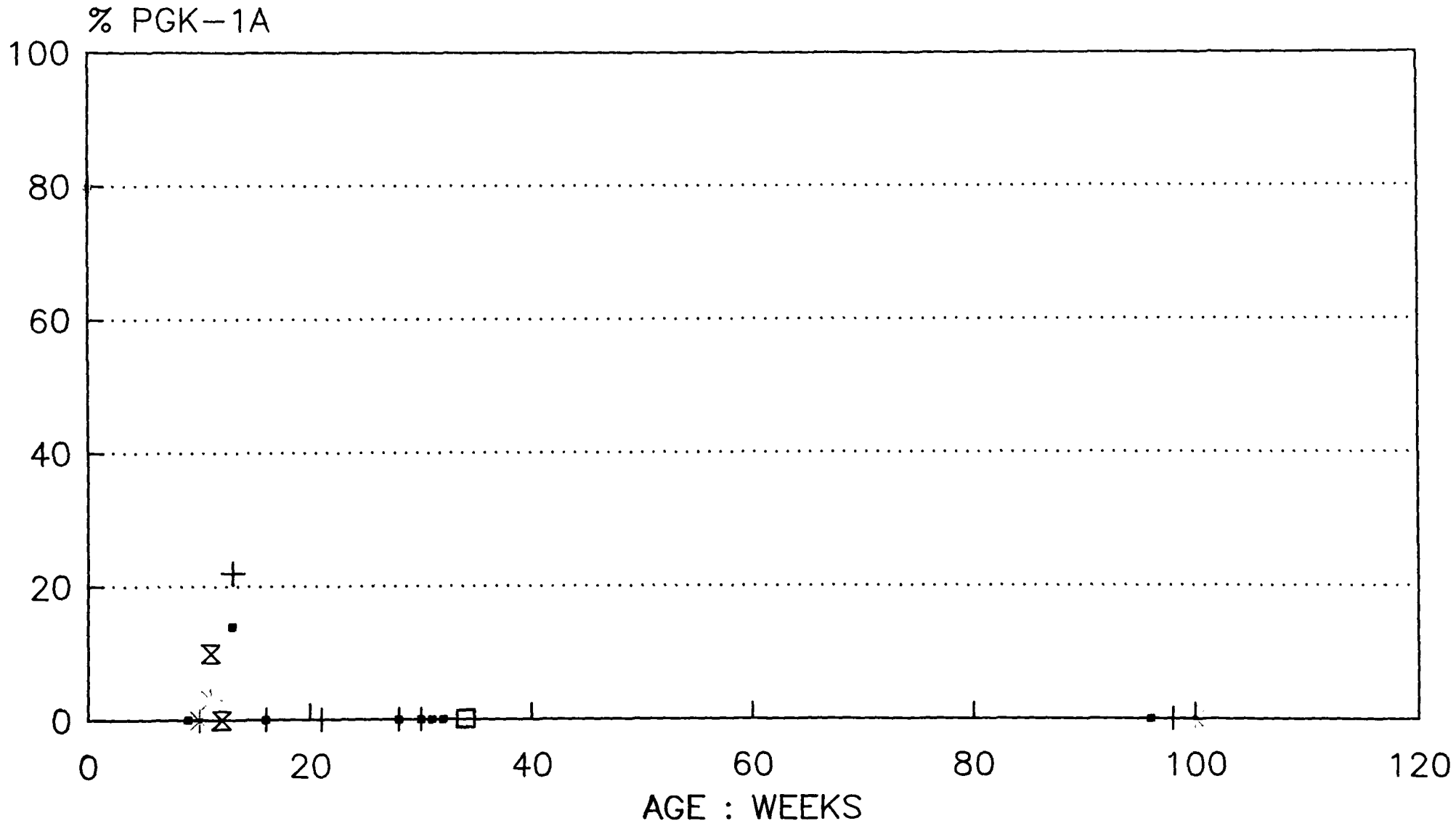


Fig. 3.19

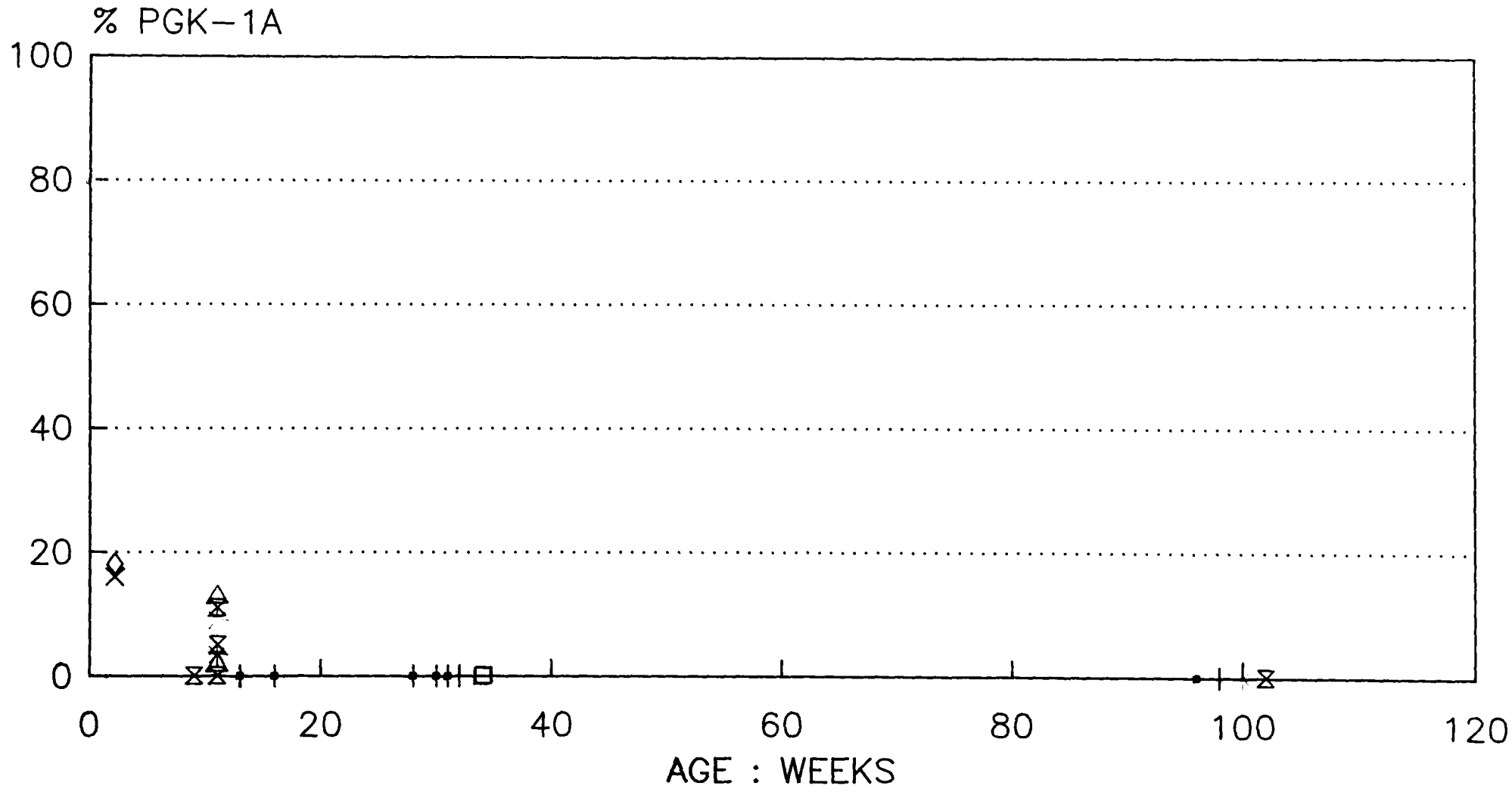
DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA)PI : SORTED LYMPH NODE



cell populations which include B cells

Fig. 3.20

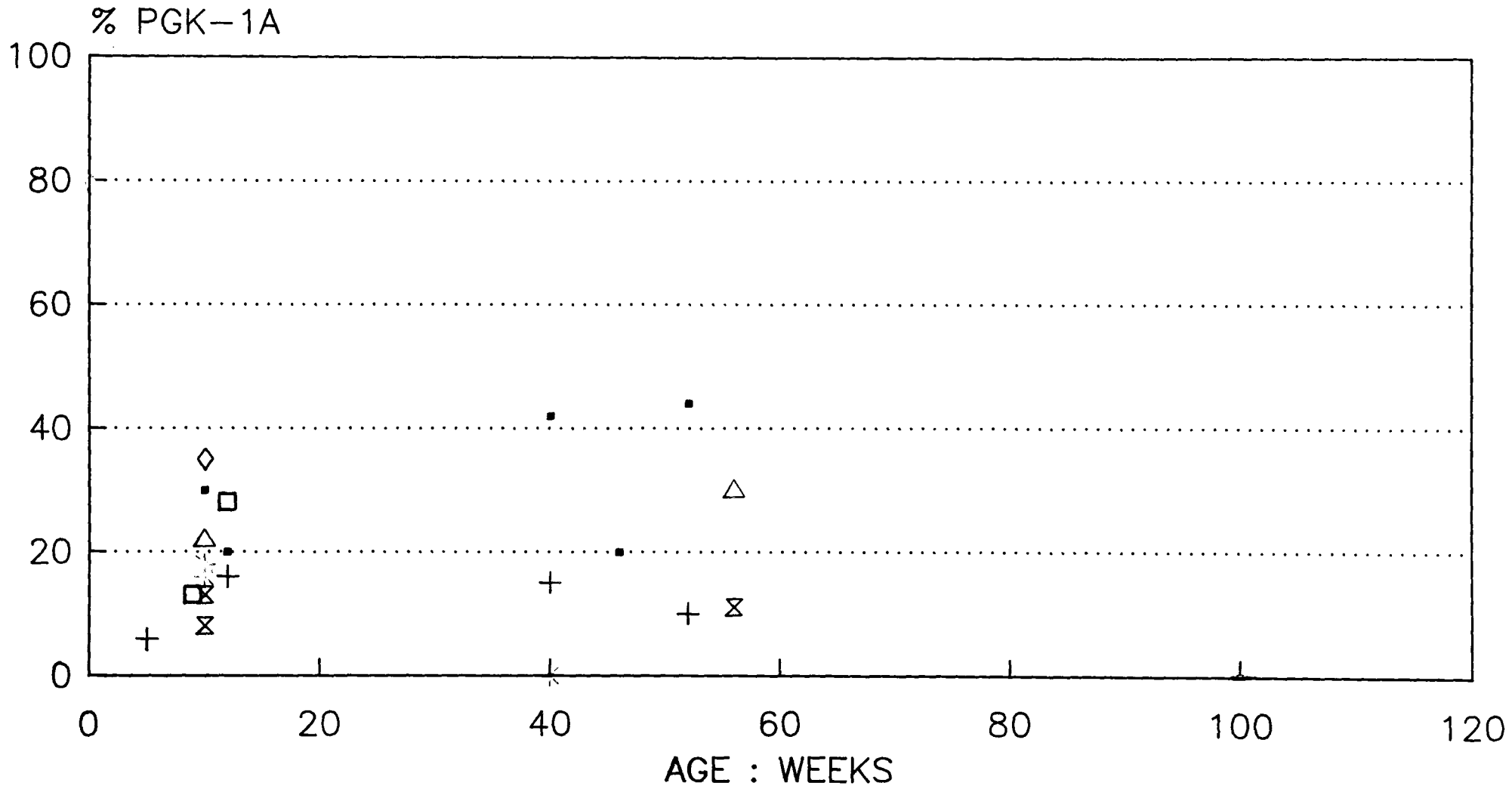
DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA)PI : SORTED LYMPH NODE



cell populations which include T cells

Fig. 3.21

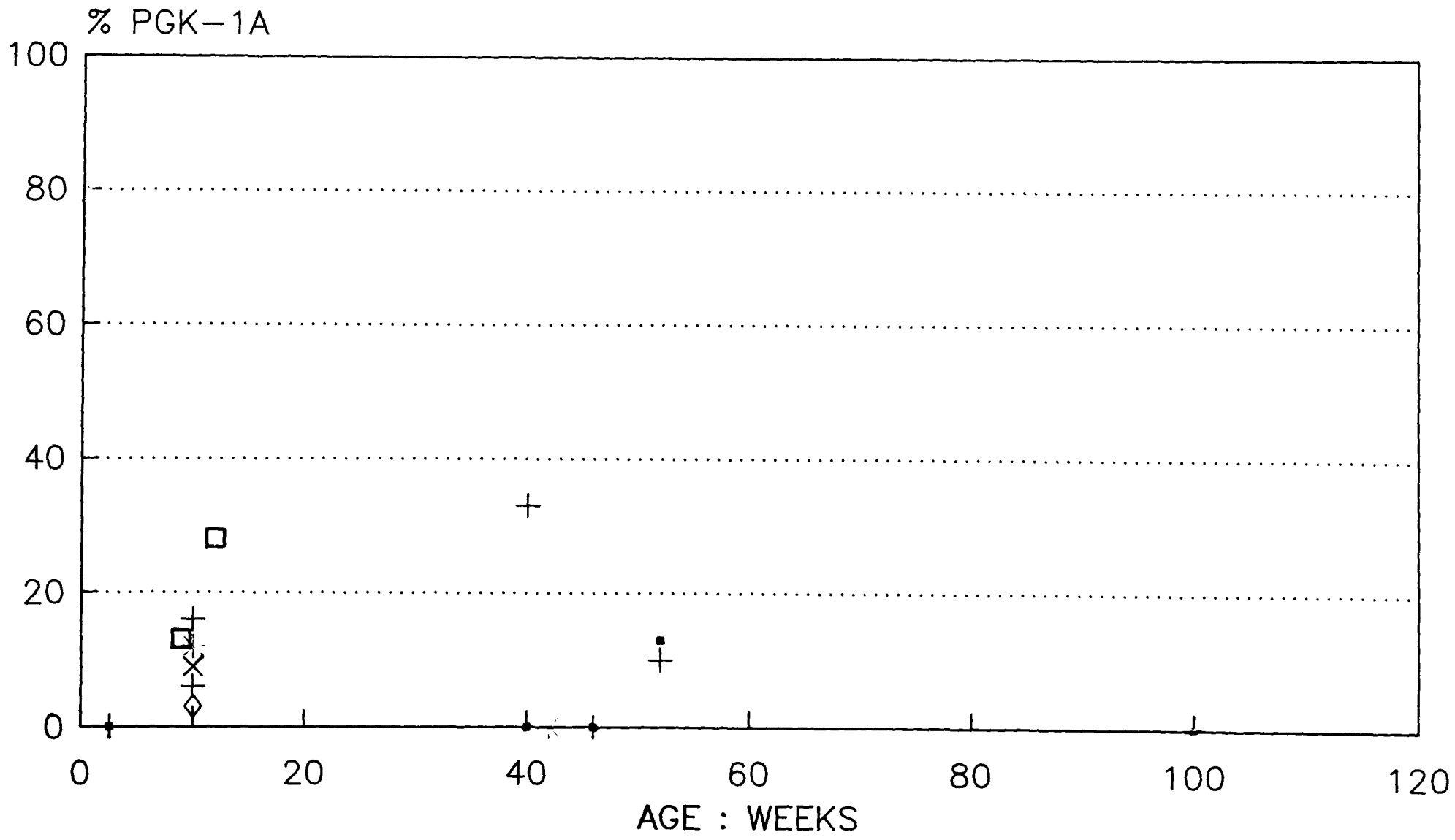
DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA/N)PIII : SORTED L.NODE



cell populations which include B cells

Fig. 3.22

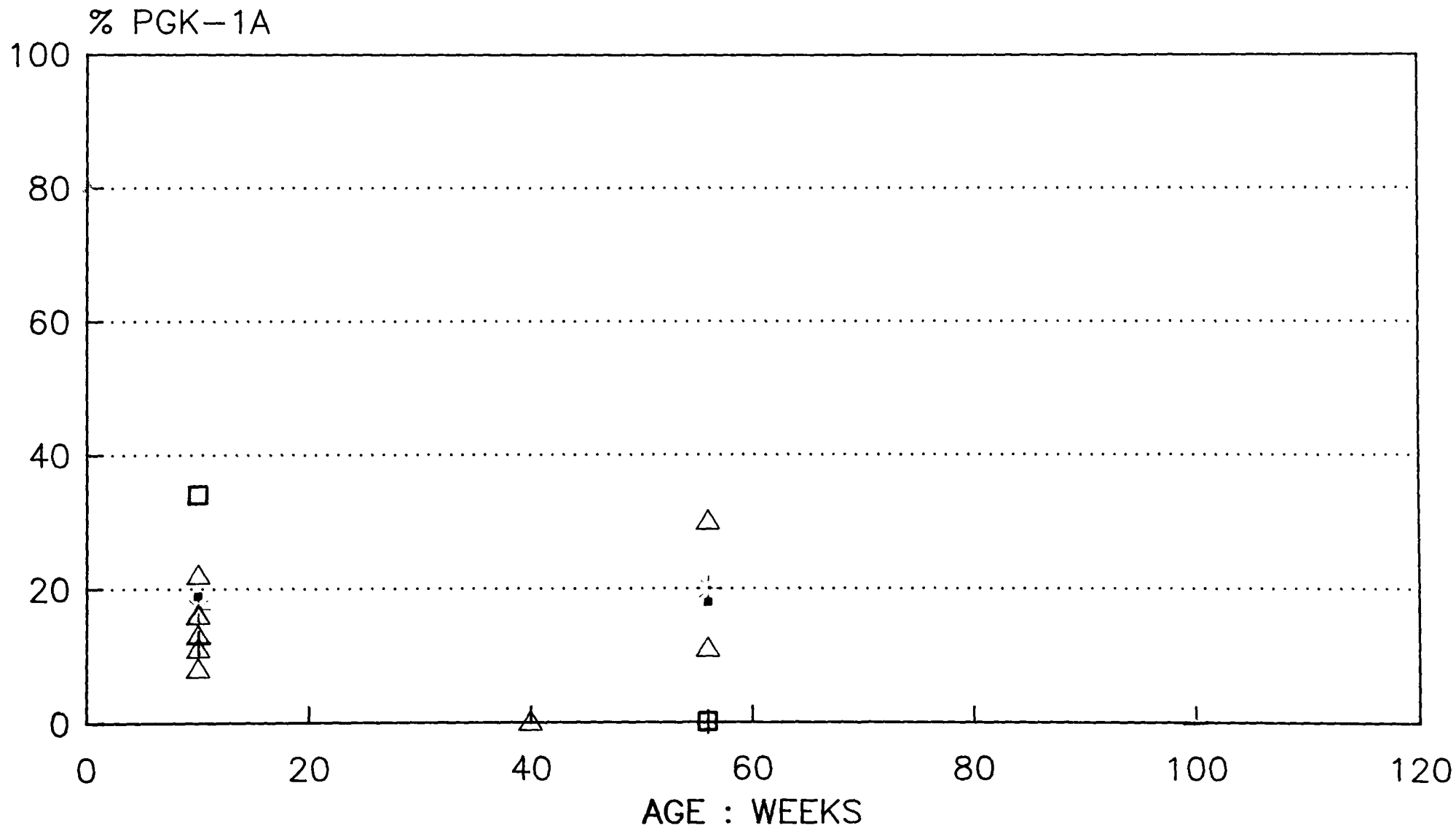
DEVELOPMENTAL PROFILE
(AT29−ENU.556xCBA/N)PIII : SORTED L.NODE



cell populations which include T cells

Fig. 3.23

DEVELOPMENTAL PROFILE
(AT29-ENU.556xCBA/N)PIII : SORTED L.NODE



cell populations which include T cells

Fig. 3.24

%PGK-1A : (AT29-ENU.556 x CBA) PI

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	35	7.09	3	15-24d
	40	11.39	24	>24d
B B+	10	13.07	7	>24d
B d	41	/	1	15-24d
OTHER B-	48	/	1	15-24d
	18	12.92	8	>24d
high scatter	62	/	1	15-24d
	44	12.51	10	>24d

Table 3.48

% PGK-1A : (AT29-ENU.556 x CBA) PI

NEONATAL LIVER : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	53	13.84	8	<15d
lymphocytes	44	7.62	4	<15d
OTHER high scatter	54	7.00	4	<15d

Table 3.49

Key to tables and figures for BONE MARROW and NEONATAL LIVER:

WHOLE = unsorted tissue

HIGH SCATTER = cells with higher right angle scatter than lymphocytes

antibody staining: B = B220; d = dull staining

%PGK-1A:(AT29-ENU.556 x CBA/N) PIII

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	42	/	1	15-24d
	40	8.06	7	>24d
B B+	22	11.19	5	>24d
OTHER B-	13	11.49	4	>24d
high scatter	39	9.72	5	>24d

Table 3.50

%PGK-1A:(AT29-ENU.556 x CBA/N) PIII

NEONATAL LIVER : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	62	16.01	4	<15d
lymphocytes	44	8.72	3	<15d
OTHER high scatter	64	/	1	<15d

Table 3.51

%PGK-1A : (AT29 x CBA)

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	59	6.11	3	)24d
OTHER				
B-	59	/	1	)24d

Table 3.52

%PGK-1A : (CBA x AT29)

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	54	8.48	2	)24d
B				
B+	47	9.90	2	)24d
OTHER				
B-	42	0.71	2	)24d
high scattering	59	12.73	2	)24d

Table 3.53

%PGK-1A : (CBA x CBA-Pgk-1a)

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	66	13.18	7	)24d
B				
B+	85	/	1	)24d
OTHER				
B-	80	/	1	)24d
high scattering	86	/	1	)24d

Table 3.54

%PGK-1A : (CBA x AT29-ENU.556) PI

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL				
WHOLE	39	2.83	2	)24d
B				
B+	0	/	1	)24d
OTHER				
B-	0	/	1	)24d
high scattering	52	2.83	2	)24d

Table 3.55

%PGK-1A : (AT29-ENU.556 x CBA) PII

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	68	6.76	8	)24d
B B+	68	/	1	)24d
OTHER B-	50	/	1	)24d
high scattering	50	/	1	)24d

Table 3.56

%PGK-1A: (AT29-ENU.556 x CBA/N)PIV

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	70	9.66	11	)24d
B B+	70	12.87	4	)24d
OTHER B-	57	18.84	5	)24d
high scattering	73	9.16	3	)24d

Table 3.57

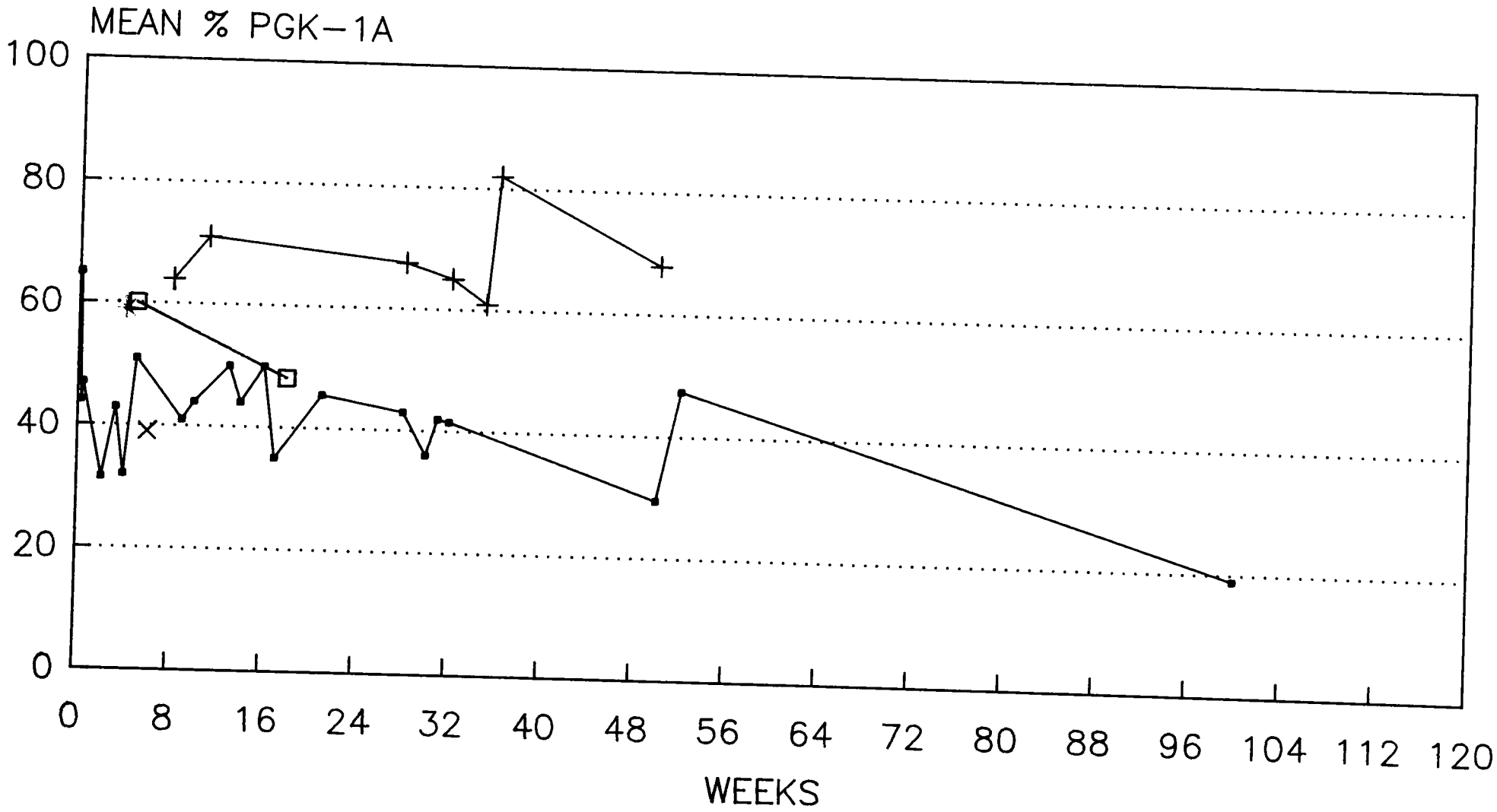
%PGK-1A : (AT29 x CBA/N)

BONE MARROW : CELL POPULATIONS

cell type	mean	st deviation	n	age
ALL WHOLE	57	9.90	2	)24d
B B+	62	/	1	)24d
OTHER B-	56	/	1	)24d
high scattering	52	/	1	)24d

Table 3.58

DEVELOPMENTAL PROFILE
TISSUE : NEONATAL LIVER / BONE MARROW



where neonatal liver was from PI mice aged 1–3 days

Fig. 3.25

DEVELOPMENTAL PROFILE
TISSUE : NEONATAL LIVER / BONE MARROW

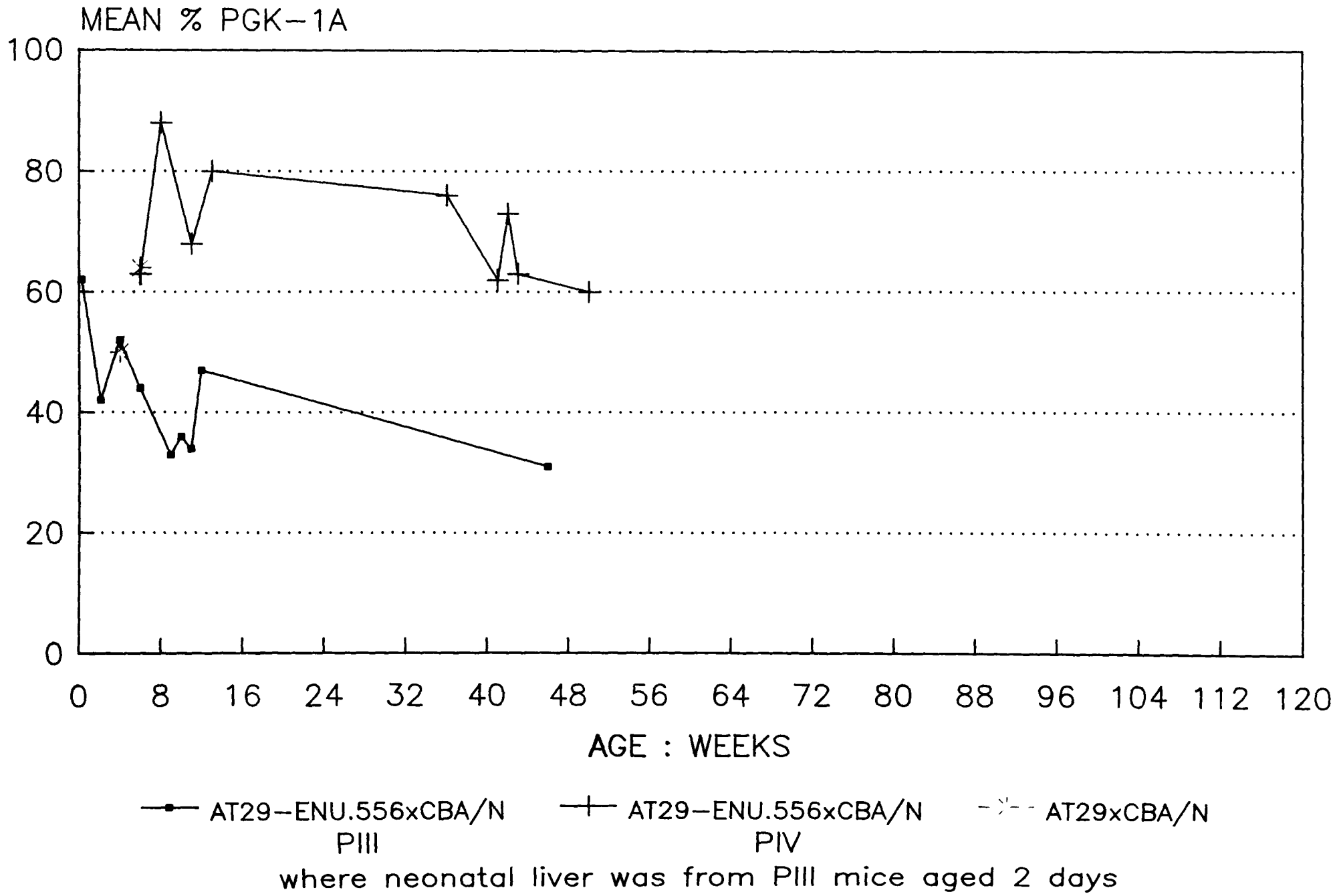
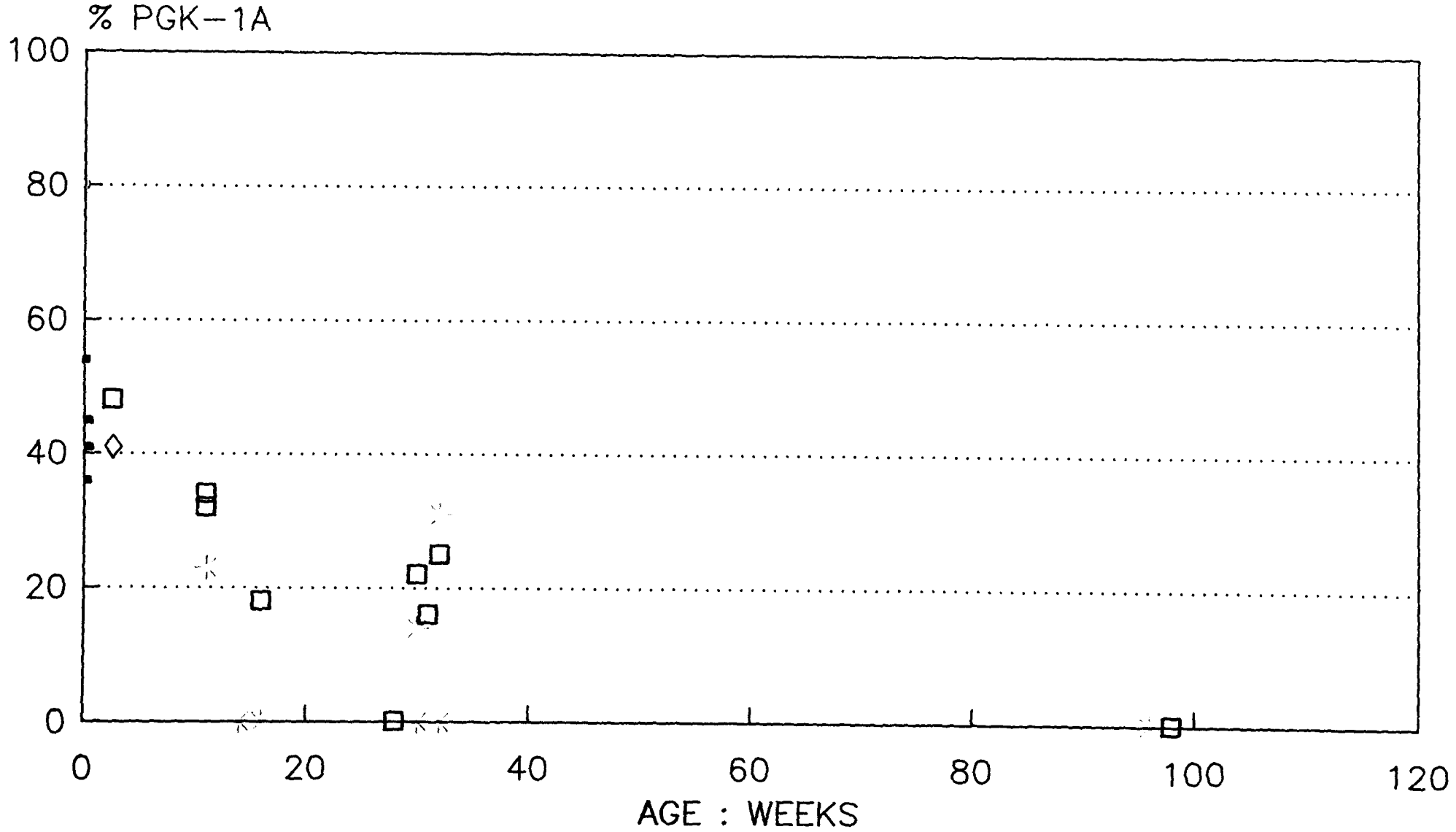


Fig. 3.26

DEVELOPMENTAL PROFILE
(AT29–ENU.556xCBA)PI : SORTED
NEONATAL LIVER AND BONE MARROW

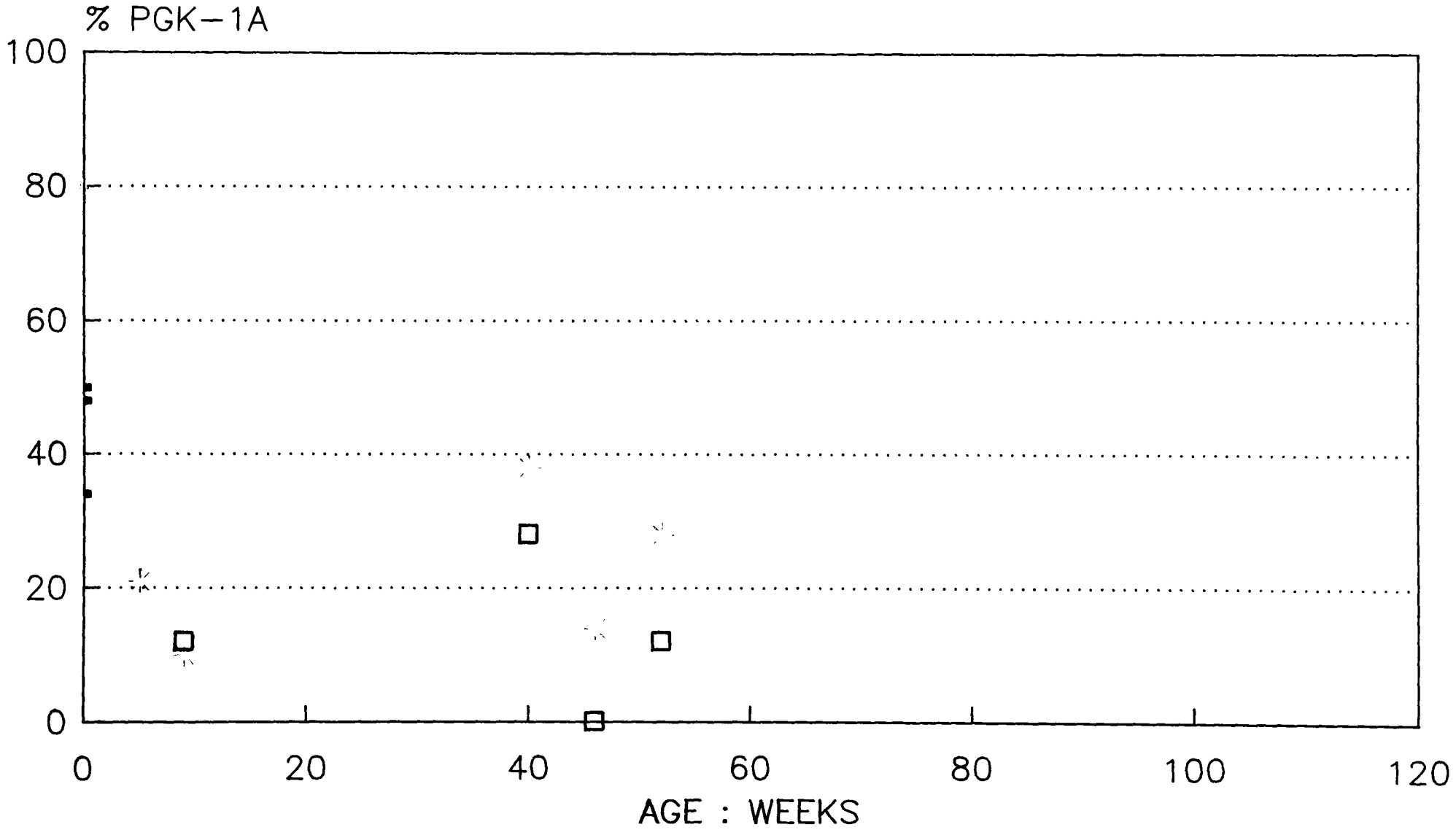


▪ N.L. : lymphocytes ✳ B.M. : B+ □ B.M. : B– ◇ B.M. : B d

where sorted neonatal liver was from mice aged 1–3 days

Fig. 3.27

DEVELOPMENTAL PROFILE
(AT29–ENU.556xCBA/N)PIII : SORTED NEONATAL LIVER AND BONE MARROW



▪ N.L. : lymphocytes ✳ B.M. : B+ □ B.M. : B–

where sorted neonatal liver was from mice aged 2 days

Fig. 3.28

CHAPTER 4

Phenotypic Variation In Female Offspring From AT29-ENU.556 Crosses

Studies of X-chromosome inactivation patterns in female offspring of (1) AT29-ENU.556 x CBA, (2) CBA x AT29-ENU.556 and (3) AT29-ENU.556 x CBA/N crosses revealed two distinct phenotypes for cross (1) and (3), defined in Chapter 3 as PI and PII for cross(1) and PIII and PIV for cross (3), respectively.

Phenotype Of AT29-ENU.556 x CBA Female Offspring (PI, PII).

The phenotypes of PI and PII mice have been described in Chapter 3. Briefly, females with the PI phenotype had unbalanced patterns of PGK-1 expression in favour of CBA for skeletal muscle, erythrocytes and lymphocytes. In contrast, PII females termed non-mutant, did not show any selection against cells carrying the AT29-ENU.556 X chromosome and the patterns of PGK-1 expression for skeletal muscle, erythrocytes and lymphocytes were similar to that in control females.

Phenotype Of AT29-ENU.556 x CBA/N Female Offspring (PIII, PIV).

The phenotypes of PIII and PIV mice have been described in Chapter 3. Briefly, females with the PIII phenotype had unbalanced patterns of PGK-1 expression in favour of CBA/N

for skeletal muscle, erythrocytes and certain lymphocyte populations. As both the ENU and xid mutations result in defective lymphocytes, the patterns of PGK-1 expression for the females carrying both these mutations will depend on:

(a) whether the mutations affect the same or different lymphocyte populations

(b) the stage at which each mutation takes effect

(c) the selective effects of the mutations

For example, if both mutations affect the same population to the same degree although not necessarily in the same way, the cells will be equally defective and the PGK-1 patterns of expression would be similar to those in control females.

In general, in PIII females there was selection against T cells carrying the AT29-ENU.556 X chromosome. For B cell populations, the patterns of PGK-1 expression tended to a random pattern, depending on the tissue and the age of the individual.

In contrast, PIV females did not show any selection against cells carrying the AT29-ENU.556 X chromosome for skeletal muscle, erythrocytes or lymphocytes; rather, B cell populations carrying xid were selected against.

The phenotypic distribution of the female progeny from

various crosses is summarised in table 4.1, where 1703, 2261, 2199, 1998, 2115 and 2203 were homozygous AT29-ENU.556 mice and all female progeny of a given pair from each cross had the same phenotype, namely PI or PII and PIII or PIV.

Table 4.1 showing the phenotypes of female progeny

CROSS	PHENOTYPE OF FEMALE PROGENY OF THE PAIRS IN EACH CROSS			
	PI	PII	PIII	PIV
(1703 x CBA)	1	0	/	/
(2261 x CBA)	1	0	/	/
[(2199 x 1998) x CBA]	3	1	/	/
(2203 x CBA)	0	1	/	/
(CBA x 2115)	1	0	/	/
(* x CBA)	3	0	/	/
[(2199 x 1998) x CBA/N]	/	/	1	3
(* x CBA/N)	/	/	2	0

* $\{[(CBAx2115)x(2199x1998)]x[(CBAx2115)x(2199x1998)]^{2}\}^{2}$

the male parent is given first

In this discussion the alleles of Pgk-1 will be represented by a and b and the mutant and wildtype allelic forms of the xid-mutation in CBA/N mice will be represented by xid and xid^{+}, respectively.

As the ENU mutation affected both the skeletal muscle and the haematopoietic system and not one without the other, in all PI and PIII mice, it is assumed that one locus controlling both systems is involved, represented by M with its allele M^{+}.

Possible genotypes for the X chromosome in the homozygous strains studied, applying the definitions stated above,

are presented in table 4.2.

Table 4.2 showing X-chromosome loci of the strains studied

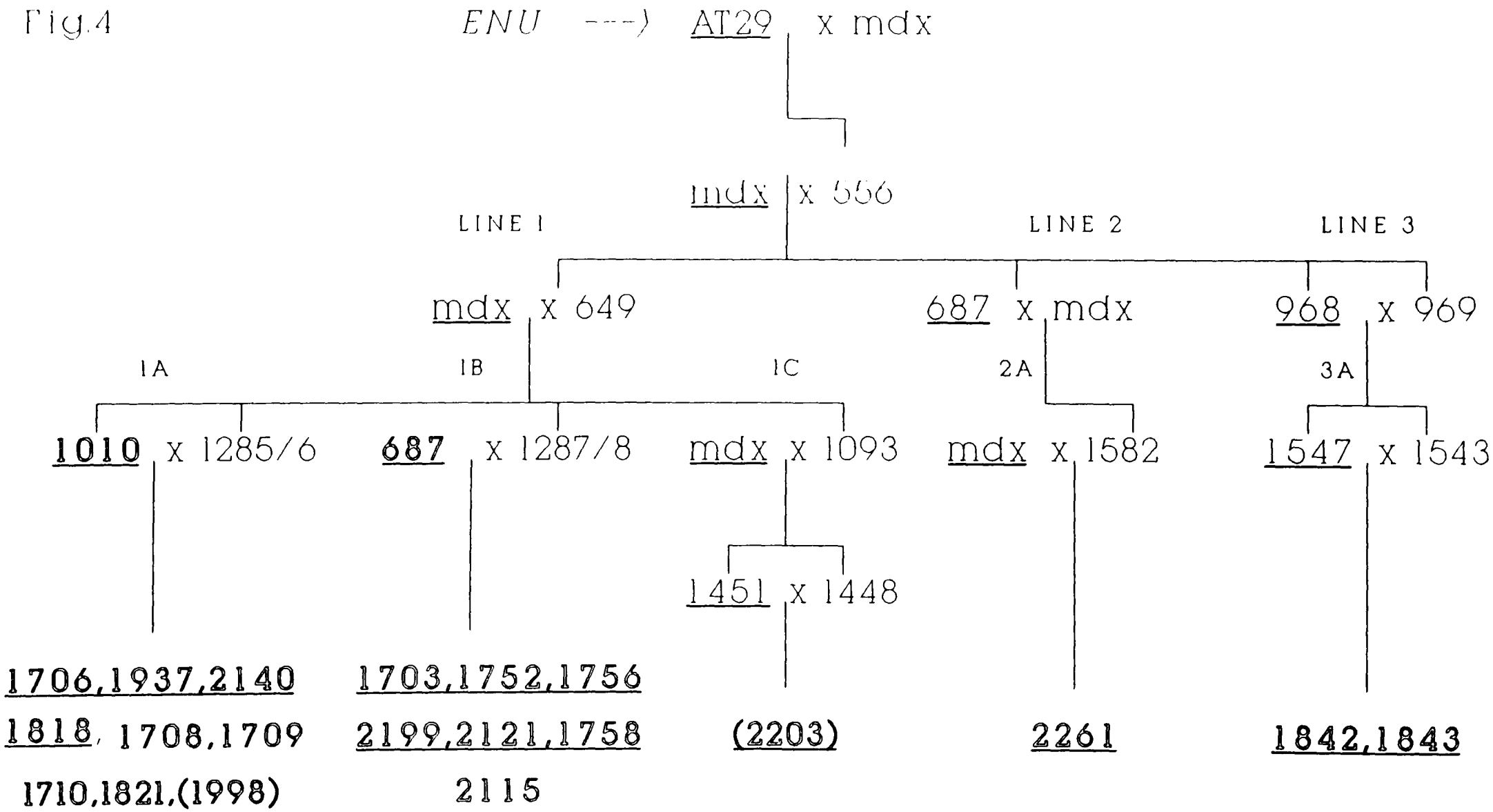
STRAIN	X-CHROMOSOME LOCI
AT29	M^{+} ,xid^{+} ,a
AT29-ENU.556	$\underline{M}^{+}$,xid^{+} ,a
CBA-Pgk-1a	M^{+} ,xid^{+} ,a
CBA	M^{+} ,xid^{+} ,b
CBA/N	M^{+} ,$\underline{xid}$,b

The results of the patterns of PGK-1 expression for the females derived from the male 2203 suggest that M^{+} was inherited by the female offspring giving PII mice and the results for the females derived from the male (2199x1998) suggest that $\underline{M}$ was inherited by half the offspring giving PI and PIII mice and that M^{+} was inherited by the remainder giving PII and PIV mice (table 4.1).

This suggests that the male 2203 was hemi/homozygous for M^{+} and that the female 1998 was heterozygous for the loci M^{+} and $\underline{M}$ although homozygous for $\underline{Pgk}$-1a and $\underline{Hprt}$a, as defined in Chapter 2. This is discussed further below.

Hence/

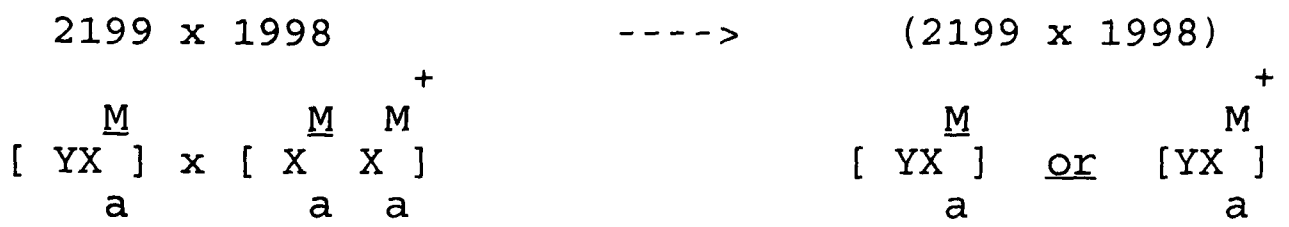
Fig.4



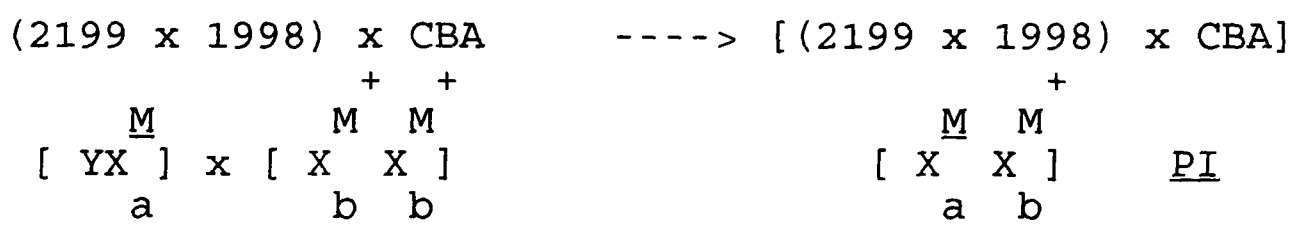
AT29-ENU.556 PEDIGREE

MICE USED IN PRESENT STUDY ARE IN BOLD; MALE MICE ARE UNDERLINED
( ) IS CARRYING WILDTYPE ALLELE

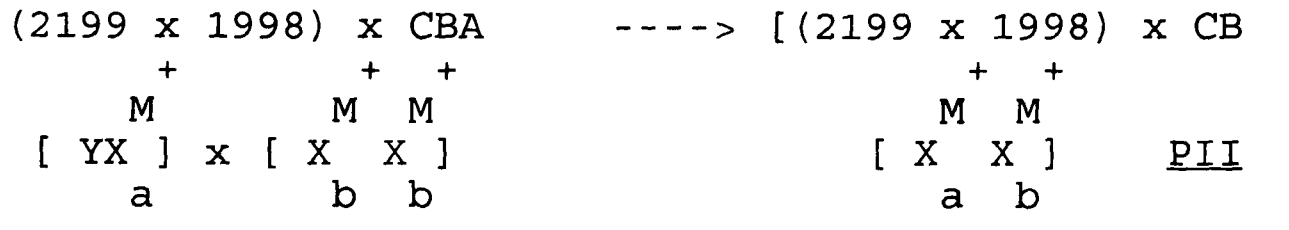
Hence,



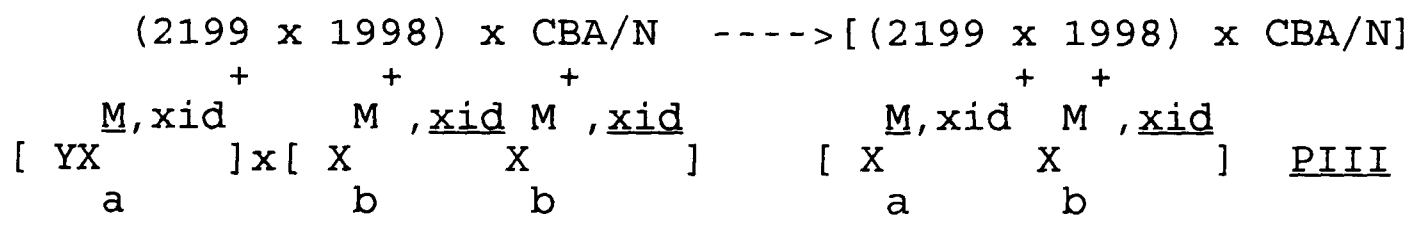
and,



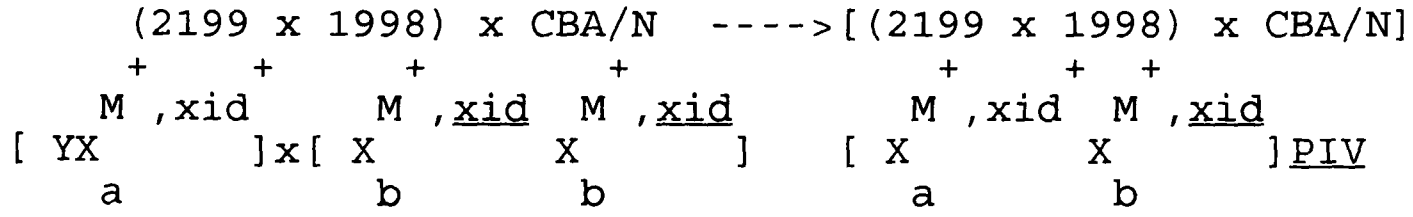
also,



and,



also,



With reference to the pedigree (Fig. 4), males 2199, 1703 and female 2115 were derived from line 1B and male 2261 was derived from line 2A. All were hemi/homozygous for M, as determined by the patterns of PGK-1 expression of their female progeny.

The male 2203 and female 1998 were derived from lines 1C and 1A, respectively. Hence, from the progeny analysed, some mice of line 1 and, more specifically, 1A and 1C carried the allele M^{+}.

As the ENU-mutant mice (M) were selected on the basis of expression of PGK-1A which is discussed in Chapter 2, the introduction of the M^{+} allele to the pedigree, which gave rise to PII and PIV mice, could be explained by the process of recombination such that the mice 2203 and 1998, which were screened as hemi/homozygous for Pgk-1a, carried the allele M^{+}.

The process of recombination is the rearrangement of genetic material at crossover points or chiasmata on nonsister chromatids of homologous chromosomes during meiosis (Morgan, 1910; Tease and Jones, 1978). This results in the formation of different arrangements of alleles in the gametes such that the original arrangements of alleles are called the parental chromosomes and the two new combinations are called recombinants. Sturtevant, 1913 suggested that the percentage of recombinants is a quantitative index of the linear distance between two genes such that the greater the distance between genes on a chromosome the greater the chance that nonsister chromatids cross over in the region between the genes and hence the greater the proportion of recombinants that would be produced.

If a cross over had occurred between the loci M and Pgk-1 then the recombinant X chromosome would carry M^{+} and Pgk-1a and the X chromosome with the parental combination would carry M and Pgk-1a as the mice were screened for expression of PGK-1A (Chapter 2).

The hypothesis states that the production of PII and PIV females results from the inheritance of the recombinant X chromosome through the male 2203 (line 1C) and through the female 1998 (line 1A) and the production of PI and PIII females results from the inheritance of the X chromosome with the parental combination of loci through the female 1998 (line 1A) (Fig. 4).

Recombination could have occurred in female 649 involving at least two gametes that affected line 1 or in females, 1285/1286 and 1093 involving at least one gamete per female that affected line 1A and 1C, respectively (Fig. 4).

DISCUSSION

This hypothesis offers an explanation for the phenomenon of phenotypic variation in AT29-ENU.556 crosses. It depends on :

(1) the assumption that there is one locus controlling the haematopoietic and skeletal muscle systems as both systems are affected in all cases

(2) the production of at least one gamete carrying the

recombinant X chromosome in females 1285/1286 and 1093 or the production of two gametes carrying the recombinant X chromosome in female 649 only (Fig. 4)

The question to be addressed is whether recombination occurred in one or several females. There is evidence of a high frequency of recombination on the X chromosome (Davies et al, 1985; Grimm et al, 1989). The frequency of recombination has been shown to increase by various recombinagenic agents such as mutagens which affect DNA metabolism, repair and recombination (Kunz and Haynes, 1981). Another possibility worth mentioning, is that the ENU mutation induced a meiotic-like recombination event during mitosis of the germ line of female 649. Inter-chromosomal recombination could explain the production of at least two gametes carrying the recombinant X chromosome in female 649.

Before formation of the recombinants, females 649, 1285/1286 and 1093 carried a paternally-derived X chromosome which was of C57BL/10.mdx background with the alleles Pgk-1b, M^+ and mdx and a maternally-derived X chromosome which was of AT29-ENU.556 background with the alleles Pgk-1a, M and mdx^+. To speculate further, the recombinant X chromosome in PII and PIV mice could have been of C57BL/10.mdx background with the alleles Pgk-1a, M^+ and mdx or of AT29-ENU.566 background with the alleles Pgk-1a, M^+ and mdx^+ (Fig. 4).

The genotype of the recombinant X chromosome in PII and PIV mice might be assessed by the identification of traits specific to C57BL/10.mdx or AT29-ENU.556 mice. For example, measuring the levels of creatine phosphokinase (Chapman et al, 1989) or probing for mdx might indicate the presence of mdx on the recombinant X chromosome.

This hypothesis may reflect the positional relationship of the loci M and Pgk-1 where the greater the distance between loci on a chromosome the greater the chance that nonsister chromatids cross over in the region between the loci and hence the greater the proportion of recombinants produced.

Linkage mapping utilises the process of recombination to map loci in relation to each other as the percentage of recombinants is a quantitative index of the linear distance between two loci.

CHAPTER 5

X-Chromosome Inactivation Studies In Normal Tissues Of AT29 And AT29-ENU.556 Hybrids

X-chromosome controlling elements have been shown to produce non-random inactivation of the X chromosome (Cattanach and Williams, 1972; West and Chapman, 1978). The murine locus (*Xce*) was mapped to the XD region, between *mdx* and *Pgk*-1 (Cattanach, 1970; Cattanach *et al*, 1970). Allelic variants of this locus influence X-chromosome inactivation such that an X chromosome expressing the Xce^{a} allele has a greater chance of being inactivated than an X chromosome carrying the Xce^{b} allele which, in turn, is more likely to be inactivated than an X chromosome carrying the Xce^{c} allele (Cattanach, 1972; Johnston and Cattanach, 1981). It has also been shown that the parental source of the *Xce* allele determines which X chromosome is inactivated (Rastan and Cattanach, 1983). For example, in females heterozygous for *Xce* (b/c), the X^{M} chromosome bearing the Xce^{c} allele is less likely to be inactivated than the X^{P} chromosome carrying the same allele (Forrester and Ansell, 1985). Further evidence of parental effects on expression of the X chromosome, in addition to the possible existence of a fourth *Xce* allele have been demonstrated (Fowlis, 1988; Fowlis *et al*, 1991).

Studies of X-chromosome inactivation patterns using PGK-1 alloenzyme analysis were used to examine variation of PGK-

1 expression to determine the nature of the Xce in AT29 and AT29-ENU.556 mice and to examine the parental effects in the hybrids of these and CBA and CBA/N mice.

The following hybrids were considered:

Hybrid 1: AT29 x CBA

Hybrid 2: CBA x AT29

Hybrid 3: AT29-ENU.556 x CBA (PI)

Hybrid 4: CBA x AT29-ENU.556 (PI*)

Hybrid 5: AT29 x CBA/N

Hybrid 6: AT29-ENU.556 x CBA/N (PIII)

CBA mice carry the Pgk-1b and Xce^{a} alleles. It is assumed that CBA/N mice (Pgk-1b) also carry the same Xce allele as CBA mice as xid was originally a mutation in CBA stocks and it is assumed that Pgk-1 and Xce tend to cosegregate as they are closely linked and any recombination between these loci would be expected to be infrequent (Cattanach et al, 1970; Franke and Taggart, 1980; Forrester and Ansell, 1985). Similarly, the Xce allele of AT29 and AT29-ENU.556 mice (both Pgk-1a) is expected to be the same. This would be expected to be Xce^{c} which is associated with Pgk-1a. C57BL/6 lines have been shown to carry Xce^{b} (Cattanach et al, 1970).

PGK-1 alloenzyme analysis was performed on tissues unaffected by either the xid or the ENU mutation. These included brain of all hybrids and skeletal muscle of hybrids 1, 2 and 5. Individual variation in PGK-1

expression may depend on whether cells seeding different tissues originated from the same precursor pool and whether the sampling events concerned with seeding the tissue involved few cells. For example, similar PGK-1 levels have been noted for blood granulocytes, erythrocytes and lymphocytes (Gandini and Gartler, 1969). Similarly, Gornish et al, 1972 and Micklem et al, 1987 showed that cells of the thymus, bone marrow, spleen and blood originated from the same precursor pool.

In this study, PGK-1 expression was determined for skeletal muscle with mesodermal origin and brain with ectodermal origin. Genotypes and mean %PGK-1A values of hybrids are presented in table 5A. Student's t-test was used to examine possible differences in the mean %PGK-1A values of the following hybrids:

reciprocal hybrids:

1 versus 2; 3 versus 4.

AT29 compared with AT29-ENU.556 hybrids:

1 versus 3

CBA compared with CBA/N hybrids:

1 versus 5; 3 versus 6.

It was expected that there would be no significant difference between:

(1) the AT29 and AT29-ENN.556 hybrids (1 versus 3) or the CBA and CBA/N hybrids (1 versus 5 and 3 versus 6) as the Xce allele for the respective groups is assumed to be the

same.

(2) the reciprocal hybrids assuming AT29 and AT29-ENU.556 mice carry the $\underline{Xce}^{c}$ allele. Studies in hybrids carrying the Xce^{a} and Xce^{c} alleles showed no reciprocal cross differences in the mean %PGK-1A values as there are no parental effects for this combination of alleles and demonstrated high mean values of %PGK-1A for both the c/a and the a/c hybrids (Johnston and Cattanach, 1981; Forrester and Ansell, 1985).

Table 5: Genotypes and mean %PGK-1A values of hybrids

		HYBRID					
		1	2	3	4	5	6
MALE PARENT		AT29	CBA	AT29-ENU.556	CBA	AT29	AT29-ENU.556
Pgk-1 ALLELE		a	b	a	b	a	a
Xce ALLELE on X	P	?	a	?	a	?	?
FEMALE PARENT		CBA	AT29	CBA	AT29-ENU.556	CBA/N	CBA/N
Pgk-1 ALLELE		b	a	b	a	b	b
Xce ALLELE on X	M	a	?	a	?	a	a
		MEAN %PGK-1A					
BRAIN		**69**±10 (4)	**55**±7 (4)	**63**±13 (34)	**64**±6 (5)	**60**±9 (4)	**64**±12 (14)
MUSCLE		**61**±12 (4)	**46**±7 (4)	/	/	**56**±7 (4)	/

showing mean %PGK-1A values ± standard deviation and sample number in brackets

X^{P} = paternally-derived X chromosome

X^{M} = maternally-derived X chromosome.

Results and Discussion

There was no significant difference in the mean %PGK-1A values between CBA and CBA/N hybrids for brain (1 versus 5 and 3 versus 6) and for skeletal muscle (1 versus 5). This is consistent with the assumption that the Xce allele for CBA and CBA/N is the same, as discussed above.

There was no significant difference in the mean %PGK-1A values between AT29 and AT29-ENU.556 hybrids for brain (1 versus 3) which is consistent with the assumption that the Xce allele for AT29 and AT29-ENU.556 is the same, as discussed above.

There was no significant difference in the mean %PGK-1A values between the reciprocal hybrids 1 versus 2 which is (AT29xCBA) versus (CBAxAT29) for brain and skeletal muscle and also, 3 versus 4 which is (AT29-ENU.556xCBA) versus (CBAxAT29-ENU.556) for brain. This is consistent with an $Xce^{a/c}$ genotype which has no reciprocal cross differences in the mean %PGK-1A values as there are no parental effects for this combination of Xce alleles with high levels of PGK-1A expression (60-70% PGK-1A), as discussed above (Johnston and Cattanach, 1981; Forrester and Ansell, 1985).

CHAPTER 6

Flow Cytometric Analysis Of Lymphocyte Surface Antigens From Homozygous Normal And AT29-ENU.556 Mice And Their F1 Crosses

This chapter is divided into sections:

Section I. comparisons between normal (CBA, AT29) and mutant (AT29-ENU.556) mice.

Section II. comparisons between (a) normal (AT29 and CBA) F1s and mutant (PI) F1s and (b) CBA/N (AT29 x CBA/N) and mutant (PIII) F1s and (c) mutant (PI, PIII) F1s and homozygous CBA or CBA/N, mutant (AT29-ENU.556) and AT29 mice.

It aims to identify immunological defects associated with the X-chromosome inactivation patterns described in Chapter 3 by analysing the effect of the mutation on the relative proportions of B cells and their subsets, T cells and thymocytes and their subsets and high scattering bone marrow cells. The latter are defined as high scattering, as in Chapter 3, on the basis of their relatively high right angle scatter, being structurally more complex than lymphocytes. Fig. 1, Appendix 2 shows the FACS dot plots for bone marrow lymphocytes and high scattering cells separated by their right angle scatter properties.

The B and pre-B cell marker used in this study is a surface glycoprotein termed B220 (Trowbridge et al,

1975; Coffman et al, 1981a, b; Kincade et al, 1981). B cells were also classified according to the density of surface immunoglobulin isotype (see Table 2A, Chapter 2). Population I has high IgD, low IgM; population II has high IgD, high IgM and population III with low IgD, high IgM (Hardy et al, 1982, 1984) Population III are a subpopulation of B cells termed B-1 cells which also express CD5 (Ly-1), a membrane protein found on all T cells. These constitute 1-2% of splenic (Manohar et al, 1982) and 25-50% of peritoneal (Hayakawa et al, 1986) B cells in normal CBA mice. Immunodefective strains such as the NZB and motheaten viable mice have elevated levels of B-1 cells and CBA/N mice have low levels of B-1 cells (Hayakawa et al, 1983, 1986; Sidman et al, 1986). Population I B cells are also absent in CBA/N mice (Hardy et al, 1982, 1984).

Fig. 6.1 is a diagrammatic representation of the FACS analysis delineating population I-III in CBA and CBA/N mice and Fig. 6i-viii shows FACS dot plots of splenic, lymph node and peritoneal B lymphocytes defined as region 1 (R1) which were separated according to the density of surface IgD (FL1) and IgM (FL2). Lymphocytes (R1) were distinguished from a population of cells named high scattering (R2) which had relatively high forward scatter being larger than the lymphocytes in R1. It is believed that the R2 population were dividing lymphoblasts.

Murine T cell markers used in this study include Thy1.2

which is an alloantigen of Thy1 and L3T4 and Lyt2 (see Table 2A, Chapter 2). Thy1.2 is present in high density on cortical thymocytes (80-85% of thymus) and low density on medullary thymocytes (15% of thymus) and peripheral T cells (Fathman et al, 1975; Shortman et al, 1979; Mulder, 1986). $CD4^+$ (murine $L3T4^+$) T cells interact with cells expressing class II MHC antigens and $CD8^+$ (murine $Lyt2^+$) T cells with cells expressing class I MHC (Swain, 1983; Wilde et al, 1983). Although there is evidence that $CD4^+$ ($L3T4^+$) T cells can have cytolytic activity (MacPhail et al, 1987), it is considered that the helper subset is marked by the $CD4^+$ ($L3T4^+$) antigen and the cytolytic subset by the $CD8^+$ ($Lyt2^+$) antigen (Ceredig et al, 1983; Dialynas et al, 1983). Thy1.2 expression should be studied in relation to expression of markers, L3T4 and Lyt2 since it can be dislodged from T cells (Scollay et al, 1982) and may attach to other cells.

Thymocyte subsets were classified as belonging to the inner cortex, the outer cortex and the medulla using a combination of forward light scatter measurements defining relative cell size and intensity of fluorescence measuring the density of Thy1.2 (Fig. k, Appendix 2).

SECTION I

Comparisons Between Normal (CBA, AT29) And Mutant (AT29-ENU.556) Mice

The effect of the ENU mutation on the frequency of B and T cells or thymocytes and T cell subsets was examined using FACS analysis and Student's t-test to compare the mean frequency (%) of cells staining positively for a cell marker in the mutant strain AT29-ENU.556 and normal (CBA, AT29) mice. Comparisons between the strains for each cell type, excluding B lymphocyte subsets are given in tables 6.1-6.3 which follow an account of the results. Summary tables 6.6-6.15 and the FACS dot plots showing B cell subsets, Fig. 6i-viii are presented at the end of this chapter. Representative FACS dot plots for all tissues, Fig. a-p are given in Appendix 2.

Ia The Normal Phenotype: Comparison Between CBA And AT29 Mice

It was important to characterise the phenotype of AT29 mice in terms of the frequency of lymphocyte subsets constituting various haematopoietic tissues as the AT29-ENU.556 mutant strain was derived from the AT29 line. The well-characterised strain, CBA was used for comparison.

B Lymphocytes

AT29 mice had significantly less mean % of B cells ($B220^+$) within the spleen and bone marrow and significantly more

in lymph node than CBA mice. The greatest difference in the mean % of B cells was for lymph node. There was no significant difference between the two strains for blood (table, 6.1, 6.6, 6.8, 6.13, 6.14).

T Lymphocytes or Thymocytes

AT29 mice had a significantly less mean % of T cells ($Thy1.2^+$) within the lymph node than CBA mice which is consistent with the data for B cells above. There was no significant difference in the mean % of T cells for spleen, blood or thymocytes of thymic cortex (C) or medulla (M). However, by splitting the cortex into inner (IC) and outer (OC) parts, AT29 mice had significantly less mean % of thymocytes within the inner cortex and significantly more within the outer cortex than CBA mice (table 6.1, 6.6, 6.8, 6.11, 6.12, 6.14).

T Lymphocyte Subsets

There was no significant difference in the means between CBA and AT29 mice for $Lyt2^+$ cells of all tissues and $L3T4^+$ cells of all tissues with the exception of the lymph node for AT29 mice which had a significantly less mean % of $L3T4^+$ cells than CBA mice. This latter result is consistent with the data for the frequency of B and T cells, discussed above (table, 6.1, 6.7, 6.9, 6.10, 6.15).

High Scattering Cells

Bone marrow high scattering cells were separated from lymphocytes by virtue of their relatively high right angle scatter, being structurally more complex. The bone marrow of AT29 mice had a significantly greater mean % of high scattering cells than CBA mice (table, 6.1, 6.13).

B Lymphocyte Subsets

The B cell subsets, population I-III were present in the spleen and peritoneum of AT29 and CBA mice. Populations I and II, but not III, were present in the lymph node of AT29 and CBA mice. For peritoneum of both strains, the high scattering compartment (R2) which was distinguished from lymphocytes in R1 by virtue of its relatively high forward scatter being larger, contained more population III cells than populations I or II cells (Fig. 6.1, Fig. 6i, v, viii).

DISCUSSION

The results demonstrate several differences in the frequency of leukocyte populations between the CBA and AT29 normal strains. There was a significant difference in in the frequency of B cells for the spleen which was down 15% in AT29 mice (Table 6.1, 6.6) and for lymph node which was up 30% in AT29 mice (Table 6.1, 6.8) and for bone marrow which was down 9% in AT29 mice (Table 6.1, 6.13). There was a significant difference in the frequency of T

cells, particularly $L3T4^+$ cells for the lymph node which was down 30% in AT29 mice (Table 6.1, 6.9). Furthermore, within the marrow, AT29 mice had 20% more high scattering cells than the CBA strain (Table 6.1, 6.13). It is worth considering whether compensatory mechanisms exist between B and $L3T4^+$ cells within the lymph node and between B and high scattering cells within the marrow which might explain the relative increases or decreases of these cell types. Regarding B lymphocyte subsets, there was no apparent difference between AT29 and CBA mice in the distribution of populations I-III within the three tissues studied (Fig. 6i, 6v, 6viii).

Table 6.1/

Table 6.1: Frequency of cells positive for selected lymphocyte markers in CBA and AT29 mice

	mean frequency (%) of positive cells			
	B220	THY1.2	LYT2	L3T4
SPLEEN	**45**,59**	31,30	26,15	19,21
L. NODE	**44**,17**	**54**,83**	32,40	**26**,56**
BLOOD	61,57	53,45	31,16	31,39
THYMUS	/		86,85	88,92

	THY1.2			
	IC	OC	C	M
THYMUS	**49**,68**	**32**,15*	81,82	18,13

	B220	HS
MARROW	**26**,35*	**79**,61**

showing the mean frequency (%) of cells positive for the selected markers and the AT29 means underlined

significant differences, *=P<0.05, **=P<0.01 in bold

HS=high scattering cells defined as having a relatively higher right angle scatter than lymphocytes

Ib The Mutant Phenotype: Comparison Between Normal (AT29) And Mutant (AT29-ENU.556) Mice

Studies of X-chromosome inactivation patterns of heterozygous females, discussed in Chapter 3, placed homozygous AT29-ENU.556 mice into two groups:

(1) a mutant phenotype which produced PI and PIII heterozygous females following crosses with AT29-ENU.556 and CBA and CBA/N mice, respectively.

(2) a non-mutant phenotype which produced PII and PIV heterozygous females following crosses with CBA and CBA/N mice, respectively.

The frequencies of leukocytes were compared between mutant and non-mutant homozygous AT29-ENU.556 mice to identify differences due to the mutation. Comparisons between mutant and non-mutant homozygous mice showed:

(1) a narrow range of values for the frequency of B cells of spleen, lymph node, bone marrow and blood (table 6.3).

(2) a narrow range of values for the frequency of T cells or thymocytes of spleen, lymph node, blood, thymic inner cortex and medulla (Table 6.3)

(3) an overlapping range of values for the frequency of $Lyt2^{+}$ cells of spleen and lymph node (Table 6.3).

(4) a narrow range of values for the frequency of $L3T4^{+}$ cells of spleen, lymph node and blood (Table 6.3).

(5) a narrow range of values for the frequency of high scattering cells of bone marrow (Table 6.3)

Any differences that did exist between mutant and non-mutant mice may be explained by sampling effects or low sample number. For example, see values for frequency of blood $Lyt2^+$; thymus $Lyt2^+$ and $L3T4^+$ and thymus outer cortex $Thy1.2^+$ (Table 6.3).

The results showed that the range of values for the frequency of leukocytes of mutant mice overlapped or were close (within 10%) to those of non-mutant mice which suggests the mutation does not affect leukocyte frequency. Hence, for comparisons of frequency of leukocytes with other strains AT29-ENU.556 mutant and non-mutant mice shall be considered as one group.

Comparisons between AT29-ENU.556 and AT29 mice

B Lymphocytes

There was no significant difference between AT29-ENU.556 and AT29 mice in the mean % of B cells for spleen, lymph node and marrow. AT29-ENU.556 mice had significantly less mean % of B cells than AT29 mice for blood (Table 6.2, 6.6, 6.8, 6.13, 6.14).

T Lymphocytes and Thymocytes

There was no significant difference between AT29-ENU.556 and AT29 mice in the mean % of T cells for lymph node or of thymocytes for thymic cortex, inner cortex and medulla. AT29-ENU.556 mice had significantly less mean % of thymocytes than AT29 mice for outer cortex (Table 6.2, 6.8, 6.11, 6.12).

T Lymphocyte Subsets

There was no significant difference between AT29-ENU.556 and AT29 mice in the mean % of $Lyt2^+$ cells for lymph node and blood and in the mean % of $L3T4^+$ cells for spleen, lymph node and blood. AT29-ENU.556 mice had significantly less mean % of $Lyt2^+$ cells for spleen and thymus than AT29 mice and significantly less mean % of $L3T4^+$ cells for thymus than AT29 mice (Table 6.2, 6.7, 6.9, 6.10, 6.15).

High Scattering Cells

Bone marrow high scattering cells were distinguished from lymphocytes by virtue of their relatively high right angle scatter being structurally complex. AT29-ENU.556 mice had significantly less mean % of high scattering bone marrow cells than AT29 mice (Table 6.2, 6.13).

B Lymphocyte Subsets

AT29 and AT29-ENU.556 mice had populations I-III in the spleen and peritoneum and population I, II but not III in

the lymph node. For the peritoneum of both strains, the high scattering compartment (R2) contained more population III cells than population I or II cells (Fig. 6.1, 6i, 6v, 6viii).

DISCUSSION

The results demonstrate that for most cell types there was no significant difference in the frequency of leukocytes between AT29-ENU.556 and AT29 mice. For thymus $Lyt2^{+}$ and $L3T4^{+}$ cells, of both AT29-ENU.556 and AT29 mice there was great variation within the sample making comparisons difficult. This might explain the difference observed between the two strains for these cell types (Table 6.2, 6.10). Regarding B lymphocyte subsets, there was no obvious difference between AT29 and AT29-ENU.556 mice in the distribution of populations I-III within the three tissues studied.

It is interesting that those cell types in AT29 mice which differed most from those in CBA mice, differed least from those in AT29-ENU.556 mice. For example see Table 6.8 for lymph node stained for B220. This implies that the most obvious characteristics of the AT29 strain are also present in AT29-ENU.556 mice, despite the mutation.

Table 6.2: Frequency of cells positive for selected lymphocyte markers in AT29-ENU.556 and AT29 mice

mean frequency (%) of positive cells

	B220	THY1.2	LYT2	L3T4
SPLEEN	51,45	24,31*	14,26**	16,19
L NODE	42,44	51,54	29,32	23,26
BLOOD	35,61**	24,53**	19,31	19,31
THYMUS	/		72,86**	78,88*

	THY1.2			
THYMUS	IC	OC	C	M
	56,49	16,32**	75,81	20,18

	B220	HS
MARROW	22,26	69,79**

showing the mean frequency (%) of cells positive for the selected markers and the AT29-ENU.556 mean underlined

significant difference, *=P<0.05, **=P<0.01

HS=high scattering cells defined as having a relatively higher right angle scatter than lymphocytes

Table 6.3: Frequency of cells positive for selected lymphocyte markers in homozygous AT29-ENU.556 mutant(M) and AT29-ENU.556 non-mutant(NM) mice

mean frequency (%) of positive cells

	B220	THY1.2	LYT2	L3T4
SPLEEN				
M	**48-52,**50 (2)	**25-33,**29 (3)	**3-26,**16 (3)	**16-22,**17 (3)
NM	**55-73,**66 (4)	**20-31,**25 (3)	**2-13,** 9 (4)	**7-14,**12 (4)
LYMPH NODE				
M	**38-39,**39 (2)	**49-61,**57 (3)	**32-43,**37 (3)	**16-19,**17 (3)
NM	**35-55,**47 (3)	**43-49,**45 (3)	**25-37,**31 (3)	**8-18,**14 (4)
BLOOD				
M	**20-36,**31 (3)	/ **12** (1)	33-37,35 (2)	**23-31,**27 (2)
NM	**16-53,**39 (3)	**14-29,**22 (2)	/ 9 (1)	/ **21** (1)
THYMUS				
M	/		66-74,71 (3)	71-75,73 (3)
NM	/		84-87,86 (2)	/ 89 (2)

	THY1.2		
THYMUS	IC	OC	M
M	/ **69** (1)	/ 8 (1)	/ **16** (1)
NM	/ **71** (1)	/ 21 (1)	/ **8** (1)

	B220	HS
MARROW		
M	**8-11,**10 (2)	**70-81,**74 (3)
NM	**16-27,**20 (4)	**45-63,**56 (3)

showing the range of values of the frequency (%) of cells staining positive for the selected markers and the mean underlined and sample number given in brackets

HS= high scattering cells defined as having a relatively higher right angle scatter than lymphocytes

overlapping or close (within 10%) ranges are given in bold

SECTION II

Analysis Of Lymphocyte Subpopulations In Mutant (PI, PIII) Female Mice

Student's t-test was used to compare the mean frequency (%) of cells staining positive for selected markers in hybrids. Results of comparisons, excluding B lymphocyte subsets are given in tables 6.4 and 6.5 which follow an account of the results. Summary tables 6.6-6.15 and the FACS dot plots showing B cell subsets, Fig. 6i-viii are presented at the end of this chapter. Note that CBA x CBA-Pgk-1a F1 females are included in the summary tables to represent a non-AT29 heterozygous group.

IIa Comparisons Between (i) Normal AT29 x CBA F1 females And Mutant (PI) F1 Females and (ii) These F1 Females And The Homozygous Lines From Which They Were Derived

The effect of the ENU mutation on the frequency of leukocytes in the heterozygote was studied by comparing PI F1 females (PIA and the reciprocal cross PI*A, in tables) with normal F1 females ((AT29 x CBA)A and the reciprocal cross). As X-chromosome inactivation studies (Chapter 3) revealed PII F1 females to be of normal phenotype, the data from these mice was pooled with that for the AT29 x CBA F1 females (Table 6.4). The phenotype of the heterozygotes were compared with the homozygous, CBA, AT29-ENU.556 and AT29 phenotype. PI and PII F1 males (PIB and PIIB, respectively in tables) were used as

sibling controls for PI and PII F1 females, as only the CBA X chromosome is inherited. To simplify, PIB and PIIB mice were pooled with CBA mice for comparison with F1 females and the pooled group referred to as the CBA phenotype.

B Lymphocytes

There was no significant difference the mean % of B cells between normal and PI F1 females for spleen, lymph node and bone marrow. There was a significant difference between the normal and PI F1 females for blood. However, this is probably a false result since the sample number for blood was low (Table 6.4).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both normal and PI F1 females had a reduced frequency of lymph node B cells, similar to the CBA phenotype. The converse was the case for bone marrow, in which normal and PI F1 females had an increased frequency of B cells, similar to the CBA phenotype. There was no obvious difference in the mean % of B cells of the spleen between both normal and PI F1 females and their respective homozygous counterparts (Table 6.6, 6.8, 6.13, 6.14).

T Lymphocytes and Thymocytes

There was no significant difference in the mean % of T cells between normal and PI F1 females for spleen, lymph node and blood or thymocytes of thymus including medulla, cortex, inner cortex and outer cortex (Table 6.4).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both normal and PI F1 females had an increased frequency of lymph node T cells, similar to the CBA phenotype. Conversely, compared with homozygous mice, there was a reduced frequency on of cortical thymocytes in the inner cortex of PI F1 females and in the outer cortex of normal F1 females. It should be noted that for the inner cortex and outer cortex of normal F1 females, the sample number was low which may have produced a false result. There was no obvious difference in the mean % of T cells of the spleen between both normal and PI F1 females and their homozygous counterparts (Table 6,6, 6.8, 6.11, 6.12, 6.14).

T Lymphocyte Subsets

There was no significant difference in the means between the normal and PI F1 females for $Lyt2^{+}$ cells of spleen, lymph node, thymus and blood and for $L3T4^{+}$ cells of the spleen, lymph node, thymus and blood. It should be noted that for blood the sample number was low, which may have produce a false result (Table 6.4).

Compared with homozygous AT29-ENU.556 and AT29 mice respectively, both PI and normal F1 females had an <u>increased</u> frequency of lymph node $L3T4^+$ cells, similar to the CBA phenotype. There was no difference in the frequency of $L3T4^+$ cells between normal and PI F1 females and their homozygous counterparts for spleen and thymus.

There was no notable difference in the frequency of $Lyt2^+$ between normal and PI F1 females and their homozygous counterparts for spleen, lymph node and thymus (Table 6.7, 6.9, 6.19, 6.15)

<u>High Scattering Cells</u>

Bone marrow high scattering cells were separated from lymphocytes by virtue of their relatively high right angle scatter, being structurally more complex.

There was no significant difference between normal and PI F1 females in the mean % of high scattering bone marrow cells (Table 6.4).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both normal and PI F1 females had a <u>reduced</u> frequency of high scattering marrow cells, similar to the CBA phenotype (Table 6.13).

<u>B Lymphocyte Subsets</u>

There was no difference between PI F1 females and the CBA phenotype in the distribution of populations I-III within the two spleen and lymph node. For spleen there were

populations I-III present and lymph node had populations I, II but not III present (Fig. 6.1, 6ii, 6vi).

DISCUSSION

The results demonstrate that for most tissues there was no significant difference in the frequency of leukocytes between normal and PI F1 females. The most obvious characteristics of the AT29 and AT29-ENU.556 phenotype differed from the normal F1 and PI F1 phenotype, respectively. For example, see the frequency of B, T and $L3T4^{+}$ cells of the lymph node and high scattering cells of the marrow (Table 6.4, 6.8, 6.9, 6.13).

Regarding B lymphocyte subsets, there was no obvious difference between the CBA phenotype and PI F1 females in the distributions populations I-III within the spleen and lymph node (Fig. 6ii, 6vi).

The results of section II were consistent with section I, which showed that the phenotype of AT29-ENU.556 homozygous and (PI) heterozygous mice was very similar to that of AT29 mice which suggests that the ENU mutation does not affect the frequency of leukocytes quantified by flow cytometry.

Table 6.4/

Table 6.4: Frequency of cells positive for selected lymphocyte markers in normal# and PI F1 females

mean frequency (%) of positive cells

	B220	THY1.2	LYT2	L3T4
SPLEEN	**45**,52	**27**,18	**10**,10	**23**,16
L NODE	**21**,26	**72**,67	**37**,29	**44**,41
BLOOD	**56**,30*	**18**,11	**17**,8	**13**,13
THYMUS			**84**,72	**85**,84

	THY1.2			
THYMUS	IC	OC	C	M
	49,47	**19**,17	**67**,63	**22**,22

	B220	HS
MARROW	**36**,33	**53**,53

showing the mean frequency (%) of cells positive for the selected markers and the normal F1 mice underlined

normal# F1 = (AT29xCBA), (CBAxAT29), PII females pooled data

significant difference, *=P<0.05

HS=high scattering cells defined as having a relatively higher right angle scatter than lymphocytes

IIb Comparisons Between (i) CBA/N (AT29 x CBA/N) F1 Females And Mutant (PIII) F1 Females and (ii) These F1 Females And The Homozygous Lines From Which They Were Derived

The combined effect of the ENU and xid mutation on leukocyte frequencies in PIII F1 females (PIIIA in tables 6.6-6.15) was studied by comparing PIII F1 females with CBA/N F1 females ((AT29 x CBA/N)A in tables). The phenotype of PIII and AT29 x CBA/N F1 females, relative to the homozygous CBA/N, AT29-ENU.556 and AT29 phenotype was established. Note that, from X-chromosome inactivation studies discussed in Chapter 3, PIV F1 females were regarded a CBA/N phenotype, hence PIVA mice were pooled with AT29 x CBA/N F1 females (Table 6.5). PIII and PIV F1 males (PIIIB and PIVB, respectively in tables) were used as sibling controls for PIII and PIV F1 females, since only the CBA/N X chromosome is inherited. To simplify, PIIIB and PIVB mice were pooled with CBA/N mice for comparison with F1 females and the pooled group referred to as the CBA/N phenotype.

B Lymphocytes

There was no significant difference in the mean % of B cells between AT29 x CBA/N and PIII F1 females for spleen and blood. There was a significant difference between the F1 females for lymph node and bone marrow (Table 6.5)

Compared with homozygous AT29 and AT29-ENU.556 mice

respectively, both AT29 x CBA/N and PIII F1 females had a reduced frequency of lymph node B cells, tending towards the CBA/N phenotype. There was no obvious difference in the mean % of B cells of the spleen or bone marrow between both AT29 x CBA/N and PIII F1 females and their respective homozygous counterparts (Table 6.6, 6.8, 6.13, 6.14).

T Lymphocytes or Thymocytes

There was no significant difference in the mean % of T cells between AT29 x CBA/N and PIII F1 females for spleen, lymph node or thymocytes of thymic medulla, cortex, inner cortex and outer cortex. There were no comparisons made for blood (Table 6.5).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both AT29 x CBA/N and PIII F1 females had an increased frequency of lymph node T cells, tending towards the CBA/N phenotype. There was no obvious difference in frequency of T cells of the spleen or thymocytes between both AT29 x CBA/N and PIII F1 females and their homozygous counterparts (Table 6.6, 6.8, 6.11).

T Lymphocyte Subsets

There was no significant difference in the means between the crosses for both $Lyt2^{+}$ and $L3T4^{+}$ cells of spleen, lymph node and thymus. There were no comparisons made for blood (Table 6.5).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both AT29 x CBA/N and PIII F1 females had an <u>increased</u> frequency of lymph node $L3T4^+$ cells, tending towards the CBA/N phenotype. There was no apparent difference in the frequency of $L3T4^+$ cells between both AT29 x CBA/N and PIII F1 females and their homozygous counterparts for the spleen and thymus.

Similarly, there was no difference in the frequency of $Lyt2^+$ between both AT29 x CBA/N and PIII F1 females and their homozygous counterparts for the spleen, lymph node and thymus. There were no comparisons made for blood (Table 6.7, 6.9. 6.10).

<u>High Scattering Cells</u>

Bone marrow high scattering cells were separated from lymphocytes by virtue of their relatively high right angle scatter being structurally more complex. There was no significant difference between the AT29 x CBA/N and PIII F1 females in the mean % of high scattering bone marrow cells (Table 6.5).

Compared with homozygous AT29 and AT29-ENU.556 mice respectively, both AT29 x CBA/N and PIII F1 females had a <u>reduced</u> frequency of high scattering marrow cells, tending towards the CBA/N phenotype (Table 6.13)

<u>B Lymphocyte Subsets</u>

There was a difference between PIII F1 females and the

CBA/N phenotype in the distribution of populations I-III cells within the spleen and lymph node.

Spleen of PIII F1 females had a relatively small proportion of population I and II and a relatively large proportion of III and the CBA/N spleen had a relatively large population II, low III and no I.

Lymph node of PIII F1 females had a relatively small proportion of population I and II but no III and the CBA/N lymph node had a relatively large population II but no I or III.

DISCUSSION

The results demonstrate that for most cases there was no significant difference in the frequency of leukocytes between AT29 x CBA/N and PIII F1 females. Differences can be explained by sampling effects, producing false results. The most obvious characteristics of the AT29 and AT29-ENU.556 phenotype differed from the AT29 x CBA/N F1 and PIII F1 phenotype, respectively. For example, see the frequency of B, T and $L3T4^{+}$ cells of the lymph node and high scattering cells of the marrow (Table 6.8, 6.9, 6.13).

These results give further evidence that the phenotype, in terms of the frequency of leukocytes of AT29-ENU.556 and AT29 mice and their hybrids was very similar which suggests that the ENU mutation does not affect the

frequency of leukocytes quantified by flow cytometry.

The distribution of B cell subsets for CBA/N mice were consistent with previous work which showed low levels of population III (B-1 cells) (Hayakawa *et al*, 1983, 1986; Sidman *et al*, 1986) and an absence of population I B cells (Hardy *et al*, 1982, 1984).

There was a difference between the CBA/N phenotype and PIII F1 females in the distribution of populations I-III for spleen and lymph node.

For spleen, the small population III, large II and an absence of I seen in CBA/N mice tended towards a large population III and small II, I in PIII F1 females. For lymph node, the large population II and an absence of I tended towards a small population II and I in PIII F1 females (Fig. 6iii, 6iv, 6vii).

These results suggest that, despite the ENU mutation, the AT29-ENU.556 cells have corrective ability within the IgD/IgM B cell subset in the ENU/*xid* heterozygote.

Table 6.5/

Table 6.5:Frequency of cells positive for selected lymphocyte markers in normal# and PIII F1 females

mean frequency (%) of positive cells

	B220	THY1.2	LYT2	L3T4
SPLEEN	**46**,39	**28**,28	**17**,12	**21**,18
L NODE	**26**,15*	**65**,73	**34**,35	**50**,38
BLOOD	**39**,35	/	/	/
THYMUS	/		**82**,76	**84**,74

	THY1.2			
THYMUS	IC	OC	C	M
	48,43	**22**,23	**69**,66	**18**,22

	B220	HS
MARROW	**32**,17*	**49**,51

showing the mean frequency (%) of cells positive for the selected markers and the normal F1 mice underlined

normal# F1 = (AT29xCBA/N), PIV females pooled data

significant differences, *=P<0.05

HS=high scattering cells defined as having a relatively higher right angle scatter than lymphocytes

Table 6.6: Frequency of B and T cells in the SPLEENS of Normal, Mutant and Hybrid Mice

	SPLEEN					
	B220			THY1.2		
	n	mean %	SD	n	mean %	SD
PI A	16	55	13	17	18	8
PI B	11	66	7	14	23	8
PII A	3	50	14	3	12	6
PII B	1	63	/	1	23	/
PI* A	4	42	10	4	18	9
(CBAxAT29) A	3	51	2	3	35	19
(CBAxAT29) B	1	69	/	1	25	/
(AT29xCBA) A	1	15	/	1	53	/
(AT29xCBA) B	1	10	/	1	34	/
(CBAxCBA-Pgk-1a) A	5	58	14	5	30	12
CBA C	34	59	9	12	30	16
AT29 C	14	45	14	14	31	16
AT29-ENU.556 C	60	51	15	49	24	7
CBA/N C	21	47	10		/	
PIII A	6	39	11	7	28	8
PIII B	5	40	10	4	30	7
PIV A	12	49	10	12	26	5
PIV B	8	52	12	10	27	5
(AT29xCBA/N) A	3	35	12	3	38	13
(AT29xCBA/N) B	4	40	2	4	24	2

The table shows for spleen, the mean % of cells staining positive for the antigen B220=B cells and Thy1.2=T cells, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for B220 or Thy1.2 is given

A=female mice; B=male mice; C=female and male

Table 6.7: Frequency of T cell subsets in the SPLEENS of Normal, Mutant and Hybrid mice

	SPLEEN					
	LYT2			L3T4		
	n	mean %	SD	n	mean %	SD
PI A	3	10	4	4	16	5
PI B	5	22	13	4	12	6
PII A	1	15	/	1	34	/
PII B	1	25	/	1	14	/
(CBAxAT29) A	2	6	5	1	19	/
(AT29xCBA) A	1	11	/	1	15	/
(AT29xCBA) B	1	11	/	1	17	/
(CBAxCBA-Pgk-1a) A	2	6	1	5	20	5
CBA C	16	15	4	8	21	7
AT29 C	14	26	22	14	19	12
AT29-ENU.556 C	65	14	9	58	16	10
CBA/N C	17	16	5		/	
PIII A	4	12	8	4	18	4
PIII B	3	11	5	2	23	14
PIV A		/		1	3	/
PIV B		/		1	21	/
(AT29xCBA/N) A	2	17	4	2	30	5
(AT29xCBA/N) B	4	10	2	4	20	2

The table shows for spleen, the mean % of cells staining positive for the antigen Lyt2=cytolytic T cells and L3T4=helper T cells, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for Lyt2 or L3T4 is given

A=female; B=male; C=female and male mice

Table 6.8: Frequency of B and T cells in the LYMPH NODES of Normal, Mutant and Hybrid Mice

	LYMPH NODE					
	B220			THY1.2		
	n	mean %	SD	n	mean %	SD
PI A	19	26	6	17	68	10
PI B	15	27	7	13	70	7
PII A	3	23	2	3	73	2
PII B	1	31	/	1	69	/
P1* A	3	20	4	5	64	9
(CBAxAT29) A	3	24	8	3	70	12
(CBAxAT29) B	1	40	/	1	62	/
(AT29xCBA) A	1	8	/	1	78	/
(AT29xCBA) B	1	8	/	1	86	/
(CBAxCBA-Pgk-1a)A	5	15	7	5	73	10
CBA C	31	17	4	11	83	7
AT29 C	14	44	15	14	54	15
AT29-ENU.556 C	58	42	15	51	51	11
CBA/N C	20	11	3		/	
PIII A	7	15	4	7	73	11
PIII B	6	12	3	5	83	7
PIV A	10	27	9	12	64	9
PIV B	8	17	2	10	83	3
(AT29xCBA/N) A	2	17	1	2	70	16
(AT29xCBA/N) B	4	17	2	4	78	3

The table shows for lymph node, the mean % of cells staining positive for the antigen B220=B cells and Thy1.2 =T cells, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for B220 or Thy1.2 is given

A=female mice; B=male mice; C=female and male mice

Table 6.9: Frequency of T cell subsets in the LYMPH NODES of Normal, Mutant and Hybrid Mice

	LYMPH NODE					
	LYT2			L3T4		
	n	mean %	SD	n	mean %	SD
PI A	8	29	7	9	41	5
PI B	6	32	11	5	38	4
PII A	1	37	/	1	34	/
PII B	1	38	/	1	38	/
(CBAxAT29) A	2	36	9	2	40	5
(AT29xCBA) A		/		1	63	/
(AT29xCBA) B		/		1	32	/
(CBAxCBA-Pgk-1a) A	5	28	6	5	50	10
CBA C	24	40	10	9	56	3
AT29 C	14	32	17	14	26	10
AT29-ENU.556 C	64	29	12	59	23	11
CBA/N C	17	29	3		/	
PIII A	3	35	9	3	38	9
PIII B	2	26	8	2	51	14
PIV A	1	31	/		/	
PIV B	1	32	/	1	51	/
(AT29xCBA/N) A	2	36	0	2	50	3
(AT29xCBA/N) B	4	30	4	4	49	5

The table shows for lymph node, the mean % of cells staining positive for the antigen Lyt2=cytolytic T cells and L3T4=helper T cells, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for Lyt2 or L3T4 is given

A=female; B=male; C=female and male mice

Table 6.10: Frequency of thymocyte subsets in the THYMUS of Normal, Mutant and Hybrid Mice

	THYMUS					
	LYT2			L3T4		
	n	mean %	SD	n	mean %	SD
PI A	6	72	10	6	84	10
PI B	6	78	7	4	88	11
PII A	2	80	11	1	87	/
PII B	2	82	1	1	91	/
(CBAxAT29) A	2	90	1	2	84	1
(AT29xCBA) A	1	82	/	1	84	/
(AT29xCBA) B	1	79	/	1	77	/
(CBAxCBA-Pgk-1a) A	4	81	10	4	87	16
CBA C	20	85	3	6	92	2
AT29 C	14	86	11	14	88	10
AT29-ENU.556 C	42	72	17	39	78	14
CBA/N C	13	80	6		/	
PIII A	3	76	6	3	74	9
PIII B	2	73	11	2	70	16
PIV B	1	84	/	1	84	/
(AT29xCBA/N) A	2	82	11		/	
(AT29xCBA/N) B	4	81	5		/	

The table shows for thymus, the mean % of cells staining positive for the antigen Lyt2=cytolytic thymocytes and L3T4=helper thymocytes, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for Lyt2 or L3T4 is given

A=female; B=male; C=female and male mice

Table 6.11: Frequency of thymocytes in the cortex and medulla of THYMUS of Normal, Mutant and Hybrid Mice

	THYMUS CORTEX THY1.2			THYMUS MEDULLA THY1.2		
	n	mean %	SD	n	mean %	SD
PI A	13	66	13	12	20	9
PI B	10	73	15	12	22	13
PII A	3	69	4	3	14	3
PII B	1	87	/	1	13	/
PI* A	5	55	9	5	28	12
(CBAxAT29) A	3	73	14	3	19	10
(CBAxAT29) B	1	75	/	1	25	/
(AT29xCBA) A	1	40	/	1	53	/
(AT29xCBA) B	1	50	/	1	44	/
(CBAxCBA-Pgk-1a) A		/		3	23	7
CBA C	6	82	7	5	13	5
AT29 C	14	81	7	14	18	7
AT29-ENU.556 C	18	75	17	18	20	14
PIII A	6	66	9	6	22	8
PIII B	5	75	11	5	19	10
PIV A	13	67	12	10	19	7
PIV B	4	75	12	5	19	11
(AT29xCBA/N) A	2	85	8	2	12	5
(AT29xCBA/N) B	4	67	6	4	30	8

The table shows for cortex and medulla of thymus, the mean % of cells staining positive for the antigen Thy1.2= thymocytes, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for Thy1.2 is given

A=female; B=male; C=female and male; PI* is the reciprocal PI cross

Table 6.12: Frequency of thymocytes in the inner and outer cortex of THYMUS of Normal, Mutant and Hybrid Mice

	THYMUS I.CORTEX THY1.2			THYMUS O.CORTEX THY1.2		
	n	mean %	SD	n	mean %	SD
PI A	12	49	14	13	18	6
PI B	9	71	14	9	8	4
PII A	3	51	8	3	18	8
PII B	1	74	/	1	12	/
PI* A	5	41	11	5	14	4
(CBAxAT29) A	3	51	12	3	24	3
(CBAxAT29) B	1	70	/	1	5	/
(AT29xCBA) A	1	35	/	1	5	/
(AT29xCBA) B	1	43	/	1	7	/
(CBAxCBA-Pgk-1a) A	3	57	21	3	7	3
CBA C	6	68	11	6	15	8
AT29 C	14	49	12	14	32	16
AT29-ENU.556 C	13	56	19	12	16	6
PIII A	6	43	14	6	23	9
PIII B	5	59	18	5	16	8
PIV A	13	48	13	13	20	7
PIV B	4	48	24	4	21	11
(AT29xCBA/N) A	3	50	10	2	34	5
(AT29xCBA/N) B	4	52	13	4	15	8

The table shows for the inner and outer cortex of thymus, the mean % of cells staining positive for the antigen Thy1.2=thymocytes, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for Thy1.2 is given

A=female; B=male; C=female and male; PI*is the reciprocal PI cross

Table 6.13: Frequency of B and high scattering cells in the BONE MARROW of Normal, Mutant and Hybrid Mice

	BONE MARROW					
	B220			HS		
	n	mean %	SD	n	mean %	SD
PI A	7	38	9	13	56	9
PI B	4	43	8	11	60	7
PII A	2	40	10	3	49	2
PII B	1	34	/	1	49	/
PI* A	4	26	8	3	38	4
(CBAxAT29) A	3	36	6	3	65	2
(CBAxAT29) B	1	5	/	1	70	/
(AT29xCBA) A	1	26	/	1	30	/
(AT29xCBA) B	1	26	/	1	41	/
(CBAxCBA-Pgk-1a)A	4	39	13	3	74	5
CBA C	13	35	12	22	61	10
AT29 C	14	26	7	14	79	6
AT29-ENU.556 C	48	22	12	61	69	11
CBA/N C		/		16	72	10
PIII A	6	17	5	6	51	12
PIII B	5	22	4	4	68	3
PIV A	2	27	6	12	47	12
PIV B	3	23	4	8	63	8
(AT29xCBA/N) A	3	35	8	3	54	8
(AT29xCBA/N) B	4	20	19	4	67	4

The table shows for bone marrow, the mean % of cells staining positive for the antigen B220=B cells and the mean % of cells with higher right angle scatter than lymphocytes=HS cells, the sample number, n and the standard deviation; where n=1, a single value of % of positive cells staining for B220 or % high scattering cells is given; A=female; B=male; C=female and male mice; PI* is the reciprocal PI cross

Table 6.14: Frequency of B and T cells in the BLOOD of Normal, Mutant and Hybrid Mice

	BLOOD					
	B220			THY1.2		
	n	mean %	SD	n	mean %	SD
PI A	6	30	12	1	11	/
PI B	5	40	15	1	22	/
PII A	1	38	/		/	
(CBAxAT29) A	2	66	1	2	18	11
(CBAxCBA-Pgk-1a)A	1	83	/		/	
CBA C	23	57	18	4	45	13
AT29 C	5	61	9	5	53	22
AT29-ENU.556 C	49	35	15	37	24	15
CBA/N C	11	39	15		/	
PIII A	3	35	8		/	
PIII B	2	18	3		/	
PIV A	10	36	16		/	
PIV B	9	40	12		/	
(AT29xCBA/N) A	1	64	/	1	24	/
(AT29xCBA/N) B	2	31	6	2	28	8

The table shows for blood, the mean % of cells staining positive for the antigen B220=B cells and Thy1.2=T cells, the sample number, n and the standard deviation

where n=1, a single value of % of positive cells staining for B220 or Thy1.2 is given

A=female; B=male; C=female and male mice

Table 6.15: Frequency of T cell subsets in the BLOOD of Normal, Mutant and Hybrid Mice

	BLOOD					
	LYT2			L3T4		
	n	mean %	SD	n	mean %	SD
PI A	1	8	/	1	13	/
PI B	1	18	/	1	17	/
(CBAxAT29) A	2	16	2	2	13	3
CBA C	12	16	14	4	39	12
AT29 C	5	31	12	5	31	25
AT29-ENU.556 C	28	19	14	24	19	11
CBA/N C	9	13	7		/	
(AT29xCBA/N) A	1	7	/	1	25	/
(AT29xCBA/N) B	2	8	1	2	28	1

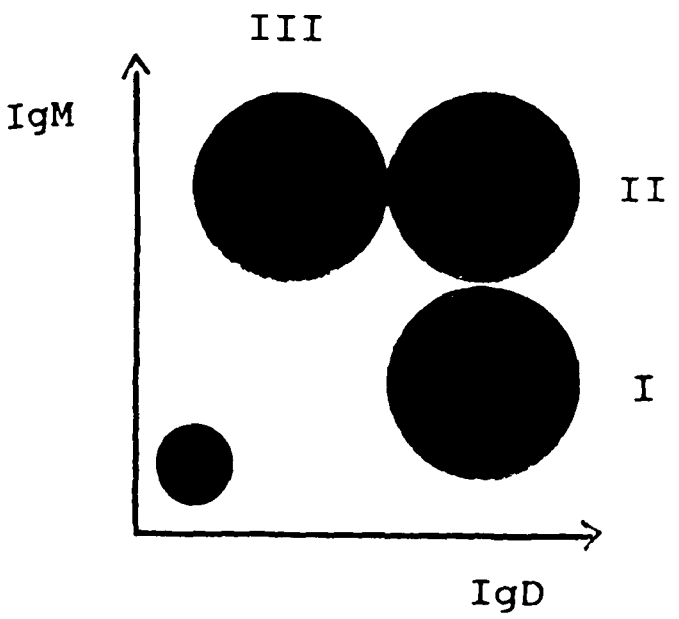
The table shows for blood, the mean % of cells staining positive for the antigen Lyt2=cytolytic T cells and L3T4=helper T cells, the sample number, n and the standard deviation

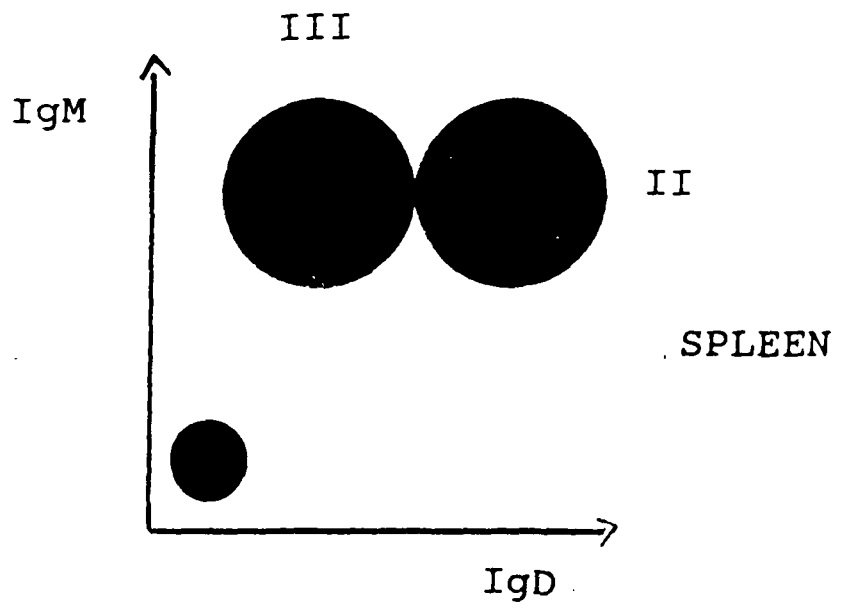
where n=1, a single value of % of positive cells staining for Lyt2 or L3T4 is given

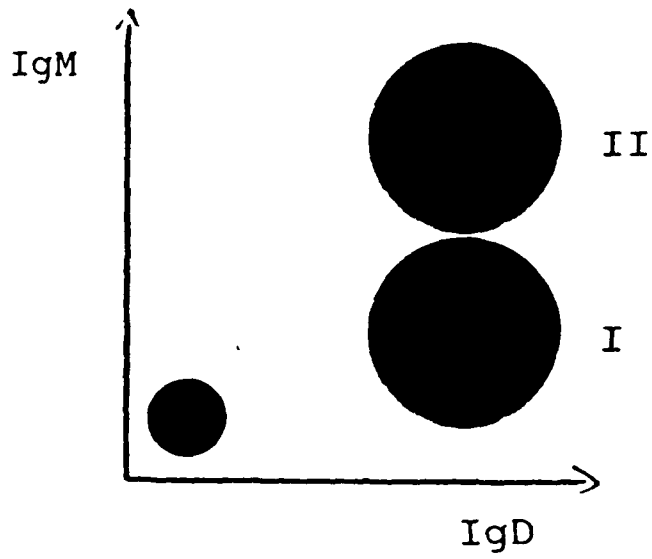
A=female; B=male mice; C=male and female

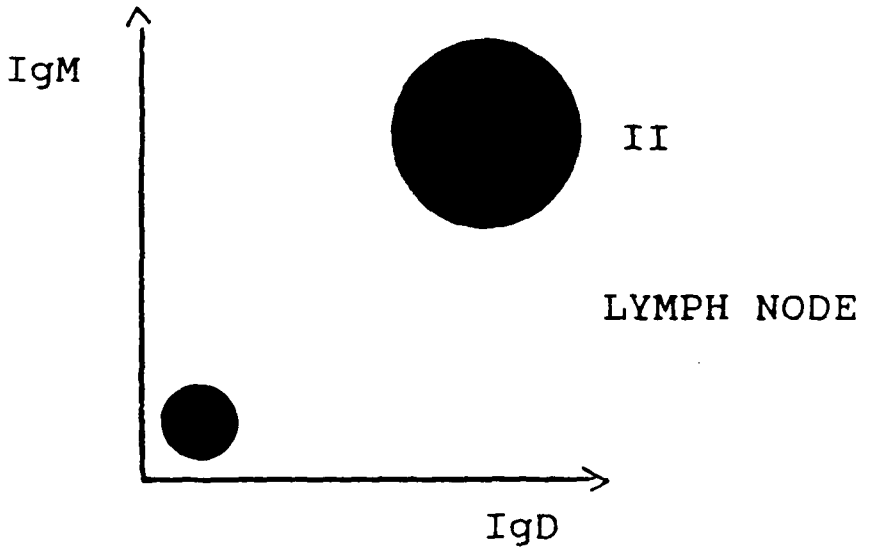
Fig. 6.1

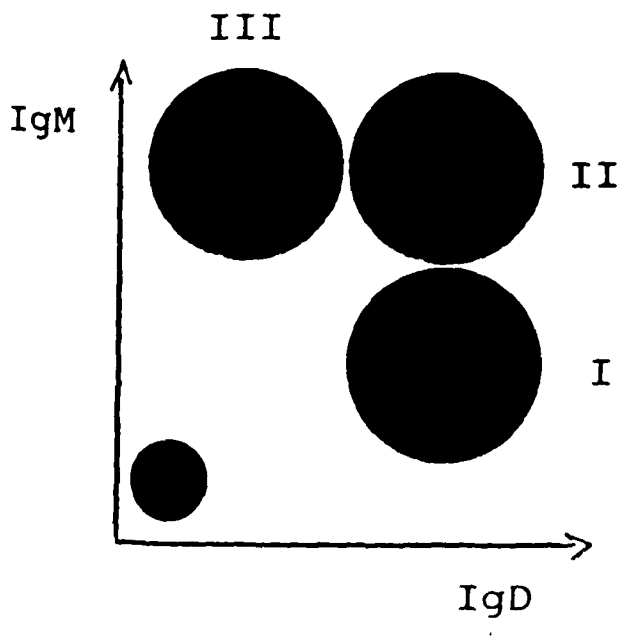
Diagrammatic representation of FACS analysis of IgD/IgM B cell subsets (Population I - III) of CBA and CBA/N mice.

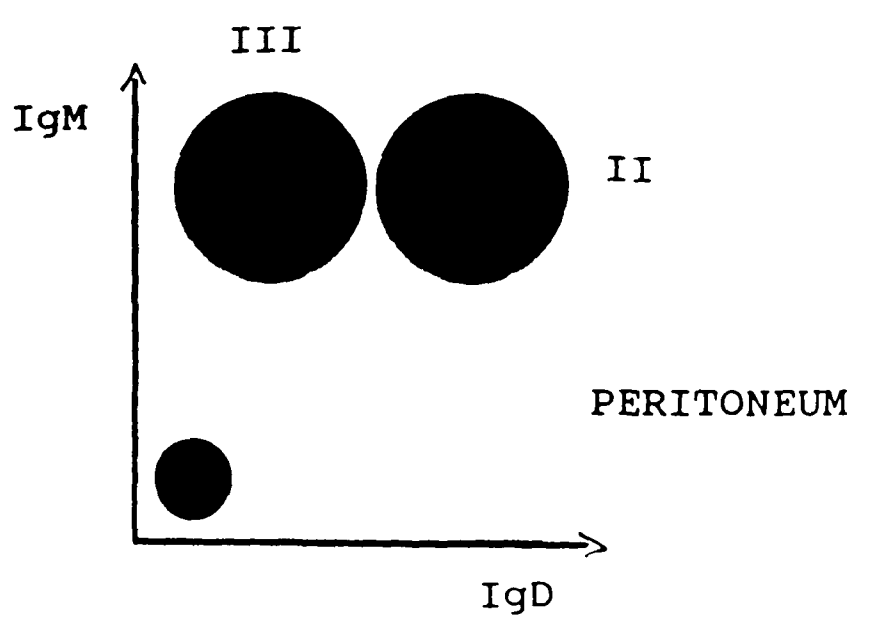












CBA

CBA/N

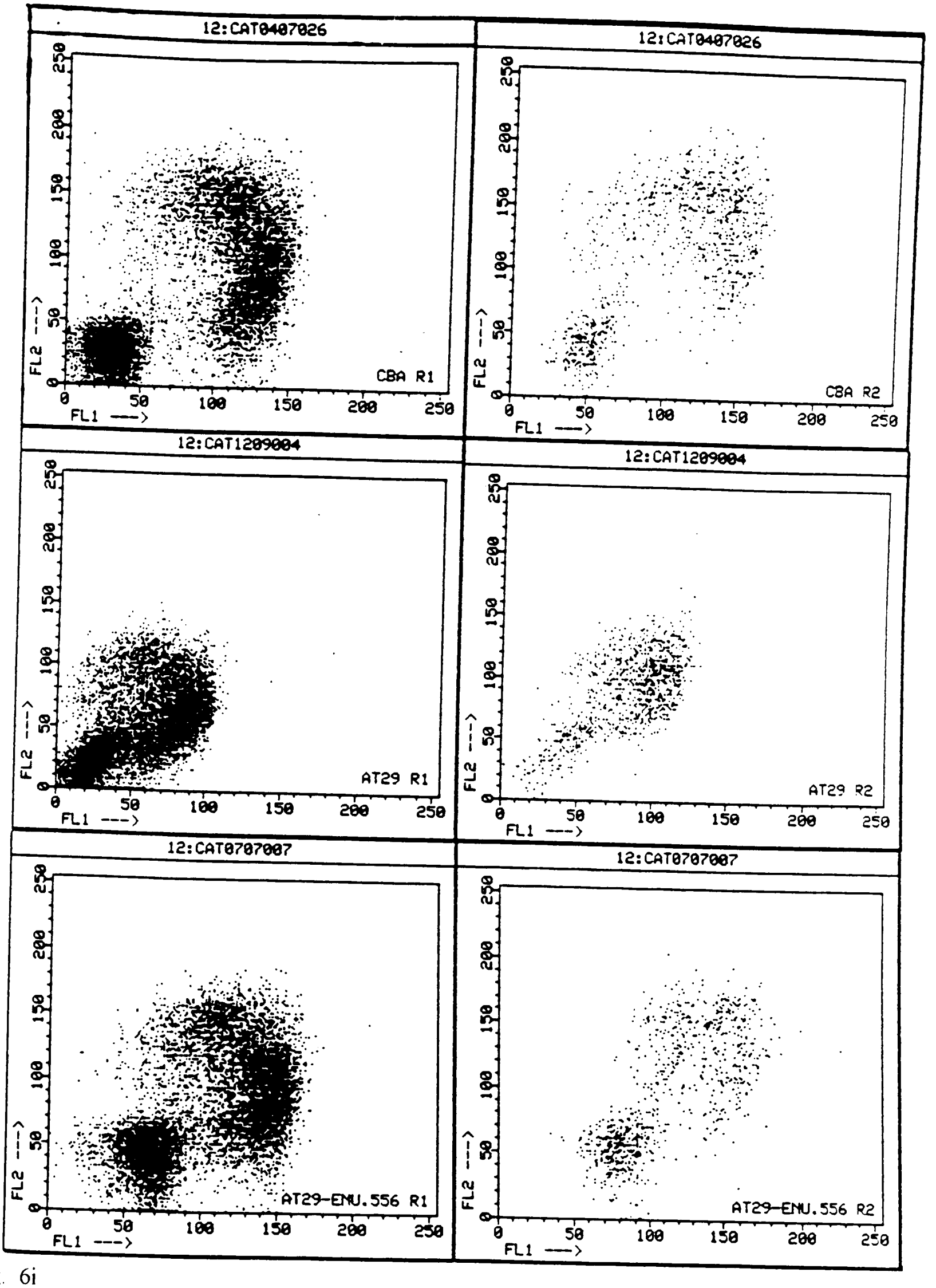


Fig. 6i

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of spleen stained for IgD (FL1) and IgM (FL2).

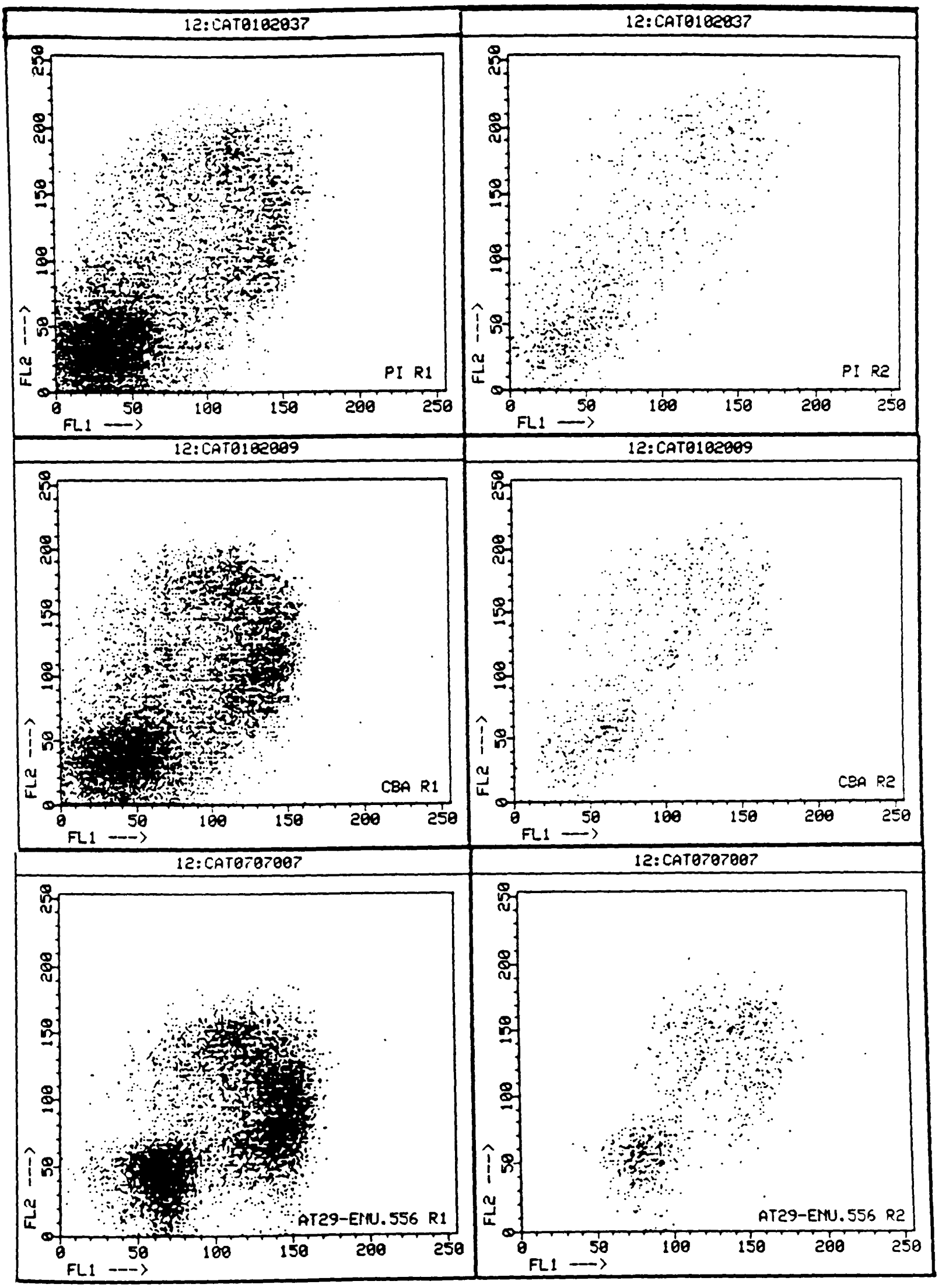


Fig. 6ii

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of spleen stained for IgD (FL1) and IgM (FL2).

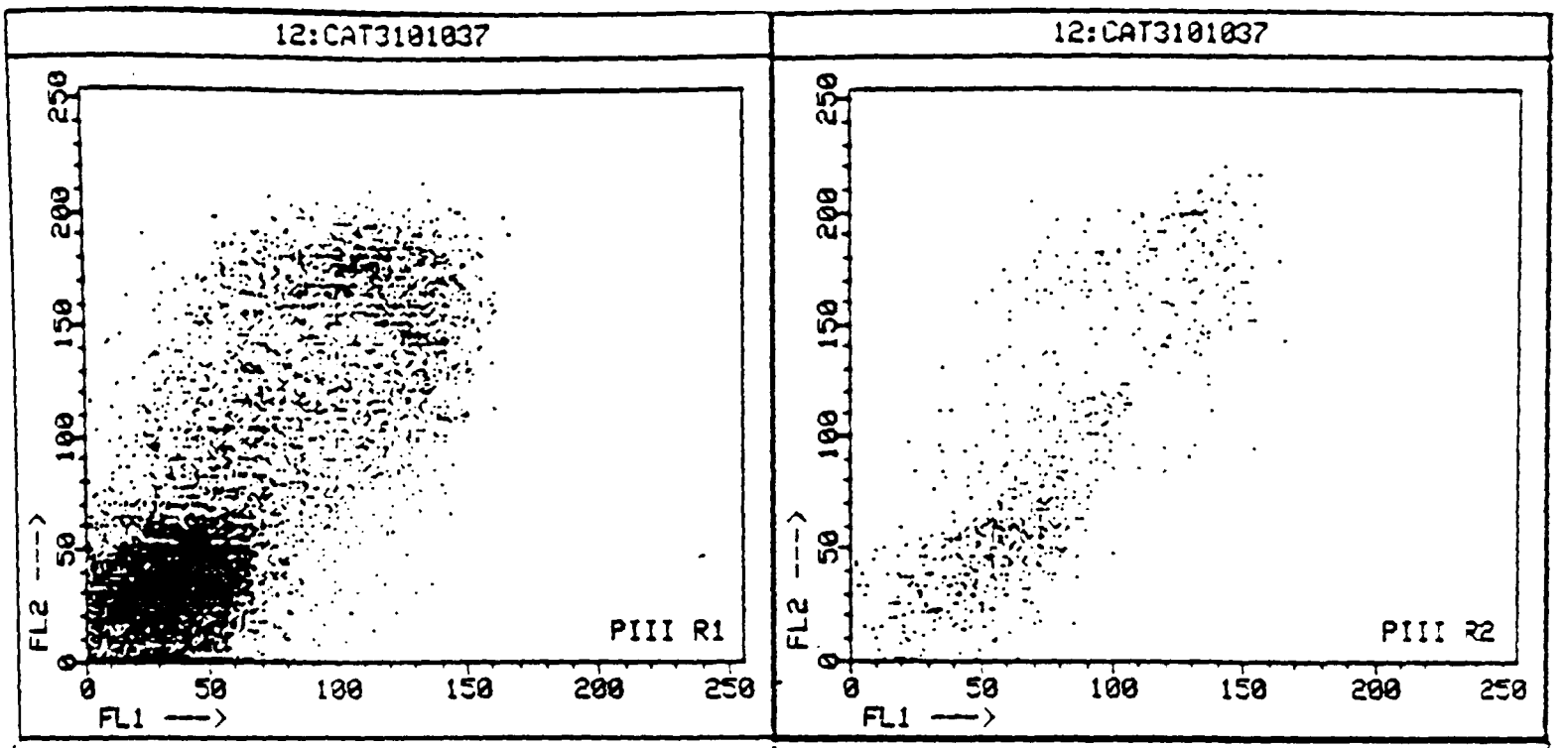


Fig. 6iii

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of spleen stained for IgD (FL1) and IgM (FL2).

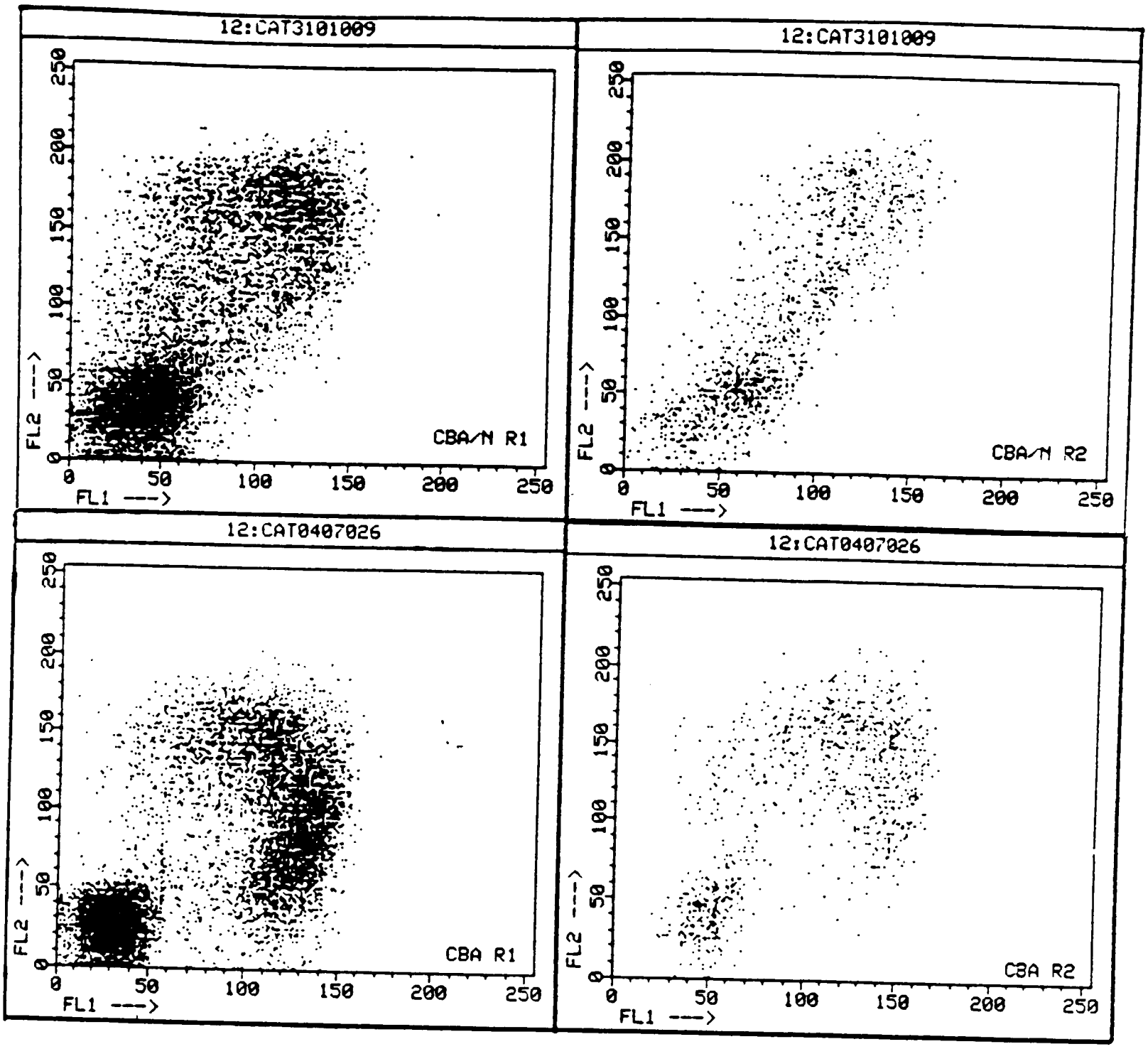


Fig. 6iv

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of spleen stained for IgD (FL1) and IgM (FL2).

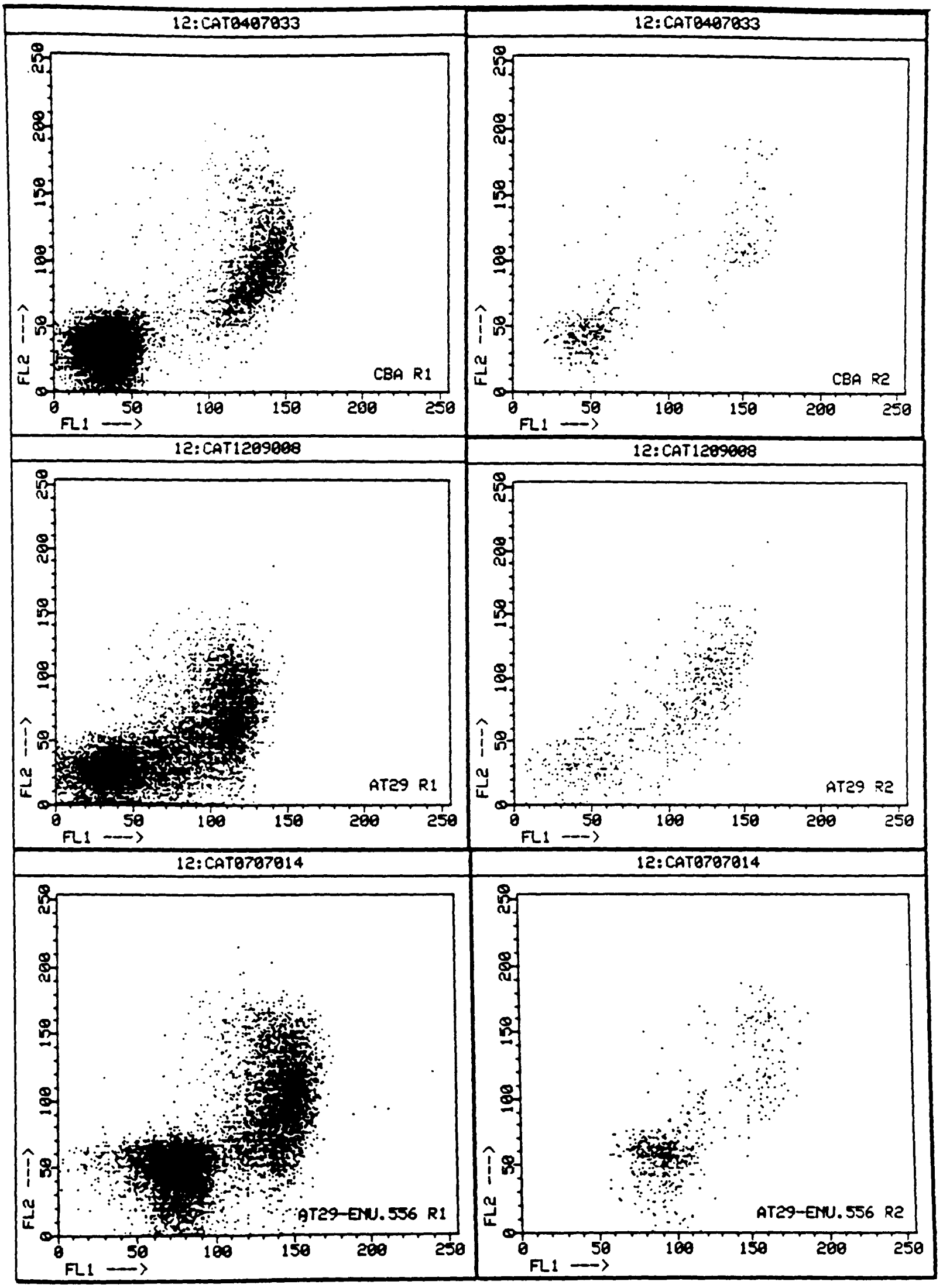


Fig. 6v

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of lymph node stained for IgD (FL1) and IgM (FL2).

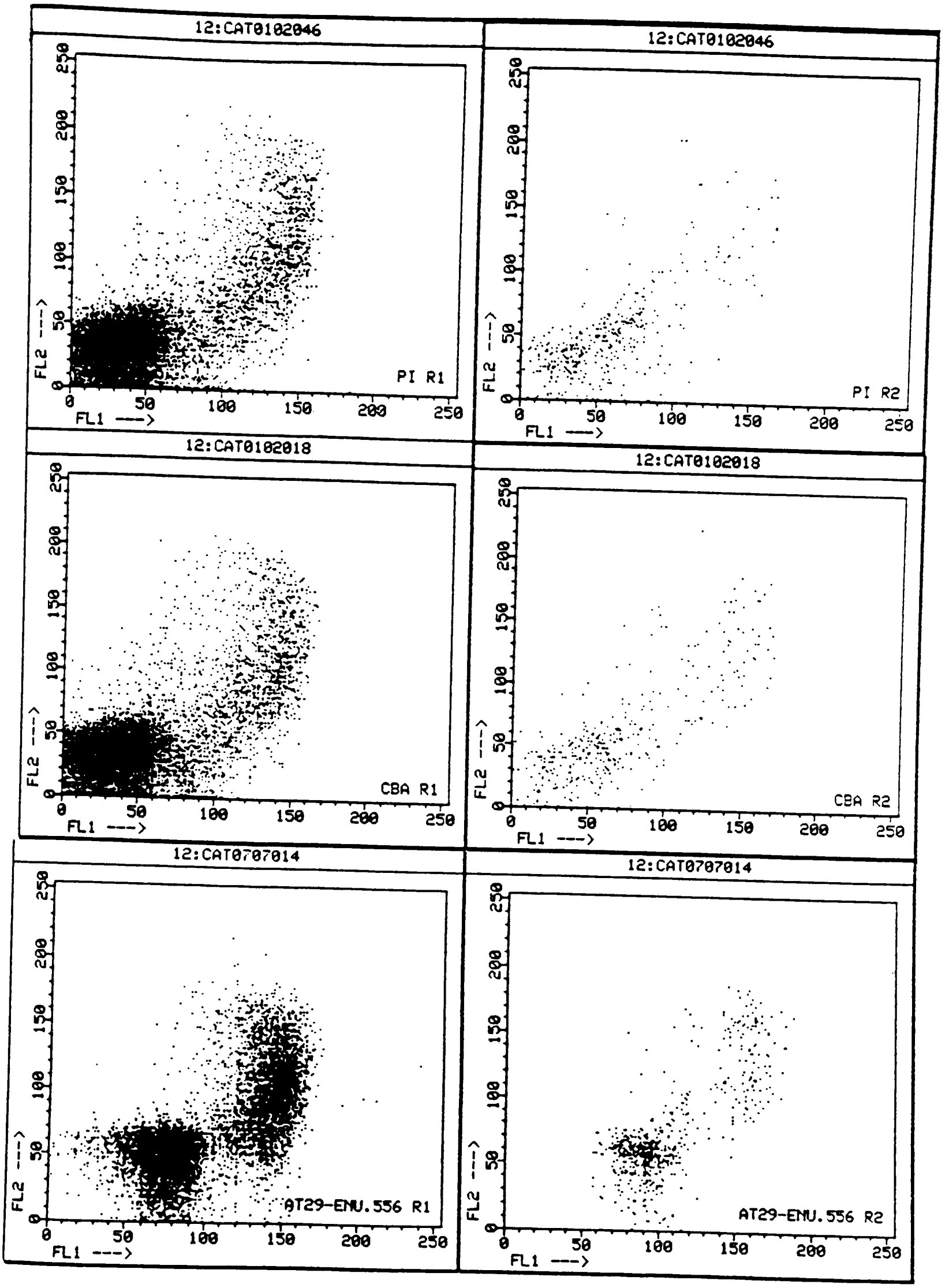


Fig. 6vi

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of lymph node stained for IgD (FL1) and IgM (FL2).

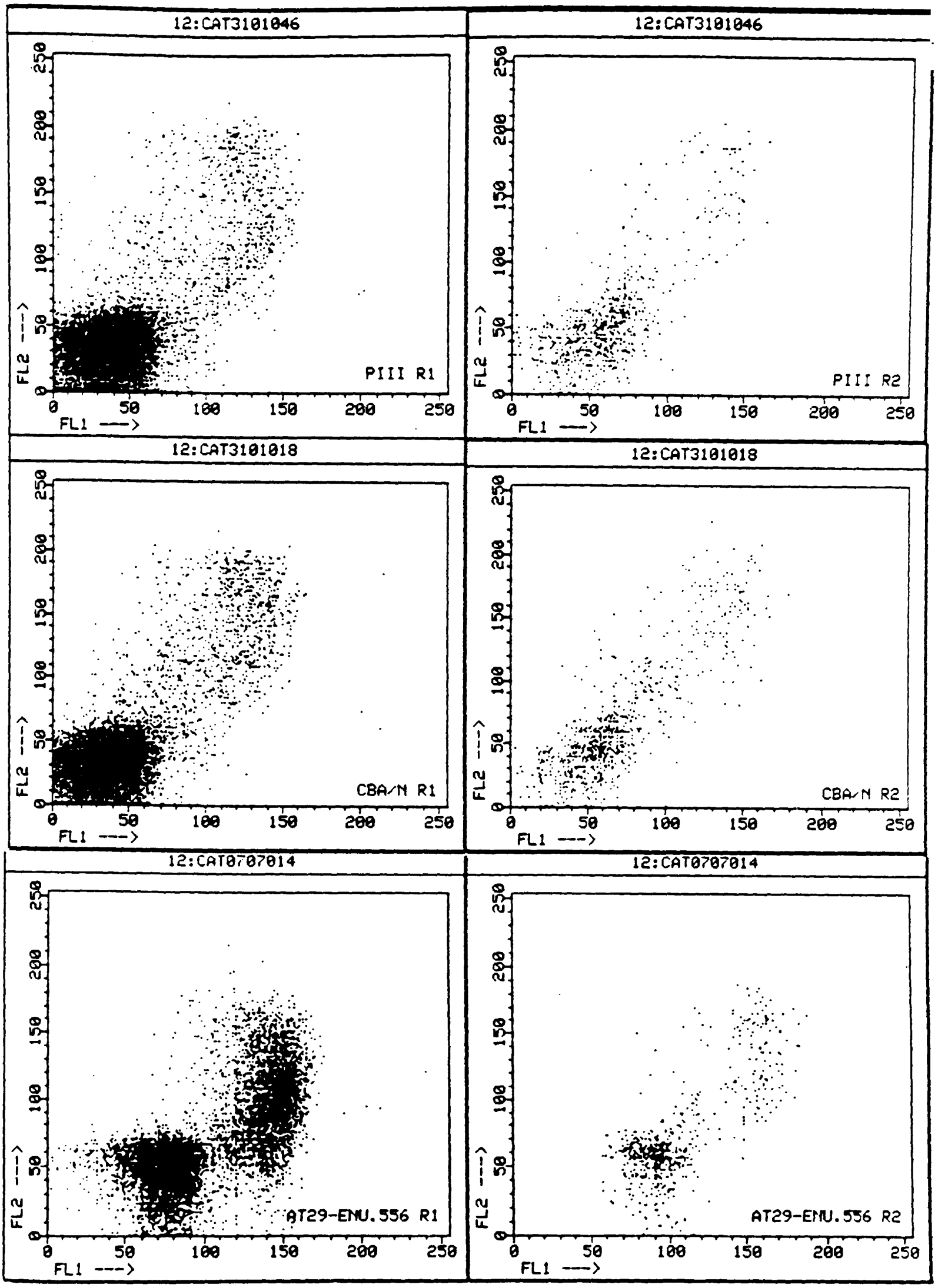


Fig. 6vii

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of lymph node stained for IgD (FL1) and IgM (FL2).

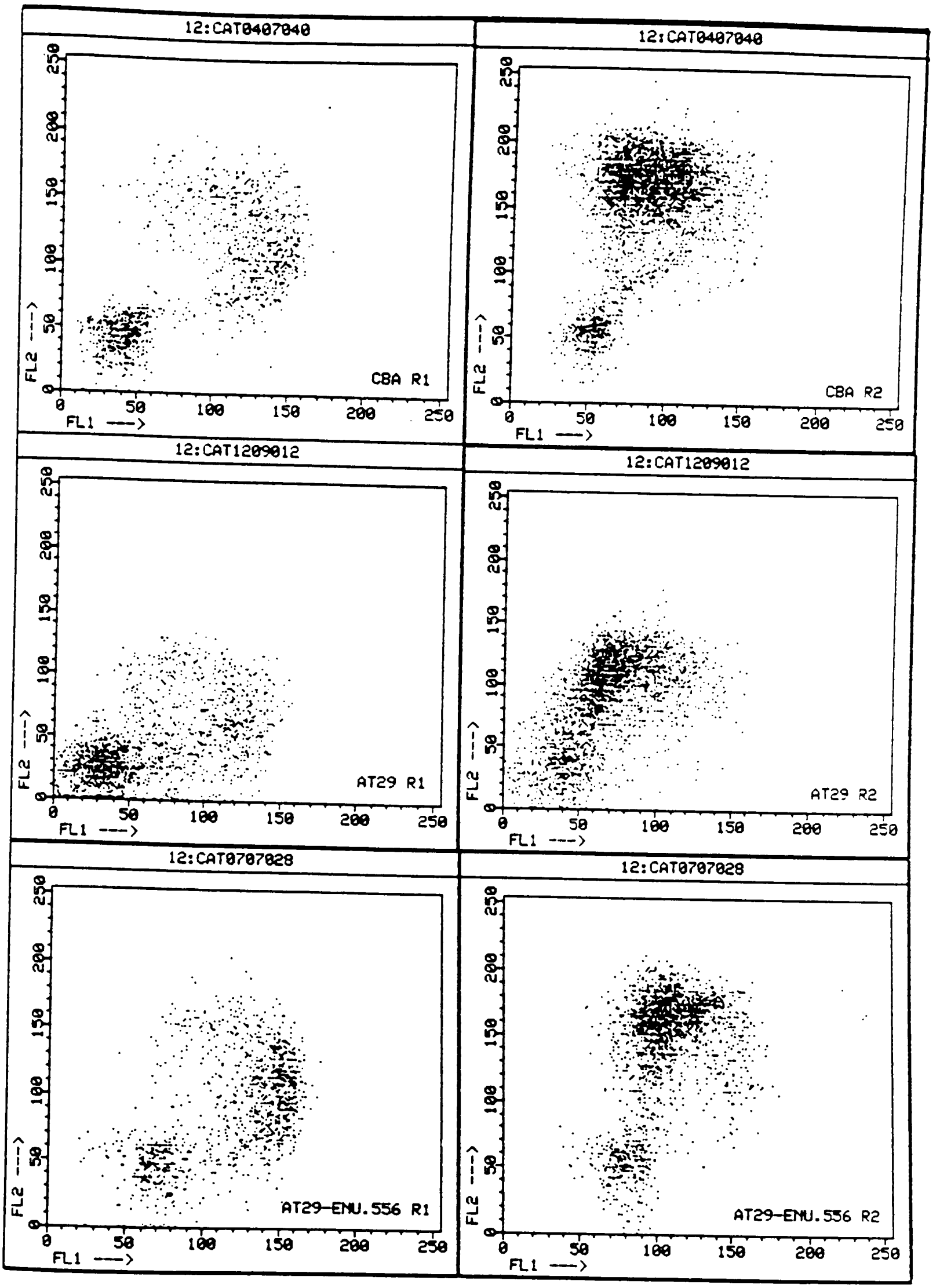


Fig. 6viii

Dotplots showing lymphocyte (R1) and high scattering cell (R2) populations of peritoneum stained for IgD (FL1) and IgM (FL2)

CHAPTER 7

The Effect Of The ENU Mutation On Immune Function: A Preliminary Study With Oxazolone

Studies of X-chromosome inactivation patterns in mice heterozygous for the ENU mutation have delineated distinct haematopoietic cell lineages that are affected by the ENU mutation. These include B and T cells, erythrocytes and possibly platelets, discussed in Chapter 3.

Preliminary experiments were carried out with the aim of characterising the effect of the ENU mutation in leukocytes in functional terms by comparing the response of homozygous AT29-ENU.556 mice and their hybrids with control mice following sensitisation with the skin-sensitising agent, oxazolone. It was expected that the leukocytes affected by the ENU mutation would be functionally defective in their response to oxazolone.

Previous work in normal mice (Davies et al, 1969) and guinea pig (Oort and Turk, 1965) showed immense proliferation of T-lineage blasts on the 4th day and proliferation of B cells which reached a peak at day 8, in the draining lymph node, and to a lesser extent, the spleen, following treatment with the skin-sensitising agent, oxazolone (Parrott and De Sousa, 1966; Pritchard and Micklem, 1972; Micklem et al, 1972).

The response following the administration of oxazolone was

assessed by analysis of the cellularity of the lymph nodes and spleens. This involved making cell counts and measuring the frequency of leukocyte populations.

Studies were made of X-chromosome inactivation patterns using alloenzyme analysis in mice that were heterozygous for the ENU mutation to assess the response of cells carrying the ENU mutation compared with cells carrying the CBA allele in PI females or compared with cells carrying the xid mutation in PIII females.

Mice of age 12 weeks were divided into an experimental group treated with oxazolone and a control group treated with ethanol. The agent was applied to a shaved area on the right side of the thorax and each group caged separately for 3 days. Spleens and brachial lymph nodes from the treated area were removed for analysis after being examined for signs of swelling.

Cell suspensions of the spleens and lymph nodes were prepared for analysis of PGK-1 expression and assessment of cellularity using the coulter counter and FACS analysis to measure the frequency of leukocytes. In FACS analysis, lymphocytes and high scattering cells were assessed separately. In this study, unlike previous sections, high scattering cells were identified by their high forward scatter, being larger than lymphocytes (see Chapter 2).

Summary tables 7.1-7.8 and representative FACS dot plots Fig. 7i-iii are given at the end of this chapter. PIA and

PIIIA refer to PI and PIII female mice and PIB and PIIIB refer to PI and PIII male mice.

Results

Cell counts and FACS analysis of AT29-ENU.556, AT29 and CBA mice

The results demonstrated the ability of AT29, CBA and AT29-ENU.556 cells of the lymph node draining the treated area, but not the spleen, to respond to oxazolone (table 7.1)

The single value for the number of cells for the lymph nodes of AT29 mice was x4 that of controls and the mean number of cells for AT29-ENU.556 mice was x3 that of controls and that for CBA mice was x9 that of controls. The results suggest that in terms of the mean number of cells, the response of CBA mice was greater than that of AT29 and AT29-ENU.556 mice and that the response of AT29 and AT29-ENU.556 mice was similar (table 7.1).

FACS analysis revealed that there were increases in the frequency of B cells and high scattering cells (defined above), almost in equal proportions (Fig. 7i).

With regard to an increase in the frequency of B cells, the response of the lymph nodes in AT29 mice was x1.3 that of controls, that of AT29-ENU.556 mice was x2.5 that of controls and that of CBA mice was x4 that of controls. The results suggest that the increase in the frequency of B

cells was greater in CBA mice than in AT29 and AT29-ENU.556 mice (table 7.2).

With regard to an increase in the frequency of high scattering cells, the response of the lymph nodes in AT29 mice was x3 that of controls, that of AT29-ENU.556 mice was x2 that of controls and that of CBA mice was x4 that of controls. The results were similar to the results for B cells where the increase in the frequency of high scattering cells was greater in CBA mice than in AT29 and AT29-ENU.556 mice and the response for AT29 and AT29-ENU.556 mice was similar(table 7.3).

There was no difference between experimental and control mice in the frequency of lymph node T cells of AT29 mice (note that no other mice were studied) or lymph node $L3T4^+$ cells of AT29, CBA and AT29-ENU.556 mice or lymph node $Lyt2^+$ cells of AT29 and CBA mice. Lymph node $Lyt2^+$ cells of AT29-ENU.556 mice appeared to respond negatively to oxazolone whereby the experimental mice had half the number of $Lyt2^+$ cells of the controls (table 7.4, 7.5, 7.6).

Although sample numbers were low, results of the mean numbers of cells and FACS analysis were consistent and showed that for the lymph nodes but not the spleen of CBA, AT29 and AT29-ENU.556 mice there were increases in the frequency of B and high scattering cells following treatment with oxazolone and the response for AT29 and

AT29-ENU.556 mice was similar.

Cell counts and FACS analysis of PI and PIII mice

The results showed that the cells of the lymph node draining the treated area but not the spleen of PI and PIII females and males responded to oxazolone (table 7.1).

There was a similarity in the mean number of cells for the lymph nodes of the experimental PI, PIII females compared with AT29 and AT29-ENU.556 mice which had a mean number of cells that was x3 or x4 that of controls and also a similarity in the mean number of cells for the lymph nodes of the experimental PI, PIII males compared with CBA mice with a mean number of cells that was x6 or x9 that of controls. The results demonstrate that in terms of the mean number of cells, the response of CBA mice was greater than that of PI and PIII females.

FACS analysis of the experimental PI and PIII mice compared with controls revealed that there were increases in the frequency of B and high scattering cells, almost in equal proportions (Fig. 7ii, 7iii where A are females and B are males).

Furthermore, for all experimental hybrids compared with controls, there was no difference in the frequency of lymph node T cells or their subsets following treatment with oxazolone (table 7.4, 7.5, 7.6,).

Although sample numbers were low, the results of the mean

number of cells and FACS analysis for the PI and PIII mice and the homozygous mice were consistent and demonstrated that the frequency of the lymph node B and high scattering cells, but not T cells, increased in response to oxazolone.

The Effects of Oxazolone-sensitisation on the Patterns of Expression of PGK-1 Alloenzymes in PI and PIII females

Studies were made of the patterns of PGK-1 expression in females that were heterozygous for the ENU mutation to assess the response to oxazolone of cells carrying the ENU mutation compared with cells carrying the CBA allele in PI females and with cells carrying the xid mutation in PIII females. Experimental mice were compared with ethanol-treated and untreated control PI or PIII females.

There was no difference in the mean values of %PGK-1A between the experimental PI females and the treated control PI females for splenic B or T cells (table 7.8).

Similarly, there was no difference in the mean values of %PGK-1A between the experimental PI females and the treated control PI females for lymph node B, T, $L3T4^+$ or $Lyt2^+$ cells. The high scattering cell population of lymph node had a mean value of 9% PGK-1A which suggests that cells within this population were affected by the ENU mutation (table 7.7).

There was a difference in the mean %PGK-1A values between

the splenic cells of experimental PIII females compared with treated control PIII females (table 7.8). For whole spleen the experimental mice had a mean value of 62% PGK-1A compared with 25% PGK-1A for the treated PIII controls. For B cells the experimental mice had a mean value of 75% PGK-1A compared with 40% PGK-1A for the treated PIII controls and 53% PGK-1A for the untreated PIII controls. There was no difference between the experimental and control PII females in the mean %PGK-1A values for splenic T cells (table 7.8).

Similarly, for B cells of lymph nodes of experimental PIII controls the mean value was 60% PGK-1A compared with the mean value of 35% PGK-1A for the treated PIII controls. There was no difference in the mean %PGK-1A values between experimental mice and the controls for lymph node $L3T4^+$ cells or $Lyt2^+$ cells (table 7.7).

The patterns of PGK-1 expression for the lymph nodes of the experimental and control PI females were similar which may suggest that following treatment with oxazolone, the cells carrying the ENU mutation expanded at the same rate as the cells carrying the CBA allele.

However, there was a difference in the patterns of PGK-1 expression for B cells, but not T cells, of the lymph nodes of the experimental and control PIII females where there was unbalanced PGK-1 expression in favour of the cells carrying the ENU mutation. This suggests that the B

cells carrying the ENU mutation may have expanded at a faster rate than the cells carrying the xid mutation following treatment with oxazolone.

DISCUSSION

The development of an immune response occurs mainly in secondary lymphoid organs such as the spleen and lymph nodes.

In general, $CD4^+$ (murine $L3T4^+$) cells are termed helper T cells and $CD8^+$ (murine $Lyt2^+$) are termed cytolytic T cells and the cell surface glycoproteins, CD4 and CD8 have specificity for class II or class I MHC proteins, respectively. These proteins have a role in the activation and effector functions of mature peripheral T cells (Swain, 1983; Parnes, 1989).

The activation of T cells results in clonal expansion, generation of memory T cells and maturation into regulatory or effector cells. Following stimulation, T cells release IL-2 initially and IL-4, Il-5, IL-6 and IFN γ appear several days later (Swain et al, 1991).

T cells interact with B cells through surface molecules. For example, cross-linking of surface CD40 on B cells by the CD40 ligand on activated $CD4^+$ T cells switches on activation and differentiation and may act as a signal for the migration of B cells into primary follicles (Armitage et al, 1992; Noelle et al, 1992). A massive proliferation

of B cells initiates the germinal centre reaction (Liu et al, 1991). Selected B cells are induced to differentiate into recirculating memory cells or plasma blasts that migrate from the germinal centres and form plasma cells that produce immunoglobulins with specific effector functions (Howard et al, 1992; Butch et al, 1993).

The combination and the relative concentrations of the various cytokines released from different subsets of $CD4^+$ TH1 and TH2 cells determines the effect on B cells in terms of the type of Ig isotype produced (Croft and Swain, 1991; Banchereau and Rousset, 1992).

In general, the generation of the $CD8^+$ cell response requires help from helper T cells and the various cytokines released from TH1 and TH2 cells have different effects on $CD8^+$ cells (Chen and Zlotnik, 1991).

Previous studies of the immune response following treatment with the skin-sensitising agent, oxazolone have demonstrated immense proliferation of T-lineage blasts on the 4th day and proliferation of B lymphocytes which reaches a peak on day 8 in the draining lymph node and to a lesser extent, the spleen (Oort and Turk, 1965; Parrott and De Sousa, 1966; Davies et al, 1969; Pritchard and Micklem, 1972; Micklem et al, 1972).

In this study, preliminary experiments were carried out with the aim of characterising the effect of the ENU mutation in leukocytes in functional terms by comparing

the response of homozygous AT29-ENU.556 mice and their hybrids with control mice following sensitisation with oxazolone. It was expected that the leukocytes affected by the ENU mutation would be functionally defective in their response to oxazolone.

In terms of an increase in the mean number of cells, leukocytes of the lymph node but not the spleen of AT29-ENU.556, AT29, CBA mice and their hybrids responded to oxazolone. The response of the leukocytes of CBA mice was greater than that of AT29-ENU.556, AT29 and PI and PIII female mice and the response of AT29 and AT29-ENU.556 leukocytes was similar (table 7.1)

FACS analysis showed that for all mice studied there was an increase in the frequency of lymph node B and high scattering cells. High scattering cells were distinguished from lymphocytes by their relatively high forward scatter profile, being larger than lymphocytes and were assumed to be dividing lymphoblasts, discussed below. There were no increases in the frequency of lymph node T cells or T cell subsets in response to oxazolone (table 7.2-7.6).

These results are consistent with previous studies which demonstrated that following oxazolone treatment there was proliferation of lymph node B lymphocytes and the formation of lymphoblasts. It was also shown that the lymphoblasts were of the T-lineage using cytological markers (Davies et al, 1969).

In the present study it is probable that the high forward scattering lymphoblasts were dividing T cells which would be consistent with other studies (Davies et al, 1969) as there was unbalanced expression of PGK-1 for the cells of this population in the lymph nodes of PI females which implies that they were affected by the ENU mutation.

If it is assumed that the lymphoblasts are large, dividing T cells, then the increase in the frequency of B cells probably reflected some B cell proliferation, being consistent with previous work (Davies et al, 1969) but may also reflect a reduction in the frequency of T cells as T cells move from the low forward scattering region to the high forward scattering region containing lymphoblasts. The B cell frequency was taken as a proportion of the number of lymphocytes, not as a proportion of the number of T-lineage cells which included lymphoblasts and T lymphocytes (Fig. 7i-iii).

Hence, the response to oxazolone was similar for AT29 and AT29-ENU.556 mice, which suggests that either the ENU mutation does not affect cell responsiveness to oxazolone or the effects of the mutation cannot be recognised by assessing cell counts or leukocyte frequencies or both. The latter is consistent with FACS data in Chapter 6.

The results of studies of the patterns of PGK-1 expression in the spleen and lymph nodes of PI females implied that the responding cells carrying the ENU mutation expanded at

the same rate as the cells carrying the CBA allele, following oxazolone treatment (table 7.7).

However, the results of studies of the patterns of PGK-1 expression for B cells of the spleen and lymph nodes of PIII females implied that the B cells carrying the ENU mutation expanded at a faster rate than the cells carrying the xid mutation, following treatment with oxazolone (table 7.7, 7.8). This suggests that B cells carrying the ENU mutation were more responsive to oxazolone than the B cells carrying the xid mutation.

Previous work noted a splenic reaction to oxazolone with regard to cell proliferation measured by radiolabelling (Asherson et al, 1971; Micklem et al, 1972). Although this reaction was to a lesser degree than that of the draining lymph node (Micklem et al, 1972), there was no evidence in the present study from cell counts or from the assessment of leukocyte frequencies of any splenic reaction to oxazolone (table 7.1-7.6).

However, the results of studies of the patterns of PGK-1 , expression in splenic B cells of PIII females suggested that following oxazolone treatment, the cells bearing the ENU mutant chromosome were more responsive than the cells bearing the xid mutation (table 7.8).

Table 7.1: Cell counts of the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE COUNT 10^7 x10	n	MEAN number of cells	st dev	n	MEAN number of cells	st dev	exptl/ control
CBA C	2	1.8	0.1	2	0.2	0.1	9.0
AT29 C	1	4.2	/	1	1.0	/	4.2
AT29-ENU.556C	3	2.1	1.1	3	0.7	0.4	3.0
PIA	2	5.4	2.6	2	1.8	0.4	3.0
PIB	2	4.7	0.1	2	0.8	0.1	5.9
PIIIA	2	3.4	0.9	2	0.8	0.1	4.2
PIIIB	2	3.5	0.6	2	0.4	0.4	8.8
SPLEEN COUNT 10^7 x10							
CBA C	2	4.4	0.7	2	5.0	1.3	0.9
AT29 C	1	7.0	/	1	7.1	/	1.0
AT29-ENU.556C	3	5.3	1.5	3	5.8	1.1	0.9
PIA	2	7.9	4.1	2	11.4	4.2	0.7
PIB	2	5.4	1.6	2	8.6	0.9	0.6
PIIIA	2	6.8	2.0	2	7.6	3.6	0.9
PIIIB	2	4.2	0.2	2	3.6	2.9	1.2

showing the mean number of cells ($x10^7$), the sample number and the standard deviation

for n=1 a single value is given

experimental mice=oxazolone-treated
control mice=ethanol-treated

the difference in the means between the experimental and control mice is given as exptl/control

A=female mice, B=male mice, C=female and male mice

Table 7.2: Frequency of B cells in the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE B220	n	mean %ofpos.cells	st dev	n	mean %ofpos.cells	st dev	exptl/ control
CBA C	2	36	3.5	2	10	3.5	3.6
AT29 C	1	70	/	1	53	/	1.3
AT29-ENU.556C	2	45	1.4	3	18	2.6	2.5
PIA	1	34	/	1	23	/	1.5
PIB	2	34	3.5	2	14	0.7	2.4
PIIIA	2	19	2.8	2	11	1.4	1.7
PIIIB	2	11	1.4	2	4	0.7	2.3
SPLEEN B220							
CBA C	2	66	2.1	2	63	1.4	1.2
AT29 C	1	54	/	1	44	/	1.2
AT29-ENU.556C	3	56	12.1	3	55	13.6	1.0
PIA	2	56	1.4	2	51	3.5	1.1
PIB	2	63	1.4	2	60	7.7	1.1
PIIIA	2	53	4.2	2	49	7.1	1.1
PIIIB	2	34	2.8	1	25	/	1.4

showing the mean % of cells positive for marker B220, the sample number (n) and the standard deviation

for n=1 a single value is given

experimental mice=oxazolone-treated
control mice=ethanol=treated

the difference in the means between the experimental and control mice is given as exptl/control

A=female mice, B=male mice, C=female and male mice

Table 7.3: Frequency of high scattering cells in the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE HIGH SCATTER	n	mean %ofcells	st dev	n	mean %ofcells	st dev	exptl/ control
CBA C	2	38	0.7	2	9	2.8	4.2
AT29 C	1	59	/	1	23	/	2.6
AT29-ENU.556C	2	30	4.9	3	15	4.4	2.0
PIA	2	21	0	2	10	5.0	2.1
PIB	2	24	2.1	2	16	2.1	1.5
PIIIA	2	14	2.1	2	6	1.4	2.3
PIIIB	2	22	9.2	2	3	0	7.3
SPLEEN HIGH SCATTER							
CBA C	2	30	7.1	2	25	4.2	1.2
AT29 C	1	32	/	1	25	/	1.3
AT29-ENU.556C	3	34	3.6	3	27	3.0	1.4
PIA	2	39	5.7	2	35	5.7	1.1
PIB	2	20	2.8	2	21	1.4	1.0
PIIIA	2	26	5	2	21	4.2	1.2
PIIIB	2	24	0.7	2	28	12.0	0.9

showing the mean % of high scattering cells, the sample number (n) and the standard deviation

for n=1 a single value is given for the % of cells

experimental mice=oxazolone-treated
control mice=ethanol-treated

the difference in the means between the experimental and control mice is given as exptl/control

high scattering cells had a relatively high forward scatter, being larger than lymphocytes

A=female mice, B=male mice, C=female and male mice

Table 7.4: Frequency of T cells in the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE THY1.2	n	mean %ofpos.cells	st dev	n	mean %ofpos.cells	st dev	exptl/ control
CBA C		/			/		
AT29 C	1	40	/	1	36	/	1.1
AT29-ENU.556C		/			/		
PIA	2	72	2.1	1	56	18.4	1.3
PIB	2	64	3.5	2	73	1.4	0.9
PIIIA	2	88	1.4	2	90	2.8	1.0
PIIIB	2	82	0.7	2	94	1.4	0.9
SPLEEN THY1.2							
CBA C		/			/		
AT29 C	1	20	/	1	35	/	0.6
AT29-ENU.556	1	13	/	1	17	/	0.8
PIA	2	12	3.5	2	16	3.5	0.8
PIB	2	18	3.5	1	25	7.1	0.7
PIIIA	2	24	1.4	2	18	2.1	1.3
PIIIB	2	29	8.5	2	37	4.2	0.8

showing the mean % of cells positive for marker Thy1.2, the sample number (n) and the standard deviation

for n=1 a single value is given

experimental mice=oxazolone-treated
control mice=ethanol=treated

the difference in the means between the experimental and control mice is given as exptl/control

A=female mice, B=male mice, C=female and male mice

Table 7.5: Frequency of Lyt2$^+$ T cells in the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE LYT2	n	mean %ofpos.cells	st dev	n	mean %ofpos.cells	st dev	exptl/ control
CBA C	2	28	7.1	2	36	2.8	0.8
AT29 C	1	33	/	1	26	/	1.3
AT29-ENU.556C	1	21	/	2	41	1.4	0.5
PIA	2	24	2.1	2	20	0	1.2
PIB	2	18	6.4	2	26	2.1	0.7
PIIIA	2	40	0.7	2	30	2.8	1.3
PIIIB	2	36	6.4	2	46	0.7	0.8
SPLEEN LYT2							
CBA C	2	18	3.5	2	15	7.1	1.2
AT29 C	1	20	/	1	20	/	1.0
AT29-ENU.556C	2	18	3.5	2	20	3.5	0.9
PIA	2	8	2.1	2	10	0	0.8
PIB	2	23	4.2	2	25	4.2	0.9
PIIIA	2	24	4.2	2	16	0.7	1.5
PIIIB	2	21	8.5	2	21	2.8	1.0

showing the mean % of cells positive for marker Lyt2, the sample number (n) and the standard deviation

for n=1 a single value is given

experimental mice=oxazolone-treated
control mice=ethanol-treated

the difference in the means between the experimental and control mice is given as exptl/control

A=female mice, B=male mice, C=female and male mice

Table 7.6: Frequency of L3T4+ T cells in the lymph nodes and spleens of oxazolone-treated and control mice

	EXPERIMENTAL			CONTROL			
L.NODE L3T4	n	mean %ofpos.cells	st dev	n	mean %ofpos.cells	st dev	exptl/ control
CBA C	2	43	0	2	56	7.7	0.8
AT29 C	1	18	/	1	22	/	0.9
AT29-ENU.556C	2	27	2.8	3	29	3.0	0.9
PIA	2	40	2.1	2	39	8.5	1.0
PIB	2	43	0	2	53	0	0.8
PIIIA	2	51	4.2	2	72	0.7	0.7
PIIIB	2	58	3.5	2	64	0.7	0.9
SPLEEN L3T4							
CBA C	2	25	5.7	2	22	0	1.2
AT29 C	1	15	/	1	22	/	0.7
AT29-ENU.556C	3	13	2.1	3	17	2.3	0.8
PIA	2	11	1.4	2	14	0.7	0.8
PIB	2	18	5.0	2	24	5.7	0.8
PIIIA	2	20	0.7	2	24	6.4	0.8
PIIIB	2	26	8.5	1	39	8.5	0.7

showing the mean % of cells positive for marker L3T4, the sample number (n) and the standard deviation

for n=1 a single value is given

experimental mice=oxazolone-treated
control mice=ethanol-treated

the difference in the means between the experimental and control mice is given as exptl/control

A=female mice, B=male mice, C=female and male mice

Table 7.7: Patterns of PGK-1A expression in the lymph nodes of oxazolone-treated and control hybrids

L.NODE PIA	n	EXPERIMENTAL mean %PGK-1A	n	CONTROL(+ve) mean %PGK-1A	n	CONTROL(-ve) mean %PGK-1A
WHOLE	2	27 (0.7)		/	26	13 (10.4)
B+	1	0	1	0	8	2 (5.0)
B-	1	0	1	0	7	0 (0.0)
L4+	1	0	1	0	3	3 (5.2)
L2+	1	0	1	0	2	6 (7.8)
HIGH SCATTER	4	9 (5.1)		/		/
L.NODE PIIIA						
WHOLE		/		/	4	23 (9.9)
B+		/		/	5	31 (11.5)
Bb	1	60	1	35		/
B-	1	18	1	6	5	13 (12.5)
L4+	1	17	1	17	2	18 (0.7)
L2+	1	30	1	34	2	15 (7.1)

showing the mean %PGK-1A , the sample number, n and the standard deviation in brackets

when n=1 a single value is given

experimental=oxazolone-treated mice
control(+ve)=ethanol-treated; control(-ve)=untreated mice

high scatter cells have a relatively high forward scatter

B cells= $B220^{+}$ and Bb is bright staining for B220

T cells= $L3T4^{+}$ and $Lyt2^{+}$ and $B220^{-}$

whole=unsorted lymph node

Table 7.8: Patterns of PGK-1A expression in the spleens of oxazolone-treated and control hybrids

SPLEEN PIA	EXPERIMENTAL n	EXPERIMENTAL mean %PGK-1A	CONTROL(+ve) n	CONTROL(+ve) mean %PGK-1A	CONTROL(-ve) n	CONTROL(-ve) mean %PGK-1A
WHOLE	/		/		27	27 (8.1)
B+	1	0	1	0	9	2 (6.0)
B-	1	22	1	24	9	4 (8.3)
SPLEEN PIIIA						
WHOLE	1	62	1	25	8	34 (9.7)
B+	/		/		3	50 (8.4)
Bb	1	75	1	40	2	53 (18.4)
B-	1	22	1	29	7	20 (10.8)

showing the mean %PGK-1A, the sample number n and the standard deviation in brackets

when n=1 a single value is given

experimental=oxazolone-treated mice

control(+ve)=ethanol-treated; control(-ve)=untreated mice

B cells=$B220^{+}$ and Bb is bright staining for B220

T cells=$B220^{-}$

whole=unsorted spleen

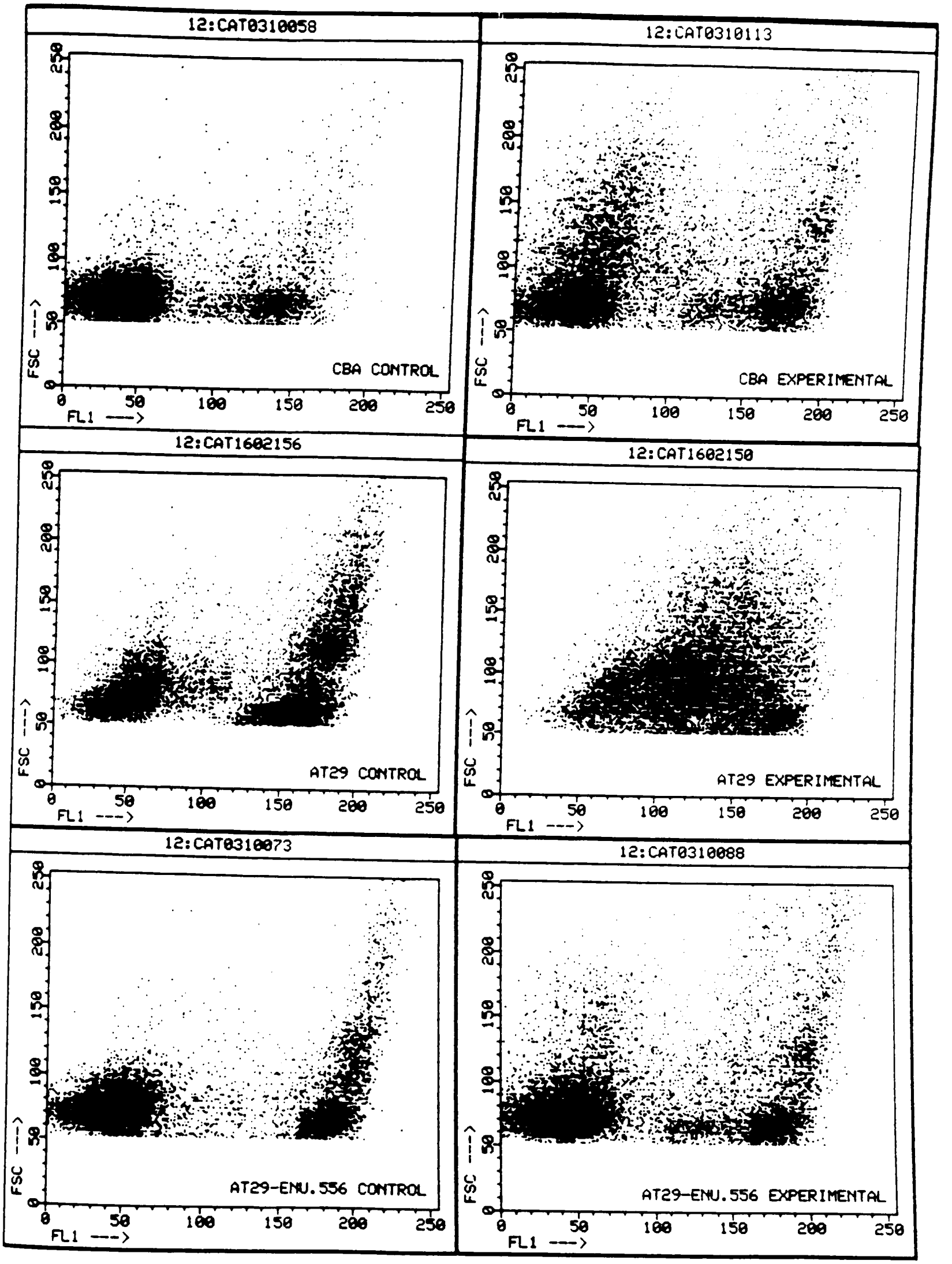


Fig. 7i

Dotplots showing lymphocyte and high scattering cell populations of lymph node draining treated side: control (alcohol treated) and experimental (oxazolone-treated) stained for B220.

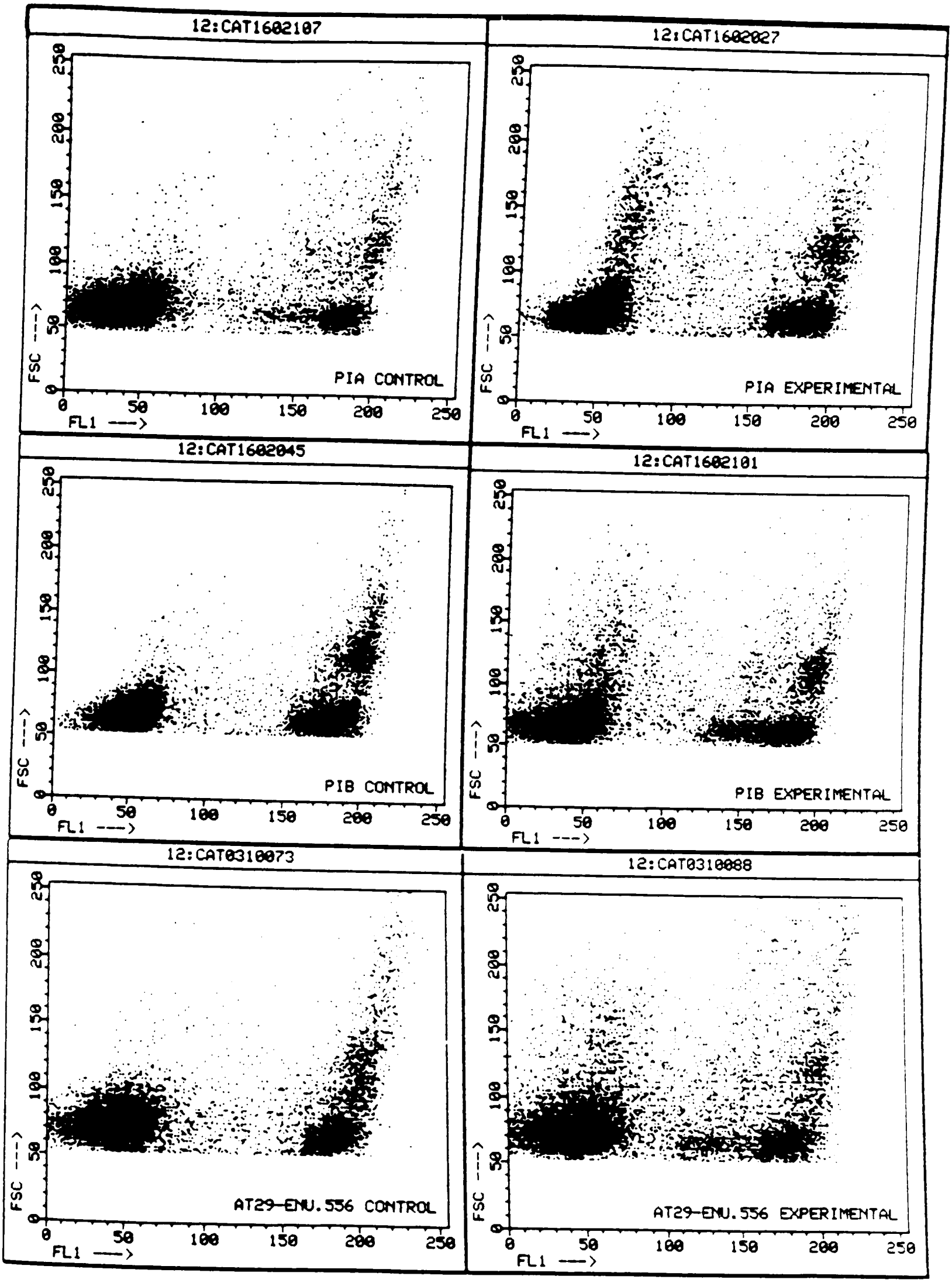


Fig. 7ii

Dotplots showing lymphocyte and high scattering cell populations of lymph node draining treated side: control (alcohol treated) and experimental (oxazolone-treated) stained for B220.

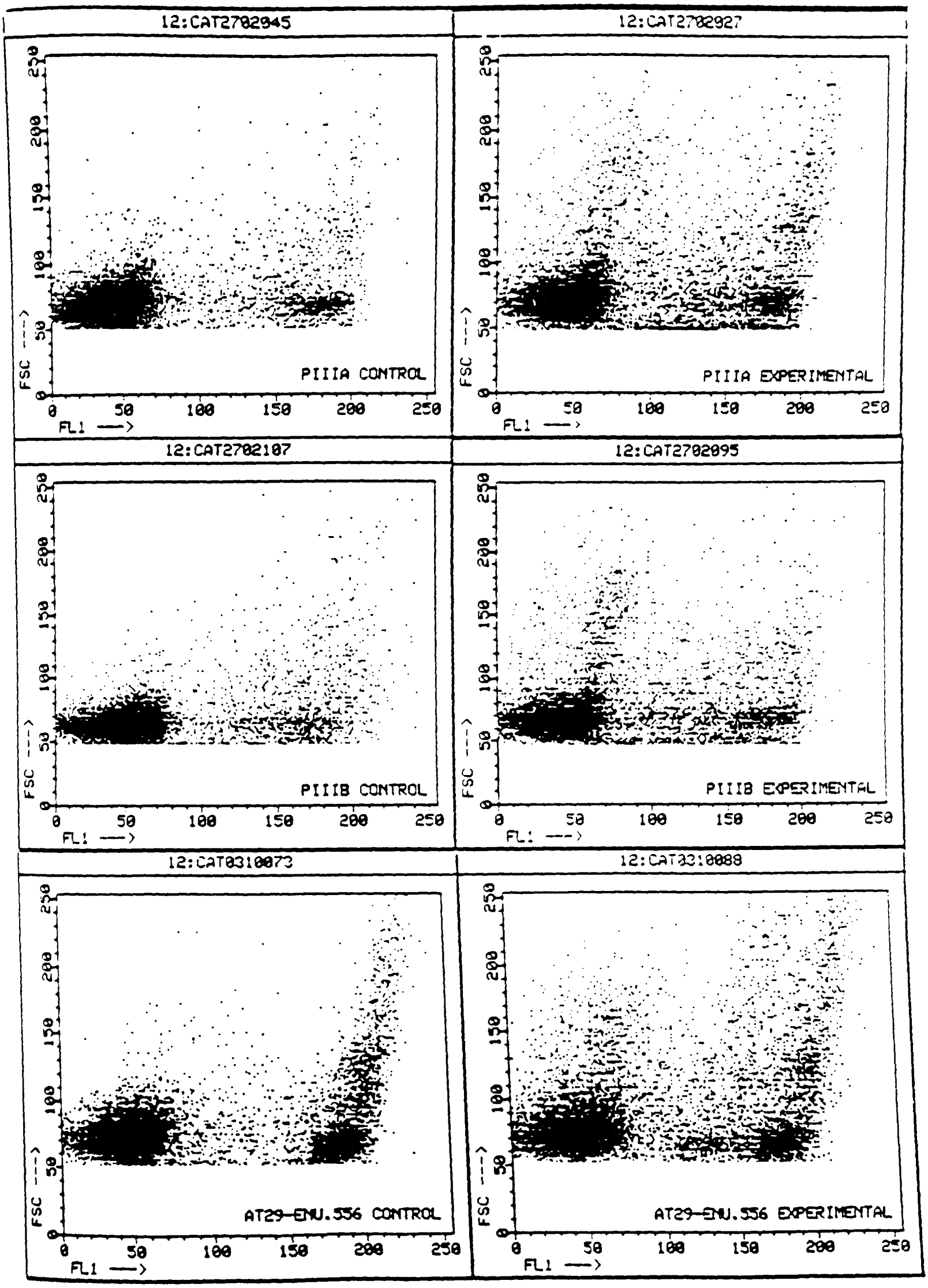


Fig. 7iii

Dotplots showing lymphocyte and high scattering cell populations of lymph node draining treated side: control (alcohol treated) and experimental (oxazolone-treated) stained for B220.

CHAPTER 8

Concluding Discussion

Animals with genetic mutations can be used to study the factors controlling normal development and function and as models for the study of genetic disease in man. Comparisons of human and murine genetic maps can indicate the chromosome regions where the genetic order is conserved and can identify mouse genetic loci which may be homologous to human mutations causing disease. In humans and mice, loci controlling the haematopoietic and muscular systems have been mapped to the X chromosome (see the comparative human and mouse X-chromosome map, Fig. 1).

The aim of this study was to characterise the phenotypic effect of a novel X-linked mutation in offspring of male mice treated with the mutagen N-ethylnitrosourea (ENU). It has been demonstrated that treatment with ENU can produce X-linked mutations affecting the haematopoietic and muscular systems (Chapman et al, 1988, 1989).

Studies of X-chromosome inactivation patterns in heterozygous females using electrophoretic alloenzyme analysis were used to characterise the phenotypic effect of the X-linked ENU mutation. The presence of cells carrying the ENU mutation was detected by the expression of the alloenzyme PGK-1A such that unbalanced expression of PGK-1A demonstrates selection against the cells affected by the mutation, the differential effect of the

mutation in cell lineages and the effect of the mutation with age.

Unbalanced expression of the X chromosome carrying the ENU mutation was seen for skeletal muscle and the haemato-poietic tissues blood, spleen, thymus, lymph node and bone marrow (summary table 3.15) and for the cell subpopulations constituting these tissues namely, erythrocytes and B and T cells and possibly platelets (table 3.19, 3.20, 3.20, 3.39).

The cells of blood, spleen and bone marrow which were defined as high scattering and were distinguished from lymphocytes by their relatively high right angle scatter, being structurally more complex, were not affected by the ENU mutation having balanced X-chromosome expression similar to that for the control tissue brain in the same mice (table 3.15) and similar to that for the same cells in control heterozygotes (table 3.21, 3.48, 3.53, 3.56).

The differential effect of the ENU mutation in tissues and cell subpopulations was demonstrated. Skeletal muscle, thymus and lymph node were affected by the mutation to a greater extent than blood, spleen and bone marrow (table 3.15). This reflected the presence of cells in these tissues that were unaffected by the mutation such as the high scattering cells defined above and also the presence of cells that were less affected by the mutation such as erythrocytes in whole blood, spleen and bone marrow B

cells, which were predominantly pre-B cells (table 3.21, 3.19, 3.48).

It was also shown that the ENU mutation had differential effects on cell subpopulations within the B cell lineage. Pre-B cells were affected by the mutation to a lesser extent than the circulating B cells of the spleen and lymph nodes (table 3.21, 3.39, 3.48). T cell subsets were similarly affected by the mutation (table 3.21, 3.30, 3.39).

Attempts to relate the ENU mutation to a candidate gene were made by assessing whether the ENU mutation was an allele of xid by studying the X-chromosome inactivation patterns in PIII females heterozygous for ENU and xid. Previous studies demonstrated that the defect in xid mice is intrinsic to B cells (Nahm et al,1983; Forrester, 1986).

Results demonstrated balanced PGK-1 expression for splenic B cells and unbalanced expression of PGK-1 in favour of cells carrying xid for B cells of the lymph node and bone marrow (table 3.22, 3.40, 3.50).

Balanced expression of PGK-1 for splenic B cells may reflect similar effects of ENU and xid in splenic B cells. Unbalanced expression of PGK-1 in bone marrow B cells was greater than that for the B cells of the spleen and lymph node which implies that ENU also acts in pre-B cells, unlike xid. These results are consistent with previous

findings in +/xid heterozygotes which showed unbalanced PGK-1 expression in favour of cells carrying the CBA X chromosome in spleen and lymph node and slightly unbalanced PGK-1 expression in the bone marrow as xid acts in circulating mature B cells and not in pre-B cells (Witkowski et al, 1985; Forrester et al, 1987).

There was also unbalanced expression of PGK-1 in T cells of the spleen and lymph node of PIII mice although this was to a lesser extent than in +/ENU (PI) females (table 3.21, 3.22 and 3.39, 3.40). This may relate to previous findings which showed defective function of a T cell helper subset in CBA/N mice (Bottomly and Mosier, 1979; Scher, 1982).

All cells affected by the ENU mutation demonstrated PGK-1 variation with age (Fig.3.2-3.28). Correlation analysis showed that for skeletal muscle, blood and lymph node of PI mice, the effect of the mutation increased with age, particularly between 0-11 weeks for skeletal muscle and between 14-100 weeks for lymph node.

Studies in PI mice showed that the effects of the ENU mutation in T and B cells were apparent in pre-adult mice and became more pronounced with age. Studies in PIII mice revealed balanced PGK-1 expression in B cells after 12 weeks of age which implies that the effects of xid became more pronounced during this period and this relates to previous studies (Forrester et al, 1987).

Balanced levels of PGK-1 expression depend on the X-chromosome inactivation patterns in the tissue progenitors (McLaren, 1972; Fialkow, 1973; West, 1975; Pappaioannou *et al*, 1981; McMahon *et al*, 1983) and on the nature of the X-chromosome controlling element (*Xce*). Allelic variants of this locus and the parental source of the allele influence X inactivation (Cattanach, 1972; Johnston and Cattanach, 1981; Forrester and Ansell, 1985; Fowlis *et al*, 1991).

Studies of X-chromosome inactivation patterns using PGK-1 alloenzyme analysis were used to examine variation of PGK-1 expression to determine the nature of the *Xce* in AT29 and AT29-ENU.556 mice and to examine the parental effects in the hybrids of these and CBA and CBA/N mice (table 5) Results were consistent with the assumption of an Xce^{a} allele for AT29 and AT29-ENU.556 mice and studies of the reciprocal crosses with CBA (Xce^{c})mice were consistent with an $Xce^{a/c}$ genotype with no parental effects for this combination of alleles (Johnston and Cattanach, 1981; Forrester and Ansell, 1985).

Flow cytometric analysis of the frequency of leukocytes demonstrated differences between the two normal strains, CBA and AT29 but not between AT29 and AT29-ENU.556 mice. Despite the mutation, both AT29 and AT29-ENU.556 mice shared the same characteristics which were highly distinguishable from those of CBA mice. Furthermore, the frequency of leukocytes in AT29 and AT29-ENU.556 F1 females was similar (table 6.6-6.15).

B cells were classified according to the density of surface immunoglobulin isotype (Fig. 6i-viii). Population I has high IgD, low IgM; population II has high IgD, high IgM and population III has low IgD, high IgM (Hardy et al, 1982, 1984). CBA/N mice have low levels of population III B cells and no population I B cells (Hardy et al, 1982, 1984; Hayakawa et al, 1983, 1986; Sidman et al, 1986).

In this study, the distribution of the B cell subsets for CBA/N mice was consistent with work described above. The corrective ability of AT29-ENU.556 cells within the IgD/ IgM B cell subset of ENU/xid heterozygotes was demonstrated (Fig. 6iii, 6iv, 6vii).

The results of flow cytometric analysis suggested that the ENU mutation does not affect the frequency of leukocytes although studies of X-chromosome expression imply the mutation may control aspects of cell function that render ENU mutant cells unable to compete with normal cells.

Preliminary experiments were carried out to characterise the effect of the ENU mutation in leukocytes in functional terms by comparing the response of homozygous AT29-ENU.556 mice and their hybrids with control mice following sensitisation with oxazolone.

In terms of an increase in the number of cells, leukocytes of the lymph node of AT29-ENU.556, AT29, CBA mice and their hybrids responded to oxazolone. The response of the

leukocytes of CBA mice was greater than that of AT29-ENU.556 and AT29 mice which had a similar response (table 7.1).

Flow cytometric studies demonstrated an increase in the frequency of lymph node B and high scattering cells (table 7.2, 7.3). High scattering cells were distinguished from lymphocytes by their relatively high forward scatter profile and were probably dividing lymphoblasts which would be consistent with other studies (Davies et al, 1969). The increase in the frequency of B cells probably reflected some B cell proliferation being consistent with previous work (Davies et al, 1969) but may also have reflected a reduction in the frequency of T cells as T cells became dividing lymphoblasts (Fig. 7i-iii).

The response was similar, in terms of cell type and degree for AT29 and AT29-ENU.556 mice. This suggests that either the mutation does not affect cell responsiveness to oxazolone or the effects of the mutation cannot be recognised by assessing cell counts or leukocyte frequencies.

The results of studies of the patterns of PGK-1 expression in the spleen and lymph nodes of PI females implied that the responding cells carrying the ENU mutation expanded at the same rate as the cells carrying the CBA allele, following oxazolone treatment (table 7.7).

However, the results of studies of the patterns of PGK-1

expression for B cells of the spleen and lymph nodes of PIII females implied that the B cells carrying the ENU mutation expanded at a faster rate than the cells carrying the xid mutation, following treatment with oxazolone (table 7.8). This suggests that B cells carrying the ENU mutation were more responsive to oxazolone than the B cells carrying the xid mutation.

Loci controlling the affected lineages have been mapped to the X chromosome. Although several loci that control muscle development are X-linked, it is rather interesting to find skeletal muscle affected by the same mutation as haematopoietic cells. Two murine X-chromosome loci can be eliminated as possibilities. These are the muscular dystrophy mutation mdx as its effect cannot be detected by unbalanced expression of PGK-1 in skeletal muscle (Chapman 1990, personal communication) and the xid mutation as, unlike the ENU mutation, it does not act in pre-B cells.

There are examples of X-linked disorders involving at least two of the cell types that are affected by the ENU mutation. Examples of these are discussed below.

XSCID is characterised by absent or depressed T lymphocytes, normal numbers of B cells and low to no levels of immunoglobulins (Rosen et al, 1966; Yount et al, 1978).

Studies of the X-chromosome inactivation patterns in XSCID delineated the cell types affected which include T and B

cells and in some cases granulocytes and natural killer cells (Goodship et al, 1991; Conley, 1992; Puck, 1993; Wengler et al, 1993).

There may be several SCID loci on the X chromosome since it is phenotypically heterogeneous (Mensink and Schuurman, 1987). One form of X-linked combined immunodeficiency, similar to but less severe than XSCID, termed thymic dysplasia with normogammaglobulinaemia, is characterised by T and B lymphocyte dysfunction with normal to slightly low lymphocyte levels and the presence of plasma cells and immunoglobulins (Nezelof et al, 1964; Fulginiti et al, 1966; Lawlor et al, 1974).

There is also evidence of human XSCID due to a membrane defect (Kersey et al, 1977; Yount et al, 1978). Affected individuals have normal levels of B cells, T cells and immunoglobulins but they have abnormal responses to concanavalin A which is characterised by an inability to "membrane cap" membrane glycoproteins (Gelfand et al, 1979).

Brooks et al, 1990 described a form of X-linked combined immunodeficiency which differed from XSCID in that serum concentrations of immunoglobulin isotypes were normal and the deficiencies in the number of T cells were less marked than in XSCID.

Another X-linked combined immunodeficiency, suggested as being either an attenuated form of XSCID or corresponding

to a separate locus has been described with T and B dysfunction but normal numbers of T cells (de Saint-Basile et al, 1992).

XLA is characterised by low numbers of B cells and normal levels of T cells and immunoglobulin levels are low but not absent (Bruton, 1952, Winkelstein, 1985; Ochs and Wedgewood, 1989).

Studies of X-chromosome inactivation patterns showed that the defect was intrinsic to B cells (Conley et al, 1986; Hendriks et al, 1989).

Vetrie et al, 1993 identified the gene, BTK which encodes protein tyrosine kinases in B cells and is mutated in XLA patients (Tsukada et al, 1993).

There have been conflicting results from studies of X-chromosome inactivation patterns to identify the defective cells in HIGM1 (Hendriks et al, 1990; Notarangelo et al, 1991).

In HIGM1, deletions or point mutations of the CD40 ligand gene results in the failure of T cells to interact with CD40 on B cells (DiSanto et al, 1993) which results in the defect in Ig switching in HIGM1.

The murine gene for the Il2r γ chain was mapped to a locus on the X chromosome consistent with the human IL2R γ locus (Noguchi et al, 1993a) and it was shown that the defect in the gene is not responsible for the xid mutation (Cao et

al, 1993).

Although xid, like XLA is characterised by a B cell lesion, the defect is less severe than that seen in XLA patients. The murine Btk has been mapped to the xid locus and xid and XLA share a similar molecular lesion despite exhibiting different disease phenotypes (Rawlings et al, 1993; Thomas et al, 1993).

The loci, Xlr-1 and Xlr-2 are expressed in both T and B lymphocytes; lymphocyte membrane antigens, Lyt-X for T cells and, Lyb-X for B cells (Lyon and Searle, 1989).

Defective membrane protein of muscle and erythrocytes has been identified in patients with Duchenne muscular dystrophy which results from abnormal lipid composition and mechanical, metabolic and morphological properties (Dhalla et al, 1975; Percy and Miller, 1975; Niebroj-dobosz, 1976; Distefano and Bosmann, 1977; Howland and Iyer, 1977; Solomons et al, 1977; Atkinson et al, 1979; Butterfield et al, 1976). Interestingly, the impairment of membrane protein capping has also been described in B and T lymphocytes of Duchenne patients (Verrill et al, 1977; Hornstein and Emery, 1980; Sensi et al, 1984; Goldsmith and Greumer, 1987; Baricordi et al, 1989) as well as, defective erythrocyte morphology (Pickard et al, 1978). This example illustrates a relationship between the four lineages affected by the ENU mutation, although mdx, the murine homologue of Duchenne has been eliminated as the

possible locus affected by ENU.

There is evidence for a common recombinase for receptors of B (Ig) and T (TCR) cells. Highly conserved DNA sequences flank V, D and J elements (Tonegawa, 1983; Kronenberg et al, 1986) and T cell receptor V region gene segments have been successfully incorporated into pre-B cells (Yancopoulis et al,1986). It has been suggested that the scid mutation in mice may adversely affect a component of the recombinase system resulting in a lack of functional B and T cells (Schuler et al, 1986) and abnormal J-associated deletions during D to J or V to J joining have been reported for these mice (Schuler et al, 1986, 1987).

In scid mice, differences have been shown in patterns of V_H gene family expression in pre-B cells such that a chromosome position-dependent bias of V_H gene expression is seen in neonatal and fetal normal mice and adult "leaky" (Bosma et al, 1983, 1988) scid (Ig+) mice and random V_H gene expression is seen in normal adult mice at 5-7 days postnatally, reaching the randomised pattern at about 2 weeks of age (Yancopoulis et al, 1988; Malynn et al, 1990).

Analysis of PGK-1 expression in females heterozygous for ENU and the normal allele has shown that selection against B and T cells carrying the ENU mutation shows a similar developmental profile. One might speculate that ENU-mutant

lymphocytes were selected against, in the presence of normal lymphocytes, because of their restricted repertoire of immunoglobulin and T cell receptor gene expression. This might also explain balanced PGK-1 expression in ENU/xid female hybrids.

The phenotypic effect of the ENU mutation was assessed. Results of analysis of X-chromosome expression indicated that B, pre-B and T cells, erythrocytes, possibly platelets and myocytes were affected by the ENU mutation. It was not possible to identify the effects of the ENU mutation in leukocytes by flow cyometric analysis or in functional terms following treatment with oxazolone, the major differences in these analyses being attributable to background genotype.

Future studies could involve a closer assessment of the B cell response in PIII hybrids as cells carrying the ENU mutation were more responsive than cells carrying xid following oxazolone treatment. This could involve studies of the response of population I-III B cells in PIII hybrids.

The effect of the mutation with age could be extended to studies of population I-III B cells in homozygous AT29-ENU.556 mice and their hybrids.

Other studies might involve functional studies of the muscular system in homozygous AT29-ENU.556 mice and their hybrids, complemented by histological and molecular

studies including gene mapping.

Studies of the X-linked mutation in AT29-ENU.556 mice may be useful as a model for X-linked disorders of the haematopoietic and muscular systems and in assessing the genetic factors that control cell development and function.

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Appendix 1: Materials And Methods

FACS medium

	Quantity
sodium azide (Sigma)	0.5g
bovine serum albumin (BDH)	500mg
EDTA	100mg

added to 500ml of PBS (below).

Phosphate-buffered saline (PBS)

	Quantity (g/l)
sodium chloride (BDH)	7.848
potassium chloride (Fisons)	0.201
disodium hydrogen orthophosphate (BDH)	1.015
potassium dihydrogen orthophosphate (BDH)	0.388

added to 1 litre distilled water; solution adjusted to pH 7.2 using sodium hydroxide or hydrochloric acid. At this pH the solution should be approximately 308 mOsm, similar to mouse plasma tonicity.

Acridine-orange/Ethidium-bromide stain

	Quantity (mg)
acridine-orange (BDH)	50.0
ethidium-bromide (Sigma)	15.0

dissolved in 1ml 95% ethanol; add 49ml distilled water, mix; store at -20 °C (1ml aliquots); dilute 1ml in 100 ml

PBS (above) and store at 4°C in dark bottle (up to one month).

Sample buffer

	Quantity (mg/100ml)
dithioerithritol (Sigma)	30.0
BSA (Sigma)	50.0
digitonin (Sigma)	200.0

added to 100ml of 50mM tri-ethanolamine hydrochloride (Sigma) at pH 7.6.

Electrophoresis buffer

	Quantity (g/l)
EDTA (BDH)	0.744
sodium citrate (Fisons)	2.94
magnesium sulphate (Fisons)	1.23
sodium barbital (Sigma)	4.12

added to 1 litre distilled water; adjust pH to 8.8.

Indicator stock

For MTT gels:

	Quantity (mg/10ml)
tri-ethanolamine hydrochloride (Sigma)	185.6
magnesium sulphate (Fisons)	320.0
glucose (BDH)	270.0
adenosine diphosphate (Sigma)	112.5

NADP (Sigma)	306.0

added to 10ml distilled water; adjust pH to 7.6 using 2M sodium hydroxide; store at -20°C (100µl aliquots).

For ^{14}C gels: as above, with 1/10 glucose.

Assay stock

	Quantity (mg)
NAD (Sigma)	32.0
trisodium fructose-1,6-diphosphate (Sigma)	650.0
dipotassium hydrogen orthophosphate (BDH)	279.0

added to 40ml electrophoresis buffer (above);stored at -20°C (400µl aliquots).

Appendix 2: Representative FACS dot-plots

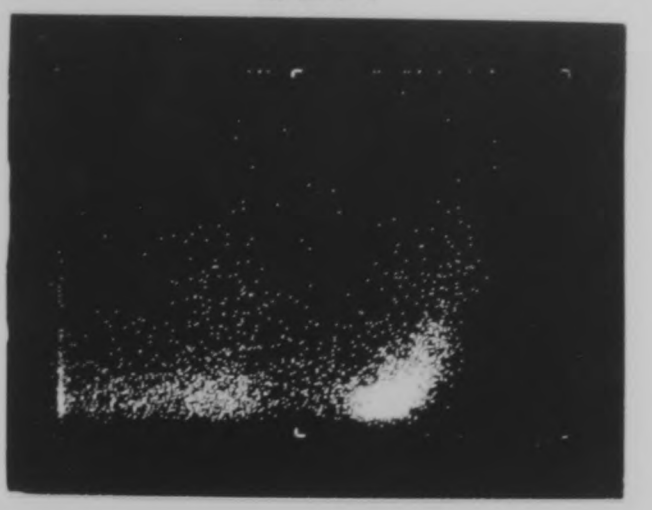
Fig. a-p are representative FACS dot-plots for Chapter 6 showing the spleen, lymph node, thymus, bone marrow and blood stained for the markers B220, Thy1.2, Lyt2 and L3T4 for homozygous CBA, AT29 and AT29-ENU.556 mice.

Lymphocytes were identified by their right angle scatter profile and lymphocyte populations were defined by quantifying fluorescence-labelled cells to assess the distribution of surface antigen.

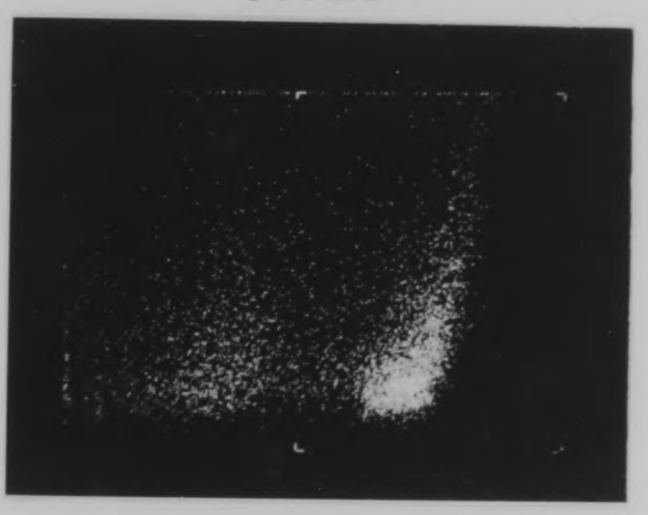
SPLEEN

STAINED FOR B220

CBA



AT29



RIGHT ANGLE SCATTER

AT29 - ENU.556

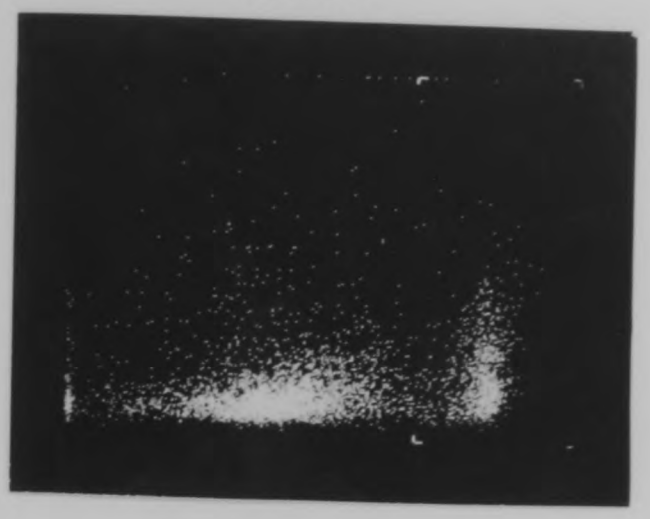
LOG FLUORESCENCE

FIG. a

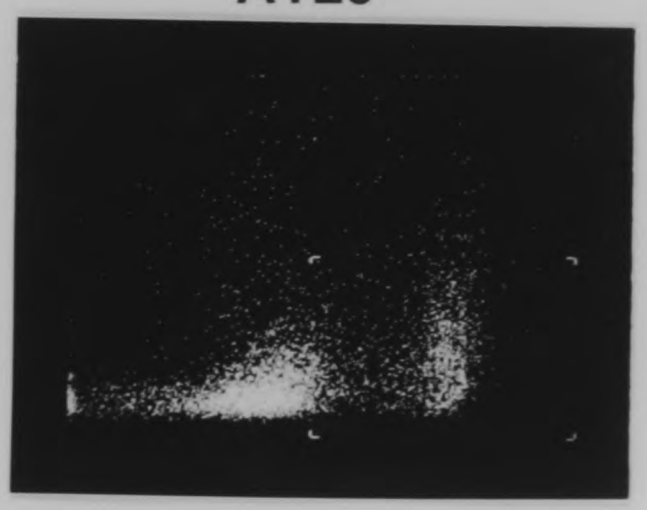
SPLEEN

STAINED FOR THY1

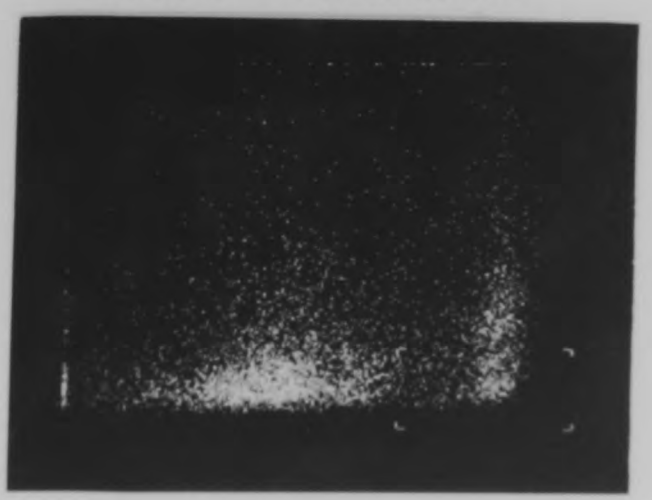
CBA



AT29



AT29 - ENU.556



RIGHT ANGLE SCATTER

LOG FLUORESCENCE

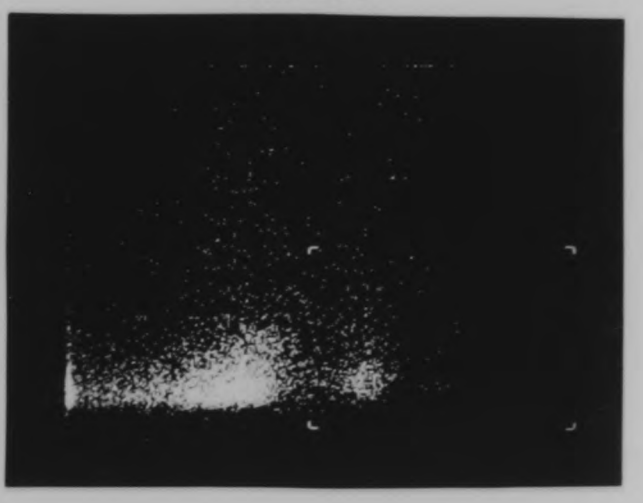
FIG. b

SPLEEN

STAINED FOR LYT2

CBA

AT29



AT29 - ENU.556

RIGHT ANGLE SCATTER

LOG FLUORESCENCE

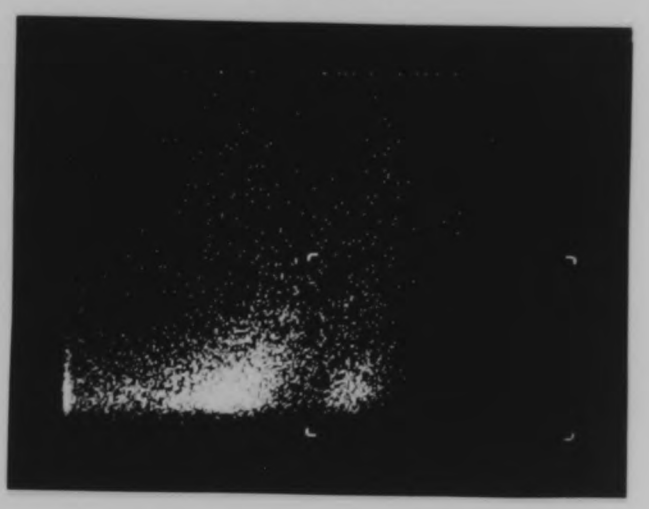
FIG.c

SPLEEN

STAINED FOR L3T4

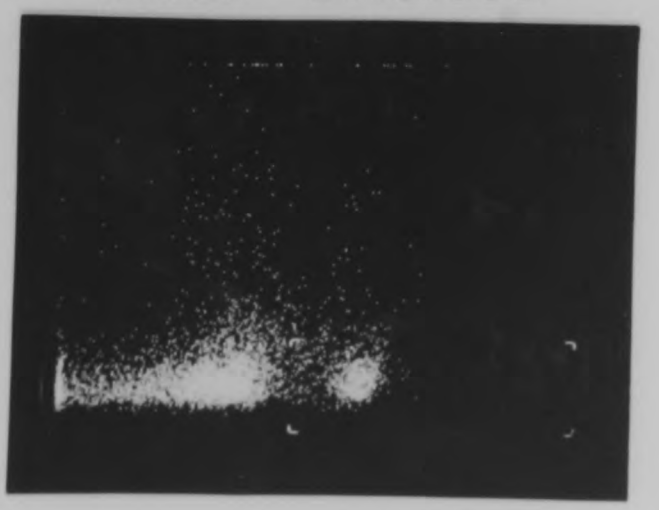
CBA

AT29



RIGHT ANGLE SCATTER

AT29 - ENU.556



LOG FLUORESCENCE

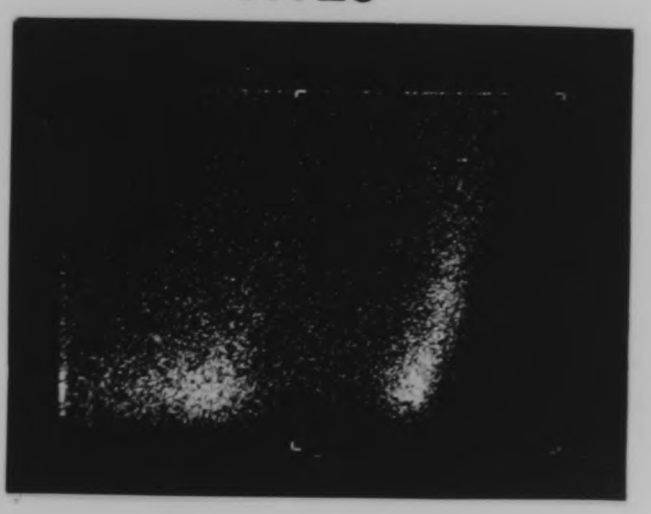
FIG.d

LYMPH NODE

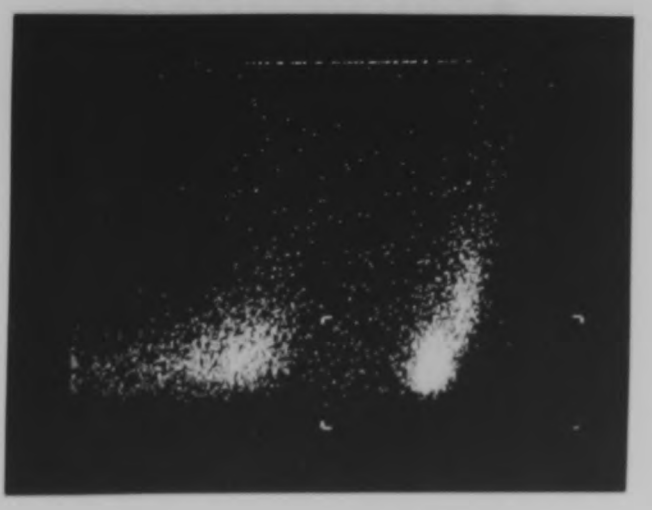
STAINED FOR B220

CBA

AT29



AT29 - ENU.556



RIGHT ANGLE SCATTER

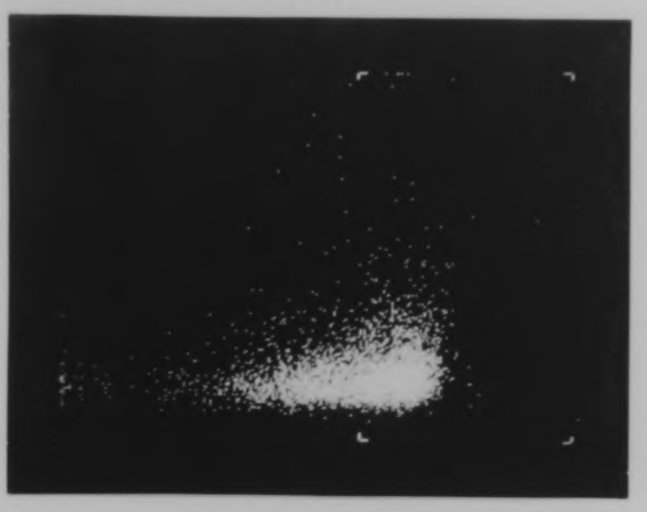
LOG FLUORESCENCE

FIG. e

LYMPH NODE

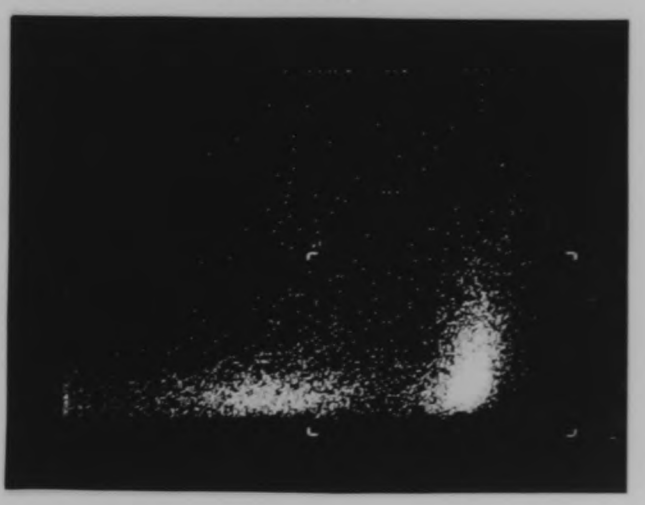
STAINED FOR THY1

CBA

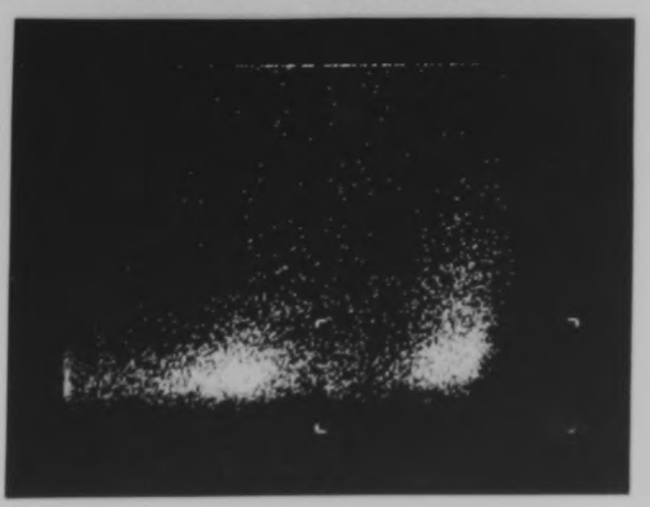


RIGHT ANGLE SCATTER

AT29



AT29 - ENU.556



LOG FLUORESCENCE

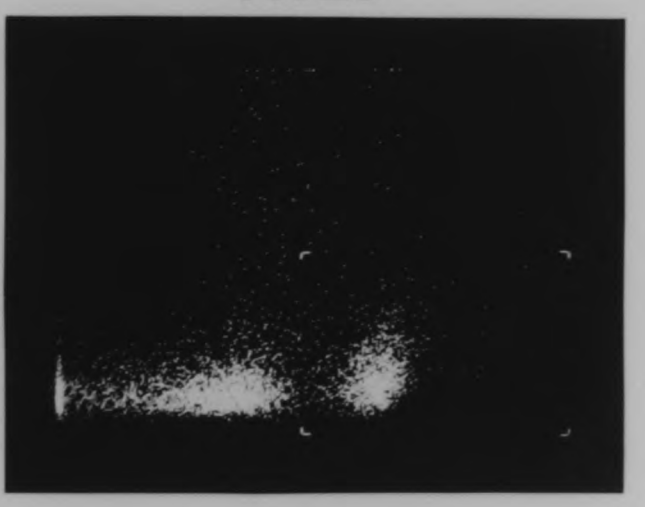
FIG.f

LYMPH NODE

STAINED FOR LYT2

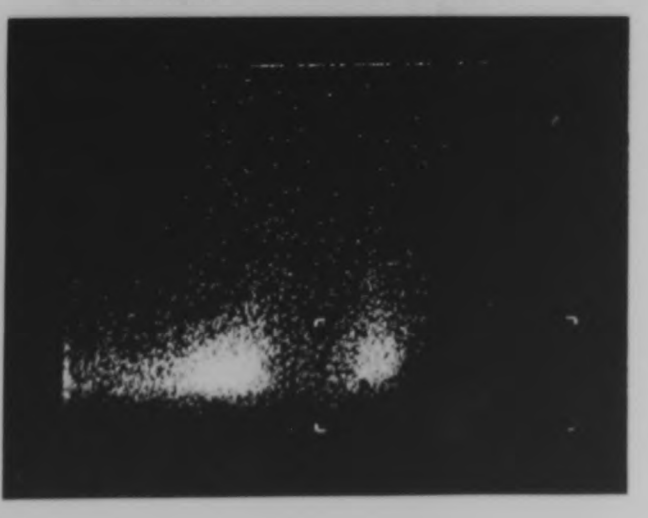
CBA

AT29



RIGHT ANGLE SCATTER

AT29 - ENU.556



LOG FLUORESCENCE

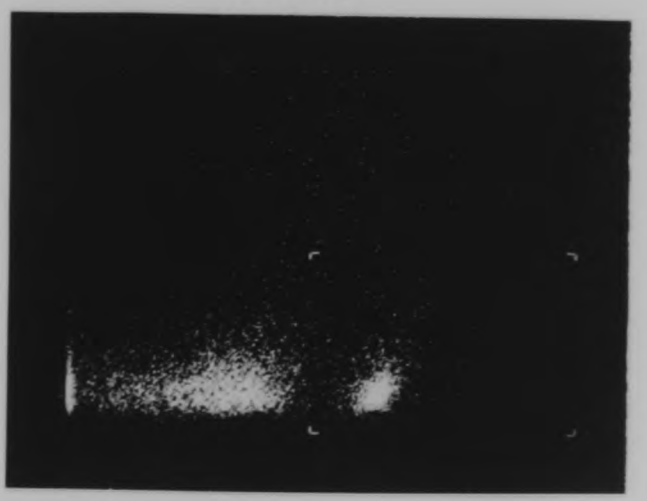
FIG. 9

LYMPH NODE

STAINED FOR L3T4

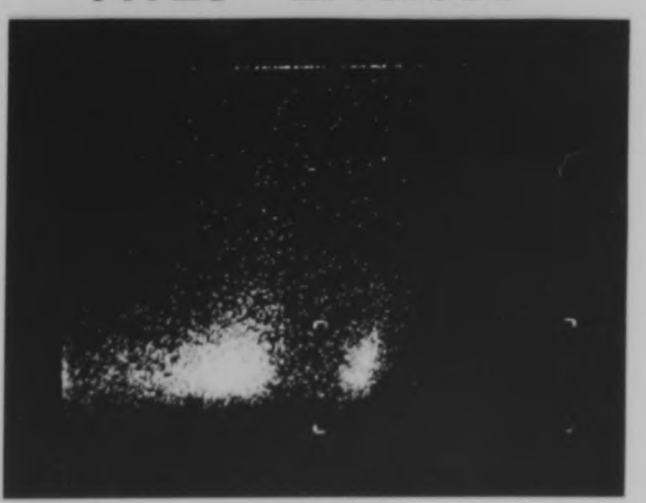
CBA

AT29



RIGHT ANGLE SCATTER

AT29 - ENU.556



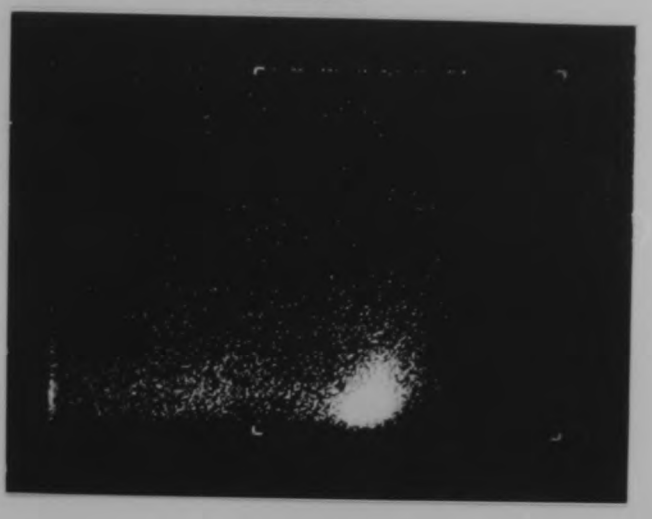
LOG FLUORESCENCE

FIG.h

THYMUS

STAINED FOR LYT2

CBA



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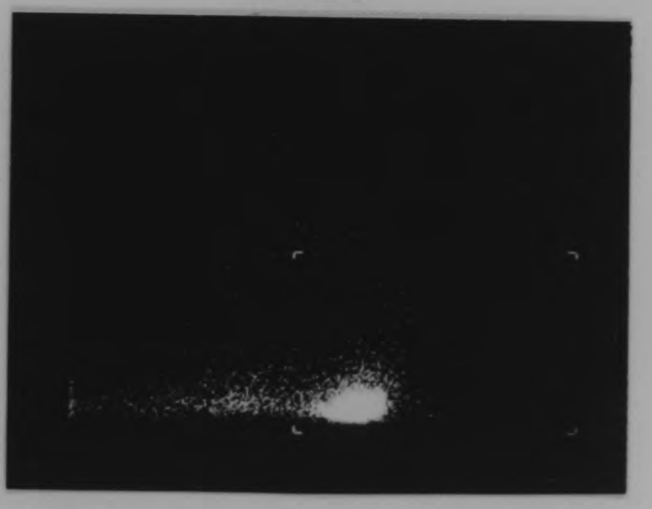

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RIGHT ANGLE SCATTER

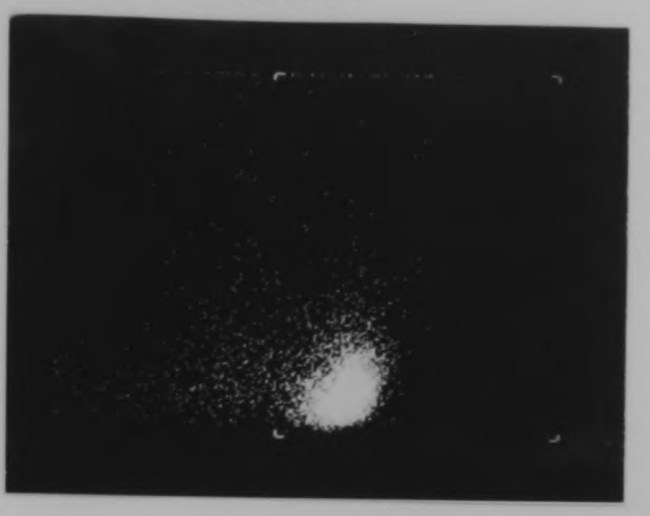
LOG FLUORESCENCE

FIG.i

THYMUS

STAINED FOR L3T4

CBA



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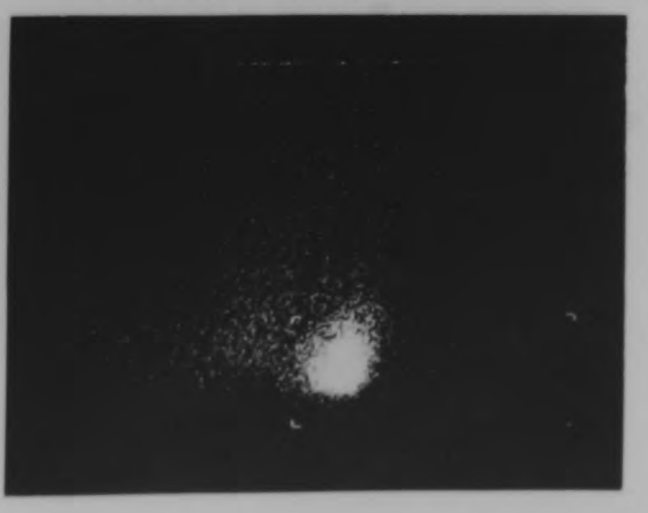


RIGHT ANGLE SCATTER

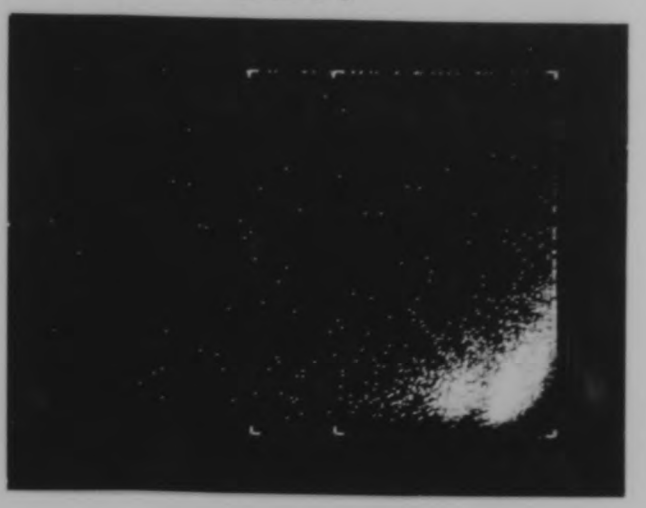
LOG FLUORESCENCE

FIG. j

THYMUS

STAINED FOR THY1

CBA



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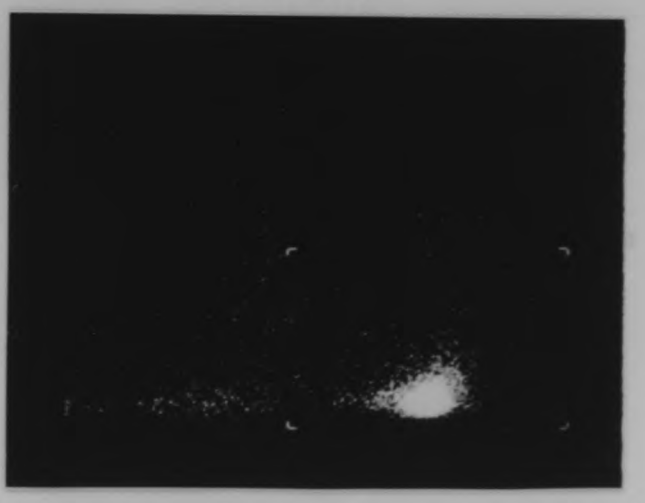
FORWARD SCATTER



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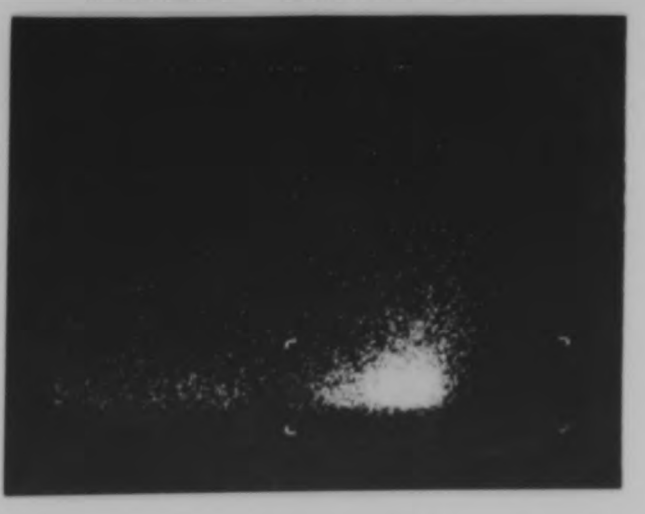


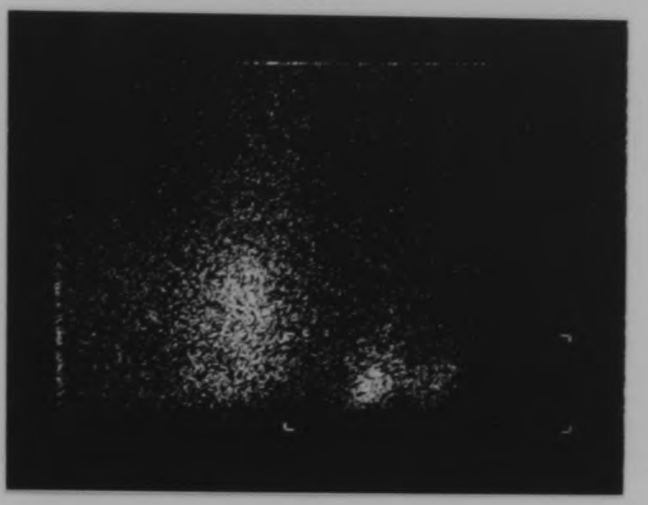
LOG FLUORESCENCE

FIG. k

BONE MARROW

STAINED FOR B220

CBA



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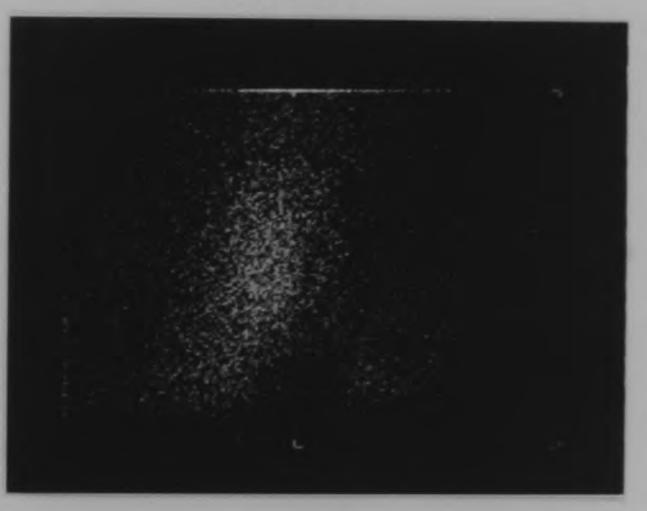

RIGHT ANGLE SCATTER

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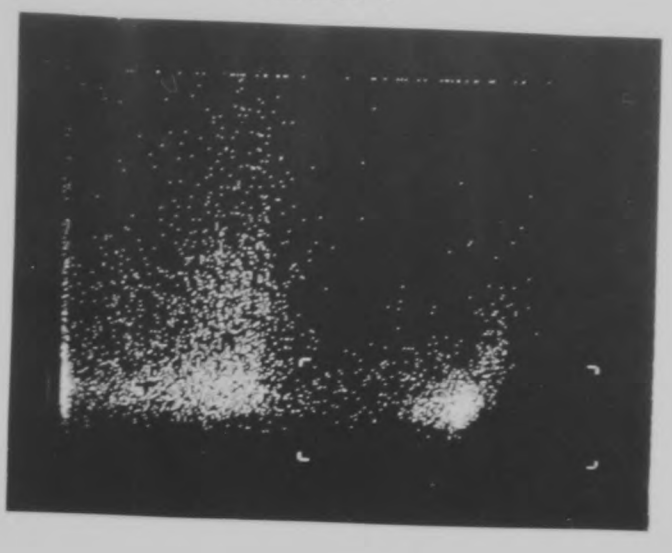
LOG FLUORESCENCE

FIG.I

BLOOD

STAINED FOR B220

CBA



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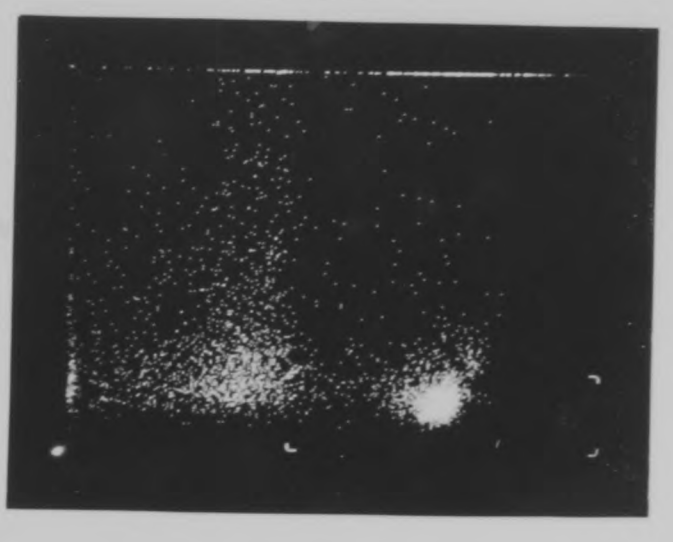
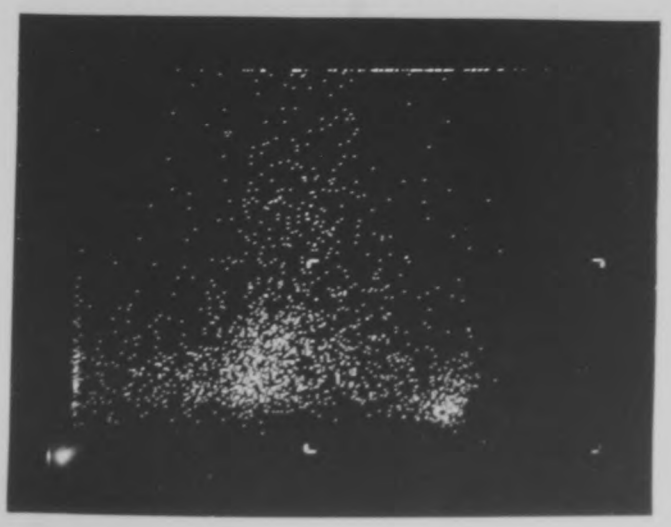


RIGHT ANGLE SCATTER

AT29 - ENU.556



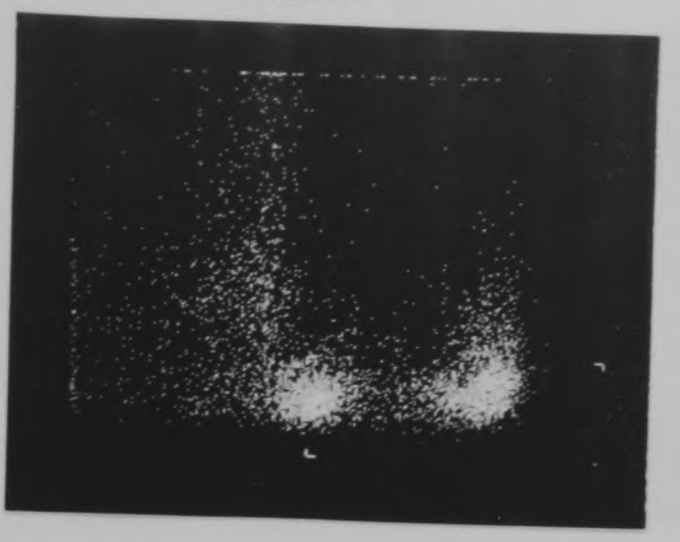
LOG FLUORESCENCE

FIG. m

BLOOD

STAINED FOR THY1

CBA



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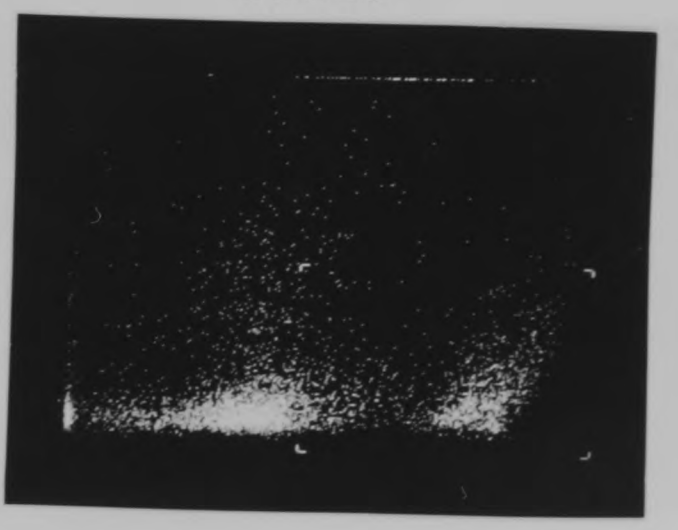

RIGHT ANGLE SCATTER

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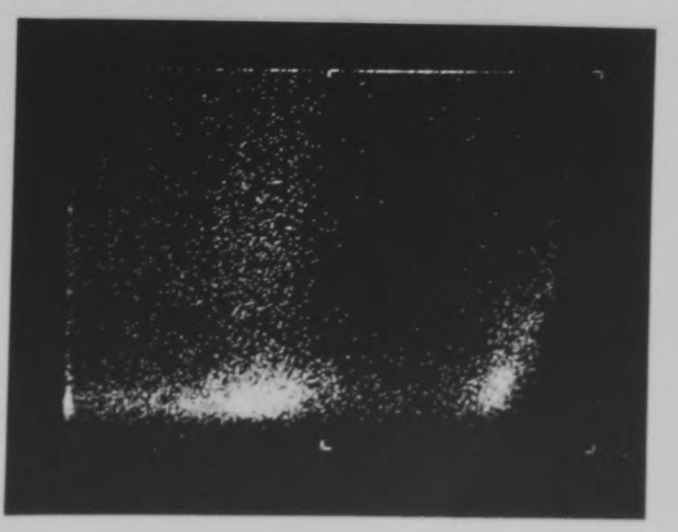


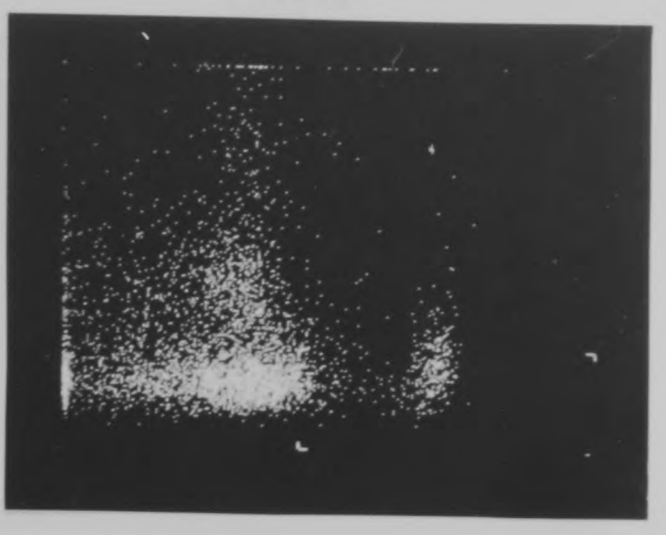
LOG FLUORESCENCE

FIG.n

BLOOD

STAINED FOR LYT2

CBA



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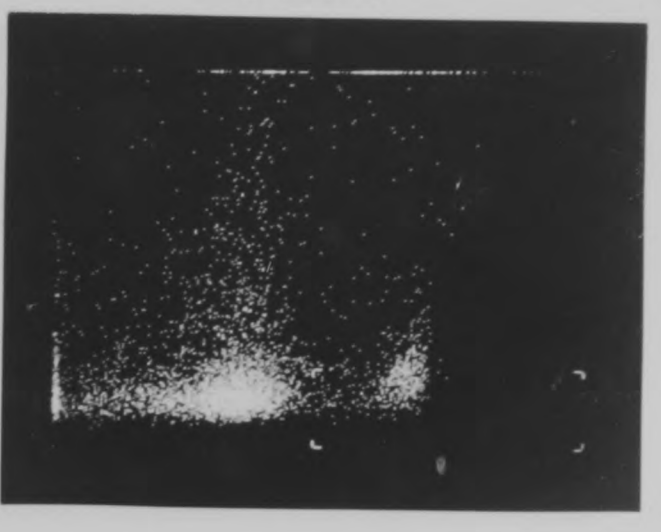

RIGHT ANGLE SCATTER

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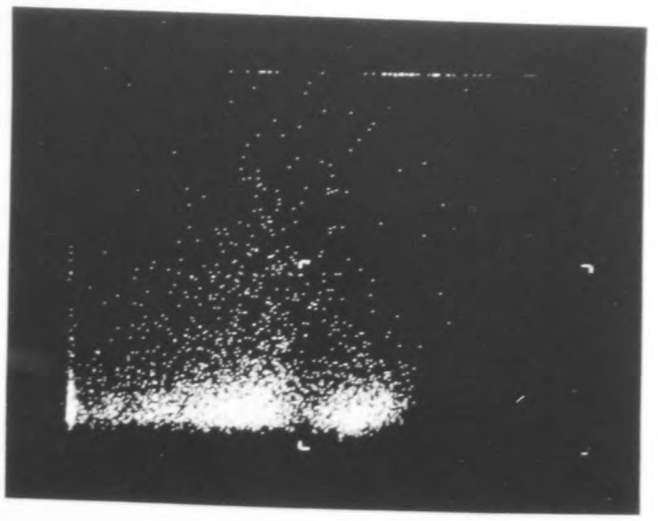
LOG FLUORESCENCE

FIG.o

BLOOD

STAINED FOR L3T4

CBA



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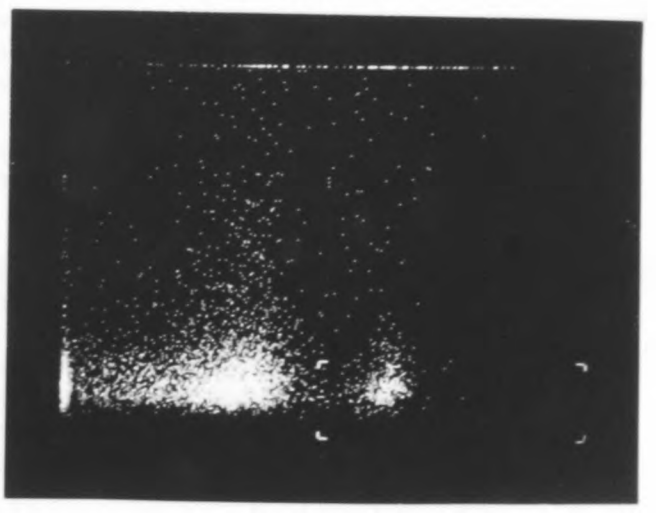

RIGHT ANGLE SCATTER

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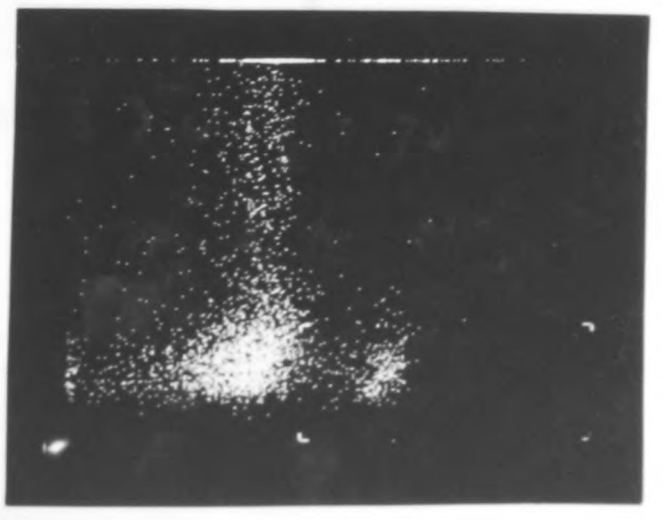

LOG FLUORESCENCE

FIG. p

Mosaic Analysis of the Effects of a Novel X-Chromosome Mutation of the Haematopoietic System

J.D. Ansell, V.M. Chapman*, L.M. Forrester, D.J. Fowlis, C. MacKenzie, and H.S. Micklem

SUMMARY

Mutations specific to the X chromosome were identified in female offspring, sired by mice treated with the mutagen ethyl nitrosourea (ENU), by alterations in the mosaic patterns of X chromosome-linked enzyme polymorphisms in blood and other somatic tissues. Flow cytometric analyses were performed on haematopoietic and lymphoid cells of mice homozygous for one such mutation. Similar studies were made on heterozygotes that were, in addition, mosaic for electrophoretic variants of the enzyme phosphoglycerate kinase (PGK-1). The PGK-1 mosaic patterns in cells of different haematopoietic lineages were used to investigate the nature of the primary lesion induced by the mutation. Preliminary results indicate that one of the ENU mutations has effects on differentiation of T lymphocytes and possibly some other haematopoietic cells.

INTRODUCTION

X chromosome-linked genes in man code for a variety of immuno-deficiency syndromes and haematopoietic disorders. Three of the major categories of immunodeficiency diseases are inherited through the X chromosome: Bruton-type agammaglobulinaemia is characterised by an absence of plasma cells, which renders patients susceptible to bacterial infections (Geha et al. 1973); in Wiskott-Aldrich syndrome (eczema-thrombocytopenia-immunodeficiency syndrome) T lymphocytes and platelets are lacking and there are defects in the afferent limb of the immune response at the level of antigen processing (Blaese et al. 1968); Swiss-type agammaglobulinaemia (thymic epithelial hypoplasia, X-linked severe combined immuno-deficiency disease) patients are vulnerable to viral and fungal, as well as bacterial, infections and suffer from lymphocytopenia and atrophy of the thymus. A profound immunodeficiency is seen in some cases despite high levels of circulating B lymphocytes, which are however incapable of terminal differentiation into plasma cells (Yount et al. 1978).

Several other less common X-linked haematopoietic disorders have been recorded. These include; immunodeficiency with increased IgM (dysgammaglobulinaemia Type 1), granulomatous disease, X-linked thrombo-cytopenia, malignant reticuloendotheliosis, acute X-linked leukaemia and Duncan disease (X-linked progressive combined immuno-deficiency, X-linked lymphoproliferative disease, familial fatal Epstein-Barr infection). X-linkage has also been noted for a rare form of hypochromic anaemia and a proliferation defect in haematopoietic cells (McKusick 1986).

In summary, there may be a number of genes on the X chromosome that play important roles in the differentiation of immune function and

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influence susceptibility to tumour development. These genes may also affect the differentiation of other haematopoietic elements. Few comparable disorders have been identified in mice or other experimental animals. The CBA/N mouse is homo- (or hemi-) zygous for the xid mutation and shows some similarity to the Bruton-type immunodeficiency syndrome (Scher 1982). However, the effect of the mutation is relatively mild. The mice do not respond to a class of 'T-independent' (T1-2) antigens and at least one population of B lymphocytes, characterised by high concentrations of membrane IgD (mIgD) and low concentrations of mIgM, is missing from the adult. Other X-linked loci in mice code for high, low or absent responses to specific antigens (Green 1981).

STRATEGIES FOR ANALYSIS OF X CHROMOSOME MUTATIONS IN MICE

Our own work in this laboratory has concentrated on the differentiation of B lymphocytes carrying the xid mutation in a heterozygous environment. The experimental strategy makes use of the phenomenon of X chromosome inactivation, an event which occurs shortly after implantation in female mammals and results in one or other of the X chromosomes in all somatic cells being functionally inactive. This is a random process, and results in an individual being a mosaic for any allelic differences on the X chromosome. Mosaicism for xid is not, in itself, detectable. However, we have been able to make use of another X-chromosome gene, Pgk-1, which has a detectable product; this is used as a marker for the presence of xid. The two alleles of Pgk-1 code for different electrophoretic forms of the enzyme phosphoglycerate kinase (PGK-1), A and B. These alloenzymes can be separated and quantified by simple electrophoretic techniques (Ansell and Micklem 1986). All somatic cells in female mice heterozygous for xid and Pgk-1 will be either PGK-1B with the xid mutant allele active or PGK-1A with the normal allelic counterpart of xid active. Any selection against cells carrying the xid mutant can be measured by looking at the relative expression of the Pgk alleles in that cell population. By separating subsets of B cells and other haematopoietic populations on a fluorescence activated cell sorter (FACS) we have been able to determine the cell types on which xid acts and the developmental stage at which it acts

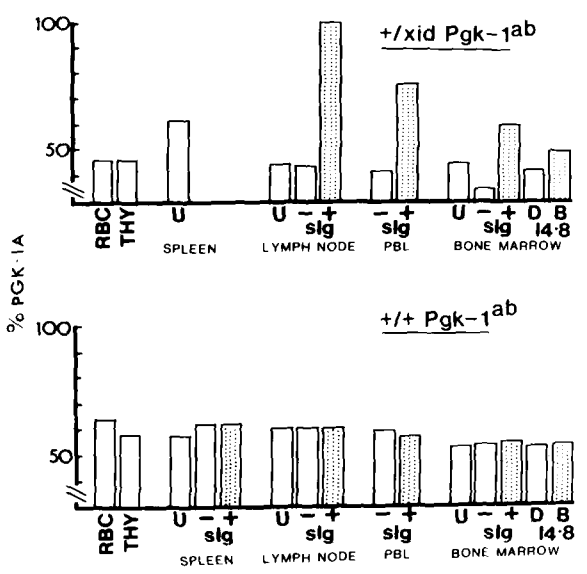

Fig. 1. Values of PGK-1A in cell lysates from a xid-heterozygous (+/xid Pgk-1ab) and a normal female (+/+ Pgk-1ab) mouse. Abbreviations : RBC, erythrocytes; THY, thymus; PBL, peripheral blood leucocytes; sIg, membrane immunoglobulin; + -, FACS-sorted cells positive or negative for sIg; 14.8, monoclonal antibody 14.8 against B220 antigen; B D, FACS-sorted cells staining brightly (B cells) or dully (pre-B cells) for B220. In the +/xid heterozygote, unbalanced mosaicism (stippled columns) is seen in peripheral B cells, but not in the bone marrow pre-B population.

(Witkowski et al. 1985, Forrester et al. 1987). An example of the data acquired by this approach is shown in Fig. 1. These and other data have shown that although the homozygous mutant is deficient only in certain classes of B lymphocyte, in the heterozygote virtually all mature B cells carrying an active mutant X chromosome are selected against. Further, this selection only operates on B cells after the stage at which membrane Ig (mIg) is expressed: earlier B-lineage cells in the bone marrow are unaffected.

This strategy can be used to analyse the effects of any X chromosome mutation at the cellular level. It has the additional advantage that genetic effects of single mutations can be analysed in a relatively normal heterozygous environment, thus enabling the nature of the primary lesion to be distinguished from other secondary effects which may constitute major parts of the syndrome associated with the homozygous (hemizygous) mutant.

ANALYSIS OF AN ENU-DERIVED MUTATION

The approach described above has been used in the analysis of an X chromosome mutation derived by ENU mutagenesis. The techniques of mutagenesis and the initial identification of mutants are described by Chapman (this volume). A female mouse was selected which showed random X chromosome expression in most somatic tissues, but selective expression of the non-mutagenised X chromosome in erythrocytes. This implied that during the course of erythropoietic differentiation, cells in which the mutant gene was active were at a selective disadvantage. In addition a preliminary experiment showed that lymph node cells had a non-mosaic phenotype. This suggested that the mutation may have been inimical to T lymphocyte differentiation, since most peripheral lymph node cells are T cells.

A number of homozygous mice derived from this original female have been analysed by flow cytofluorometry to investigate the effects of the mutation on various haematopoietic cell lineages. Female mice heterozygous for the mutant and for the A and B electrophoretic variants of PGK-1 have also been analysed and the mosaic phenotypes of sorted and unsorted haematopoietic cells investigated.

Single-cell suspensions of peripheral blood lymphocytes and from lymph node, spleen, thymus and bone marrow were prepared for FACS analysis and sorting. They were stained with monoclonal antibodies against Thy-1, Ly-3, L3T4 and B220 (Micklem 1986), a fluoresceinated goat anti-rat Ig serum being used as the second step. In addition cells of these organs as well as erythrocytes, brain and skeletal muscle, were prepared from heterozygous mice for alloenzyme analysis.

A series of dot plots taken from the FACS are shown in Figs. 2, 3 and 4, which illustrate some effects of this mutation on the haematopoietic system. Fig. 2 shows the staining of bone marrow suspensions from a normal 3 month old CBA/Ca female (A), a homozygous mutant female (B) and a heterozygous female (C), with a monoclonal antibody against the B220 antigen. Dully staining (pre-B) and brightly staining (mIg-expressing) cells usually comprise about 20% and 10% of the nucleated marrow cells respectively. In the mutant, these were reduced to 10% and 1% (mean of 5 animals). Although an approximately 1.5x increase in cellularity was seen in some mutant marrows, there appears to be some absolute reduction of B lymphopoiesis. Fig. 3 shows B220 staining in subcutaneous lymph node cells from the three types of animals. In a normal individual 14% of lymph node cells would be B cells (B220+ve). In the mutant this proportion was 47% with a proportionate decrease in the number of Thy-1+ve T cells

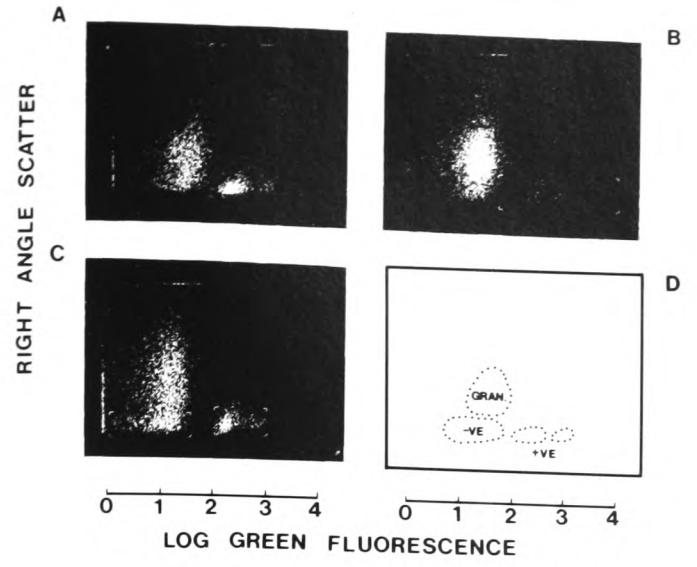

Fig. 2. FACS dot plots of bone marrow cells stained with a monoclonal antibody against B220 from a normal CBA/Ca control (A), an ENU homozygous mutant (B) and an ENU heterozygote (C). The diagram is of the cell populations in the control: the high-scattering granulopoietic and other cells (GRAN), a B220-negative population and two populations of B220-positive cells, the duller being pre-B cells and the brighter mIg-expressing B cells.

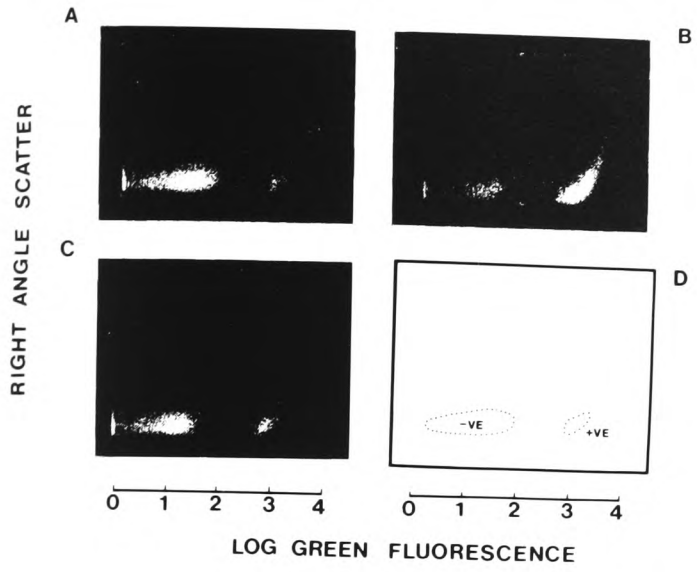

Fig. 3. Dot plots of lymph node cells stained for B220 as in Fig. 2. A, B and C are from the control, homozygous mutant and heterozygous mutant respectively. The homozygous mutant has a higher proportion of B220 +ve cells.

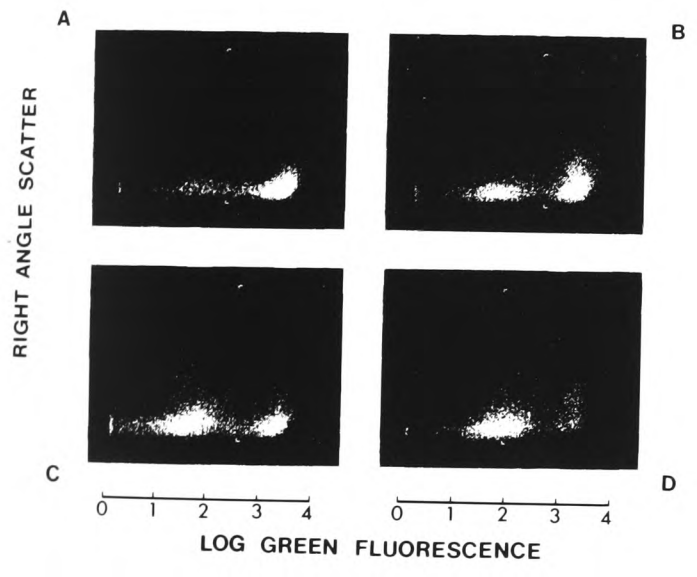

Fig. 4. Dot plots of lymph node cells (A and B) and spleen cells (C and D) stained with a monoclonal antibody against Thy-1. Thy-1 +ve cells are under-represented in the mutant (B and D) compared with the control (A and C) cell suspensions.

(Fig. 4, A and B). Similarly in the spleen (Fig. 4 C and D) the proportions of T cells in the mutant (4,D) are approximately half the normal value of 40%. Cells which stain for neither Thy-1 nor B220 usually account for 10% of splenocytes; in the mutant up to 55% of splenocytes are in this non-B, non-T category. It is apparent that one of the effects of this mutation was to alter the ratio of T to B lymphocyte numbers in the peripheral lymphoid organs. This was associated with increased granulopoiesis in the bone marrow (and a proportionate decrease in B lymphopoiesis), an increase in the numbers of granulocytes in the peripheral circulation (data not shown) and relative increases in the numbers of B lymphocytes in the peripheral lymphoid organs. Other preliminary observations on the homozygous mutant included decreased thymus size, decreased haematocrit and white blood cell count, increased bone marrow cellularity and increased size and cellularity of peripheral lymph nodes. Platelet counts were normal.

The heterozygotes appeared virtually normal. However analysis of the patterns of mosaicism for PGK-1 alloenzymes revealed defects at the cellular level in a number of organs. In normal heterozygotes all tissues would be expected to have a similar PGK-1 phenotype (McMahon et al. 1983, Micklem et al. 1987). The PGK-1 data for 5 ENU heterozygotes are summarised in Table 1. The data for skeletal muscle (SM) suggest that in these stocks two mutations may be segregating one of which has effects on the viability of muscle cells in the heterozygous environment. Since the original selection of these mutant stocks was on the basis of mosaic imbalance in blood, a double mutation would not have been detected. The mean ratio of PGK-1A:B in brain (BR) was 49:51). Against this yardstick PGK mosaicism in spleen (SPL) and bone marrow (BM) was slightly imbalanced in favour of the non-mutant X chromosome (PGK-1B in this case). This would suggest that there is no strong selection operating against B cells, or the bulk of differentiating haematopoietic cells, that carry an active mutant X chromosome. Platelets (PL) are relatively unaffected but circulating erythrocytes (E) have a greater imbalance. Selection against the mutant X is most apparent in cell suspensions from lymph node (LN) and thymus (THY). In lymph nodes of heterozygotes upwards of 80% of cells are T-cells. In thymus most cells are of T-lineage although in the preparation of cell suspensions it is possible to include varying proportions of stromal tissue. In two thymi, PGK-1A was less than 10%; in another, in which thymic lobes were analysed separately, one lobe was PGK-1B. In the one individual for which data are available so far FACS-sorted Thy-1+ve cells (T cells) from spleen and lymph node were shown to be entirely PGK-1B, i.e. devoid of cells carrying an active mutant X chromosome.

The data reviewed above indicate that the ENU mutation on the X chromosome has effects on T cell differentiation and may also have other effects on the haematopoietic system. Allozyme analysis of

Table 1. Percent PGK-1A in cells from ENU heterozygotes.

Mouse	Age (wks)	E	PBL	LN	SPL	BM	THY	PL	BR	SM	THY1+VE SPL	THY1+VE LN
2261-1	7	16	-	18	35	-	20	-	51	0	-	-
2261-2	9	28	-	16	32	-	17	-	32	18	-	-
2115-1	7	28	-	-	-	-	-	-	-	-	0	0
2261-3	12	14	9	10	20	33	14,0	-	50	-	-	-
2261-4	16	43	38	14	31	40	0	27	50	40	-	-
2115-2	7	31	-	18	46	-	8	37	60	0	-	-

FACS-sorted cell lineages as described above, coupled with functional studies of the immune system, will enable us to determine the more precise effects of the lesion on haematopoietic differentiation and immune function.

We are indebted to Kay Samuel, Andrew Sanderson and Helen Taylor for technical assistance and to Dr. Paul Kincade for a gift of 14.8 antibody. LMF, DJF and CM were supported by Medical Research Council Postgraduate Studentships.

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