Characterization of the genetic defects in patients with Severe Combined Immunodeficiency (SCID)

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Abstract

A specialised clinic for the diagnosis of primary immunodeficiency diseases was established at the Red Cross War Memorial Children's hospital (RXH) in 1982. The patient load was significant as clinic records indicated that 122 primary immunodeficiency cases were diagnosed on clinical and laboratory data, in the period between 1983-1999. More than fifty percent of these conditions were antibody deficiencies. Of the rest, nine cases were ascribed to severe combined immunodeficiency (SCID).

The aim of the project was to do (1) mutational analysis on the affected families (2)on the basis of the mutational analysis, offer genetic counselling (3) do carrier screening tests on the families studied (4) to try and find a genotype/ phenotype relationship in the gamma chain gene.

Initial work on this project concentrated on subjects with a classical X-linked SCID phenotype, caused by the common gamma chain gene. The gamma chain gene was screened for deleterious mutations in 5 patients that had come under investigation at Red Cross Hospital. Three have yielded missense mutations, these being E68K, R224W and R285Q. The 3 mutations found are not novel mutations and already appear within the SCID database. The project later expanded to include mutations in both the Rag1/Rag2 and IL-7Rα genes. It was hypothesised that the defects in the remaining 2 patients may have been due to mutations in the Rag1/Rag2 genes and IL-7Rα gene respectively. The hypothesis was strengthened after radio-labelled microsatellite analysis had been carried out. It was later confirmed that patient 4 had a mutation (an insertion of an A-adenine) in both alleles of the Rag1 gene, resulting in a truncated protein corresponding to position 859 of the cDNA and codon R259 of patient 5. Patient 4's mutation in the IL-7Rα gene still needs to be confirmed. The mutation data can now be used to offer genetic counselling and do carrier screening in the affected kindred.

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Abbreviations

α – alpha

β – beta

γ – gamma

ul- microlitre

UM – micromolar

A_{260} – absorbance at 260nm

A_{280} – absorbance at 280nm

AD - autosomal dominant

ADA – adenosine deaminase deficiency

AET- Aminoethylisothiuranium bromide hydrobromide

AIDS- acquired immune deficiency syndrome

APS - ammonium persulphate

AR-SCID - autosomal recessive sever combined immune deficiency

BSA - bovine serum albumin

btk - Bruton's tyrosine kinase

CDNA – complementary DNA

CEPH – Centre d'Etude du Polymorphisme Humaine

CGD - chronic granulomatous disease

CM – centimorgan

CVI – common variable immunodeficiency

CVID - common variable immunodeficiency

DATP - deoxyadenosine triphosphate

DGTP - deoxyguanosine triphosphate

DMSO-dimethyl sulfoxide

DNA – deoxyribonucleic acid

FCS - featal calf serum

FITC- fluoroscene isothiocynate

GDB - genome database

GvHD -f graft verusu host disease

HLA - human leukocyte antigen

HUGO - human gentics organization

HUMARA - human androgen receptor

Ig - immunoglobulin

IL - interleuken

IL2Ryc - interleuken 2 receptor gamma chain

IL7 - interleuken 7

IL7Ra - interleuken 7 receptor alpha

JAK3 - janus kinase 3

kDa - kilodaltons

LAD - leukocyte adhesion deficiency

MHC- mahor histocompatibility complex

MoAb's - monoclonal antibodies

mRNA - messenger riboneucleac acid

MW – molecular weight

NFAT - nuclear factor of activated T cells

NK - natural killer cells

OD - optical density

OS - Omenn syndrome

PAGE -polyacrylamide gel electrophoresis

PBMC's - peripheral blood mononuclear cells

PBS - phosphate buffer saline

PCR - polymerase chain reaction

PEG-ADA - polyethylene glycol adenosine deaminase deficiency

PID- primary immunodeficiency disease

PNP- purine nucleoside phosphorylase

RAG - recombination activation gene

RT-PCR - reverse transcription polymerase chain reaction

RPMI - Roswell Parker Memorial Institute

SCID- severe combined immune defiency

SSCP - single stranded conformation polymorphism

ssDNA - single stranded DNA

STR's - simple tandem repeat

TBE - tris, boric acid and EDTA

TCR - T cell receptor

TEMED - NNN'N' tetramethylethylenediamine

TH - tyrosine hydroxylase

Tris – 2-amino-2-(hydroxymethyl)-1,3-propandiol

UV – ultraviolet

VNTR's - variable number tandem repeats

XL - X-linked

XLA- X-linked agammaglobulinemia

XSCID- X-linked severe combined immune deficiency disease

ZAP 70 - zeta associated protein 70

Declaration

I, Gasna Abrahams Shaboodien, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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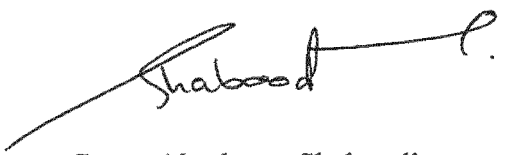

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The immune system and severe combined immunodefiency (SCID)

"The human immune system is an elegant and intricate biological defence mechanism that is unmatched in most life forms and has been evolving for more than 450 million years" (URL 9). "The immune system displays several remarkable characteristics such as specificity, memory, mobility, replicability, cooperativity and tolerance" (Claiman 1987). "When functioning properly it plays a vital role in protecting animals from invaders such as viruses and bacteria and other parasites. When it malfunctions, however, it can unleash a torrent of diseases, from allergy to arthritis to AIDS and cellular abnormalities such as cancer. The result is a sensitive system of checks and balances that produces an immune response that is prompt, effective, self-limiting and vital for the continuity and propagation of mankind" (URL 1).

1.1 Understanding the Immune System

The organs of the immune system are stationed throughout the body. They are generally referred to as lymphoid organs because they are concerned with the growth, development, and deployment of lymphocytes, the white cells that are the key operatives of the immune system. Lymphoid organs include the bone marrow and the thymus, as well as lymph nodes, spleen, tonsils and adenoids, the appendix, and clumps of lymphoid tissue in the small intestine, known as Peyer's patches. The blood and lymphatic vessels that carry lymphocytes to and from the other structures can also be considered lymphoid organs. Cells destined to become immune cells, like all other blood cells, are produced in the bone marrow. The descendants of some stem cells become lymphocytes, while others develop into a second major group of immune cells, the phagocytes (URL2).

In mammals the adaptive immune system relies on two lines of defence to detect and destroy invaders. Both parts of this system belong to a class of white blood cells called lymphocytes, found in blood and lymphoid organs. The two major classes of

lymphocytes are B cells and T cells. B cells complete their maturation in the bone marrow. T cells, on the other hand, migrate to the thymus, a multi-lobed organ that lies high behind the breastbone. There they multiply and mature into cells capable of producing an immune response, that is, they become immunocompetent. In a process referred to as T cell "education," T cells in the thymus are programmed to distinguish "self" cells from "non-self" cells; T cells that would react against "self" antigens are eliminated; T cells are divided into functional subsets that include T helper cells and cytotoxic T cells (Elgert 1996).

B and T lymphocytes perform different functions, but use similar genetic mechanisms to generate an almost unlimited number of antibodies and T-cell receptors. The human immune system is capable of producing a larger number of different antibodies and receptors than there are numbers of genes in the entire human genome. This is accomplished by using a smaller number of gene segments that can be shuffled and joined to one another to produce many distinct combinations. Each recombination essentially produces a new gene, and provides an almost infinite database of genetic information from which to generate antibodies and T-cell receptors (Janeway and Travers 1996).

The main function of B cells is to secrete antibodies, into the body's fluids, or humors (hence the term humoral immunity). Antibodies typically complex to circulating antigens on microorganisms and toxins but are unable to penetrate living cells (Figure1) (WHO Scientific group 1997, Janeway and Travers 1996).

Cytotoxic T cells, in contrast, interact directly with their targets, attacking and destroying infected body cells that have been commandeered by viruses (This is cellular immunity). Helper T cells communicate with other immune cells and instruct B-lymphocytes to synthesise antibodies (Figure2) in a few steps. The antigen is taken up by a macrophage and processed. Here the antigen reappears with the Major histo-compatibility complex

(MHC) on the surface of the macrophage where a helper T cells recognises the foreign protein and immediately sets about activating the B cells. The B cells then replicate and mature to plasma cells where they are then capable of secreting antibodies (Elgert 1996, Janeway and Travers 1996 and 1994).

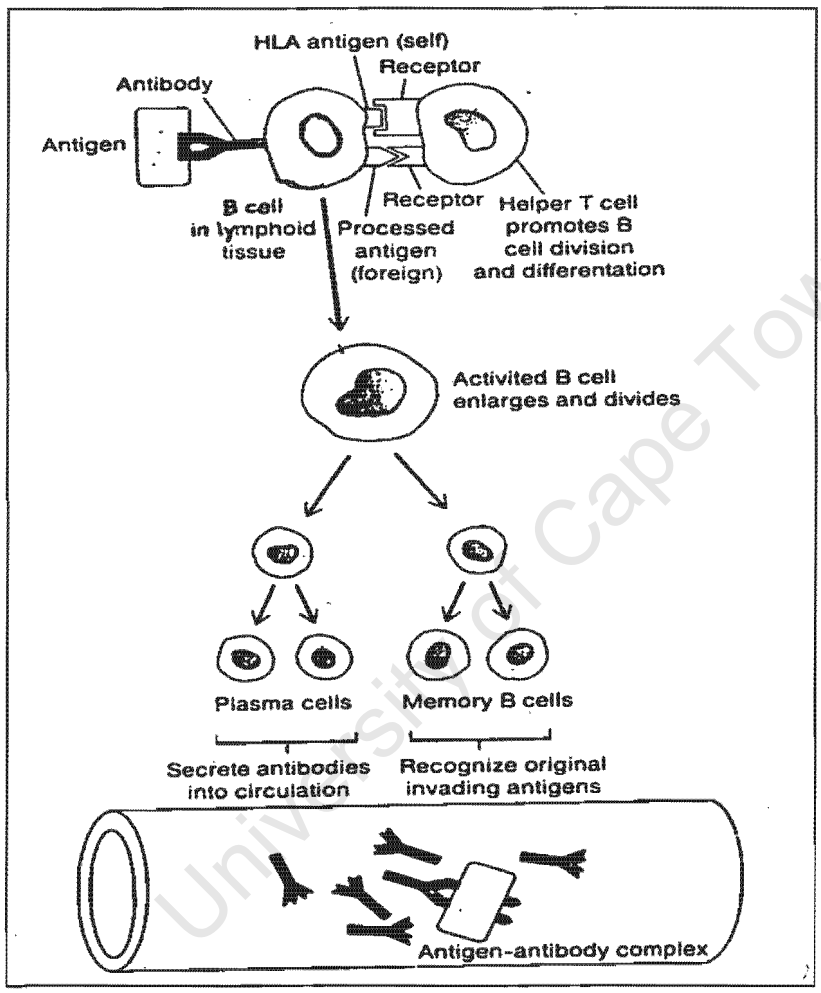


Fig 1 The role of B lymphocytes in Humoral Immunity (Travers and Janeway,1996)

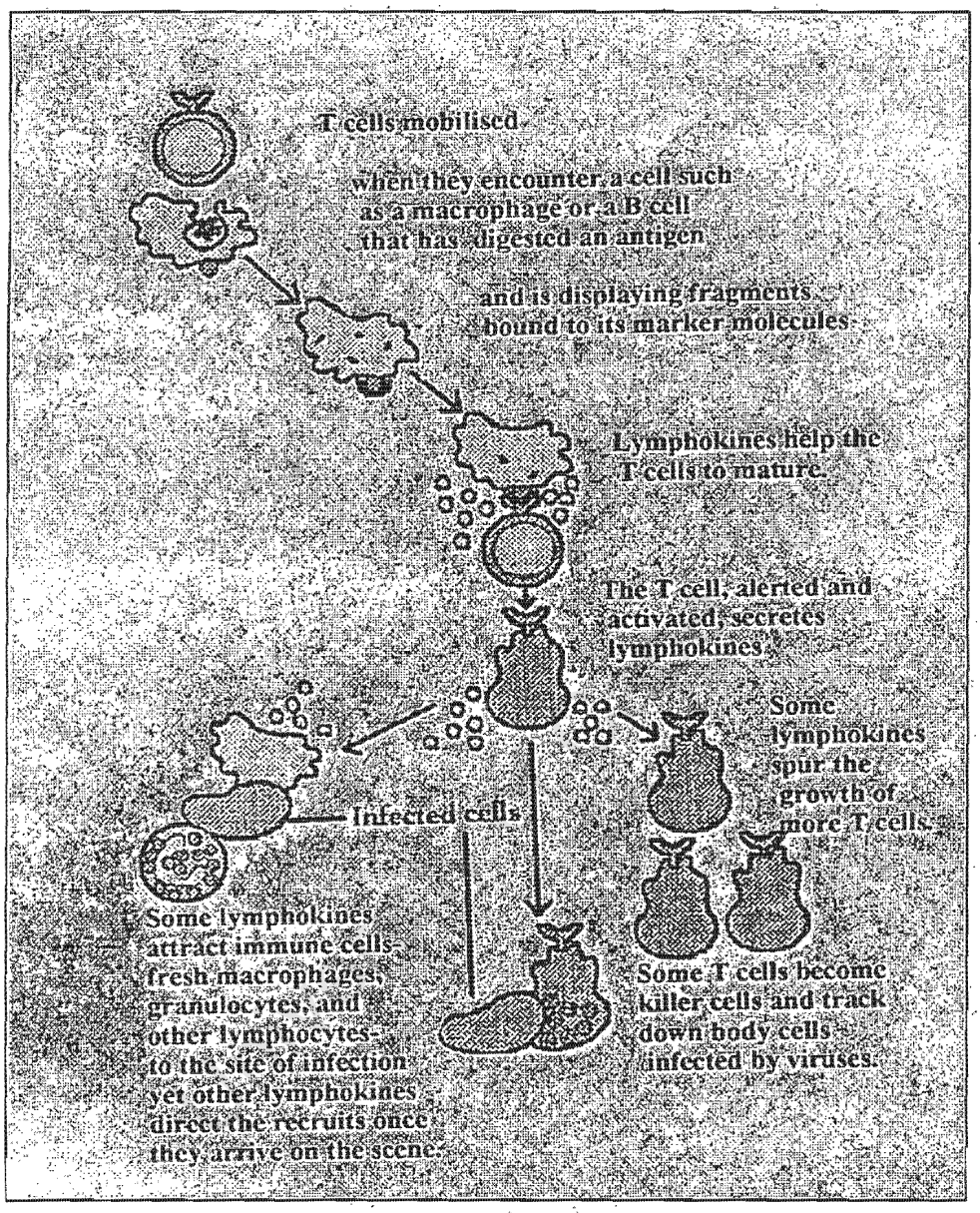


Fig 2 The role of T lymphocytes in Cellular Immunity (taken from URL 3).

1.2 What Are Primary Immunodeficiency Diseases (PID's)?

The primary immunodeficiency diseases are naturally occurring (inherited) defects of the immune system. These disorders were originally felt to be rare, to occur only in infants and young children and to be associated with severe clinical symptoms. However, as clinical

experience has grown, it has become clear that they are much more common than originally appreciated and that they can be present in older children, adolescents, and adults, and that they may cause mild clinical disease in some patients. Early detection of patients with primary immunodeficiency diseases is important, as effective therapy is available for many disorders. These therapies are most beneficial when instituted before recurrent infection or other complications has caused irreversible damage eg. chronic lung disease. Further, early recognition may lead to a precise genetic diagnosis, which may be important to the family in planning their future reproductive options (IUIS Scientific group 1999, WHO Scientific group 1997). These defects appear to be intrinsic to one or more components of the immune system.

The effects of a primary immunodeficiency can be devastating. For example, a baby born with severe combined immunodeficiency (SCID) faces death, if untreated, within a few months of birth or isolation in a sterile environment until a bone marrow transplant can be carried out (WHO Scientific Group 1997, IUIS Scientific Group 1999).

1.3 What are the causes of PID's?

PID's are a group of inherited disorders where genetic abnormalities lead to the development of these conditions. Recent progress in immunobiology and genetics has, with increasing precision, identified the causes of many PID's. Diagnosis and therapy can, as a result, be more specific and effective. Gene abnormalities are inherited, which is why in many cases several generations contain affected individuals. Some are inherited as autosomal recessive traits (e.g. autosomal recessive severe combined immunodeficiency (AR-SCID) and others as X-linked recessive traits (e.g. X-linked agammaglobulinemia (XLA). The PID's are conveniently divided into five functional categories: combined deficiencies in which both the cellular and humoral immune systems are affected, cellular deficiencies, antibody deficiencies, phagocytic disorders and deficiencies of the complement cascade (Kinnon and Gaspar 2000, Kinnon and Levinsky, 1998).

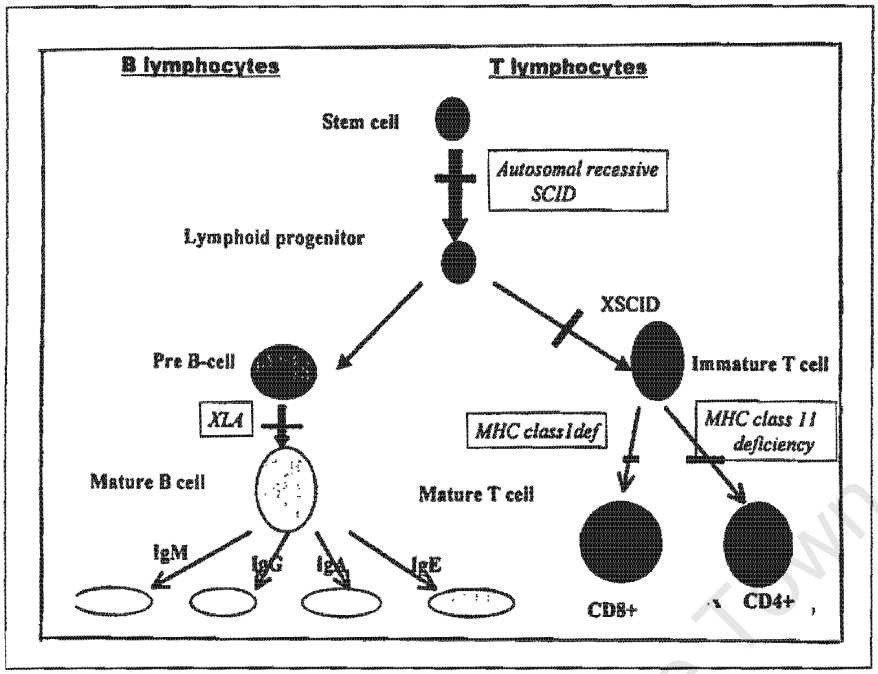


Fig3. Illustration of a few disorders in Lymphocyte development

Combined immunodeficiencies are characterised by dysfunction of both arms of the adaptive immune system i.e. T and B cell dysfunction coexists (Fig 3). Combined immunodeficiencies include SCID, purine nucleoside phosphorylase deficiency (PNP), zeta-associated protein 70 (ZAP70) deficiency and major-histocompatibility-complex class II (MHC class II) deficiency.

SCID is a group of conditions in which there is a block in T-lymphocyte development that is variably associated with abnormal development of other lymphocyte lineages i.e. B or NK lymphocyte or more rarely of the myeloid lineage. Two thirds of all SCID is caused by mutations in the gene encoding the common gamma chain, which is shared by interleuken (IL)- 2, IL-4, IL-7, IL-9 and IL-15 receptors. This condition is acquired by X-linked recessive inheritance and is referred to as X-linked SCID (XSCID) (Kumaki *et al* 1999, Puck *et al* 1997, Jones *et al* 1997). Most of the other mutated genes that cause SCID are acquired by autosomal recessive inheritance (AR), an absence of both T And B

lymphocytes is most often found. An example of AR-SCID is adenosine deaminase (ADA) deficiency. In the absence of the enzyme ADA, toxic metabolites of the purine pathway (eg. deoxyadenosine (dATP)) and the methylation pathway (S-adenosyl homosysteine) accumulate within the cell and impair proliferation. Both T and B lymphocyte functions are defective (Uribe and Weinberg 1998, Buckley *et al* 1997).

PNP deficiency also leads to build up of toxic metabolites, in this case deoxyguanosine (dGTP), which results in its accumulation within the cell and impaired proliferation. The effect of the deficiency is seen in the lymphocytes. T lymphocytes are particularly sensitive to the accumulation of dGTP and are affected to a greater degree than B-lymphocytes.

Mutations in the ZAP-70 gene, coding for a TCR associated protein tyrosine kinase that is associates with the ζ chains of the TCR-CD3 complex, produce a CD4+CD8- combined immunodeficiency, with the CD4+ cells failing to proliferate following TCR stimulation. MHC class II deficiency is characterized by T and B cells being present but non-functional (Spickett 1997, WHO Scientific group 1999).

Cellular immunodeficiencies in which T cell development is abnormal include primary CD4+ T cell deficiency, IL-2 deficiency, multiple cytokine defects and signal transduction defects. The genetics of many of these defects are, poorly understood (WHO Scientific group 1997 and 1999).

Patients with primary CD4+ T cell deficiency have a profound, persistent decrease in circulating CD4+ T cells. Immunoglobulin levels may be normal or slightly decreased. Children with Multiple Cytokine defects have T cells that lack the nuclear factor of activated T cells (NFAT). In Signal Transduction defects T cells fail to show normal calcium flux and diacylglycerol generation after antigenic stimulation. The defect can be circumvented by stimulation with aluminium tetrafluoride (AlF_4) (WHO Scientific group

1997 and 1999).

Defects in the B lymphocyte maturation pathway result in several antibody deficiencies, including X-linked agammaglobulinaemia (XLA), common variable immunodeficiency (CVID), and selective IgA deficiency (Conley *et al* 1994, Spickett 1997).

X-linked agammaglobulinaemia (XLA) is a pure B cell deficiency. There are no circulating B cells but T cell function is normal. The defect causes arrest in B cell development at the pre-B cell stage . The underlying defect in XLA is a mutation of the Bruton's tyrosine kinase (btk) gene (Buckley 2000, WHO Scientific group 1997, Janeway and Travers 1996).

Common variable immunodeficiency (CVID) is a heterogeneous group of disorders in patients who have hypogammaglobulinemia with a variable degree of T-cell dysfunction. There is no recognisable pattern of inheritance, although CVID may cluster in some families (Janeway and Travers 1996). Most patients with CVID do not manifest symptoms until the second or third decade of life; a smaller number of patients have clinical symptoms during the first decade of life (WHO Scientific group 1997).

The most common form of immunoglobulin deficiency is selective IgA deficiency and is seen in about 1 in every 700 Caucasians (in contrast to 1 in every 18,500 Japanese individuals). The genetic basis of this defect is unknown but some data suggest that a gene of unidentified function, mapping in the class III region of the MHC, may be involved (Janeway and Travers 1996, WHO 1999).

Phagocytes include white blood cells called neutrophils and macrophages that engulf and kill micro-organisms. Phagocytic disorders include defective intracellular killing mechanisms and defective locomotion. Defects in intracellular killing of ingested micro-organisms usually result from failure of production of superoxide anion, singlet oxygen, and hydrogen peroxide. These oxygen compounds are generated by the enzyme NADPH

oxidase, a multicomponent enzyme located in the plasma membrane of phagocytic leukocytes. The superoxide anion (O_2^-), when produced, may subsequently be converted to hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and other anti-microbial products in the phagosomes that contain the ingested microorganisms. Failure to produce reactive oxygen radicals results in the most severe form of phagocytic cell deficiency known as chronic granulomatous disease (CGD) (Roos *et al* 1996, WHO Scientific Group 1997). Biochemically, CGD is characterised by the inability of phagocytic leukocytes to generate reactive oxygen compounds, which are needed for the intracellular killing of phagocytized micro-organisms (Smith and Curnutte 1991).

The complement system consists of a group of more than 30 proteins interacting in a cascade that leads to a variety of biological processes important in host resistance to infection and the inflammatory response. Functions of the complement system include lysis of invading pathogens, chemotaxis, anaphylatoxin activity and enhancement of phagocytosis. Lysis of bacterial or animal cells requires the activation of the terminal complement proteins C5, C6, C7, C8 and C9 (IUIS Scientific group 1999).

The abovementioned diseases are a representative sample of the most common primary immunodeficiencies. Further examples are listed in Table 1(Kinnon and Gaspar 2000, Kinnon and Levinsky 1998, WHO Scientific group 1997 and 1999).

1.4 Treatment options for PID's

The need to develop effective treatments for these patients has been one of the greatest challenges for clinical immunology. Early attempts at treatment included the controversial confinement of patients to a germfree environment where all food, water, and air are sterilized. David, the "bubble boy" from Houston, was diagnosed as having XSCID in 1969 and survived this way until he died at the age of 12 after an unsuccessful bone marrow transplant. Quite a number of advances have been made recently. These include improved outcome after bone marrow transplantation., safe immunoglobin and enzyme replacement therapy and gene therapy.

Table 1: Disorders of the Lymphocyte maturation pathway

Functional Compartments	Designation	Immune defect	Inheritance	Chromosome Location
B lymphocyte system	XLA	Profoundly decreased B cells	XL	Xq21.3-22
	Non-X linked hyper IgM	IgM and IgD bearing cells present, other B cells absent	?	-
	Ig heavy-chain gene deletions	Normal 7or decreased B cells	AR	14q32
	Kappa chain deficiency	Normal or decreased kappa bearing cells	AR	2p11
	Selective IgG deficiency	Normal or immature B cells	Unknown	14q32
	Antibody deficiency with normal Ig's	Normal B cells	Unknown	-
	CVID	Normal or decreased B cells	Variable	-
	IgA deficiency	Normal or decreased sIgA+	Variable	-
T lymphocyte system	Primary CD4 deficiency	Persistent decrease in T lymphocytes	Unknown	Unknown
	Primary CD7 deficiency	Unknown	-	-
	Multiple cytokine defect	T cells affected	Unknown	-
	Signal transduction defect	T cells affected	Unknown	-
(Variable)	Di George syndrome	Normal or decreased T cells	de novo or AD	22q11
Combined (T & B cells) Immunodeficiencies	X-linked SCID	B cells-normal or increased, T cells markedly decreased	XL	Xq13.1
	ADA deficiency	B cells decreased, T cells Progressively decreased	AR	20q12-13.11
	PNP deficiency	B cells normal, T cells progressively decreased	AR	14q11.2
	MHC class 11 deficiency	B cells normal, T cells markedly decreased	AR	-
	Reticular dysgenesis	decreased B cells, decreased T cells	AR	-
	CD3 deficiency	Normal B and T lymphocytes	AR	11q23
	CD8 deficiency	Normal B cells, decreased CD8, normal CD4	AR	2q12
(Variable)	Wiskott-Aldrich syndrome.	Normal B cells, decreased T cells	XL	Xp11

Bone marrow transplantation. Bone marrow transplants have revolutionized the treatment of patients with severe immunodeficiencies. Transplantation of bone marrow cells from human leukocyte antigen (HLA) genotypically identical donors, T cell depleted HLA-haploidentical parental donors or cord blood sources has led to complete immunological reconstitution of many patients with SCID (Buckley *et al* 1999). Bone marrow transplantation has also been successful in other PID's including leukocyte adhesion deficiency (LAD), MHC class II deficiency, chronic granulomatous disease, and Chediak-Higashi syndrome (Blaese 1995, WHO Scientific Group, 1997, Stephan *et al* 1993).

Immunoglobin replacement therapy. It is now accepted that patients with primary specific immunodeficiency characterized by diminished serum IgG levels and/or demonstrated defects in antibody production should receive IgG replacement therapy. The severity and frequency of infections as well as the serum IgG concentration initially determine the dose required. Experience has shown that immunoglobin replacement therapy is life saving if started early (IUIS Scientific Group 1999, Chapel 1994). Optimal treatment controls the cycle of recurrent infection and arrests the progression to chronic lung disease.

Enzyme replacement therapy. Partial replacement of enzymes through transfusion with frozen irradiated red blood cells has been attempted in infants with ADA and PNP deficiency, with little success. The amounts of purine degradation enzymes within the red blood cells were not sufficient to permit efficient degradation of toxic metabolites within lymphocytes. Partial enzyme replacement in ADA deficiency has also been attempted by the intravenous infusion of bovine ADA modified by conjugation with polyethylene glycol (PEG-ADA). Repeated weekly administration of the conjugated enzyme has resulted in marked clinical and immunological improvement of several patients (WHO Scientific Group, 1997 and 1999). This treatment is not curative, but enables the patient to be kept alive until bone marrow transplantation or gene therapy can be undertaken.

Gene therapy. ADA deficiency was the first PID for which gene therapy was attempted. The normal ADA gene was transferred to mature T lymphocytes using a retroviral vector (Blaese *et al* 1995). Frequent treatments were necessary to maintain a sufficient pool of functional cells. Another research group attempted to transfer the normal gene into hematopoietic stem cells derived from ADA deficient patients but the level of transfection was too low to correct the deficiency (Spickett 1997). ADA gene therapy was attempted with blood stem cells removed from the umbilical cords of three newborn babies who were known to have homozygous ADA deficiency (Kohn *et al* 1995). These patients were simultaneously treated with PEG-ADA infusion. Five years after the gene therapy, when the PEG-ADA was stopped in one patient, plasma ADA became undetectable and erythrocyte deoxyadenosine metabolites increased to levels that had been present at birth. Discontinuation of exogenous PEG-ADA caused a decline of both transduced and non-transduced antigen-specific peripheral blood T lymphocytes and a loss of in vitro antigen specific function. When PEG-ADA was resumed, erythrocyte deoxyadenosine metabolite levels decreased, circulating NK and B cells normalized and antigen specific T lymphocyte function returned. These results, while promising, defined the limits of gene therapy during the late 1990's. To improve the outcome of gene therapy new vectors, that express transduced genes in non-dividing cells and that increase the frequency of stable human stem cell transduction, are needed (Parkman *et al* 2000).

One of the successes of gene therapy involved two infants suffering from inherited X-SCID. Gene therapy was initiated, based on the use of complementary DNA containing a normal gamma chain gene, which was then transfected into a Moloney retrovirus-derived vector with ex vivo infection of CD34+ cells. The transduced stem cells were then transfused back into the patients. After a 10-month follow-up period, γc transgene-expressing T and NK cells were detected in both patients . T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls (Cavazzana-Calvo *et al* 2000). Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit.

Gene therapy could be the treatment of choice for many severe immunodeficiencies as the disadvantages of bone marrow transplantation such as graft-versus-host disease (GvHD), increased risk of viral infections after transplantation and the rarity of finding an HLA identical bone marrow donor.

1.5 Severe combined Immunodeficiency (SCID)

Severe combined immunodeficiency is characterised by a profound deficiency of both humoral- and cell- mediated immunity that leads to death in the absence of successful bone marrow transplantation or gene therapy. Without a functional immune system, SCID patients are susceptible to bacterial, viral, protozoal and fungal infections. If untreated they usually die before the age of two years. Bone marrow transplantation may save the majority of patients with SCID. Within this disease classification are several distinct disorders that have different patterns of cellular deficiency (Fig 4). All forms of SCID are inherited, with as many as two thirds of SCID cases being X-linked. In the 51 years since the initial description of SCID, it has become evident that the genetic origins of these conditions are quite diverse (Ting *et al* 1999, Puck *et al* 1997, Buckley *et al* 1997, Puck *et al* 1995, Fischer *et al* 1990, de Saint Basile *et al* 1987).

1.6 Classification of SCID

Severe combined immunodeficiency can be conveniently divided into 2 major categories depending on the presence or absence of circulating mature T and B cells within the vascular compartment ie : (i) condition in which both T and B cell numbers are extremely low or absent and (ii) the condition in which T cells are absent but B cell numbers are normal or increased. These two categories are further subdivided depending upon the presence or absence of circulating natural killer (NK)cells. Within each subdivision one or more genetically distinct diseases have been located. The $T^-B^+NK^-$ immunophenotype is the most common form of SCID, caused primarily by mutations in the gene encoding the common gamma chain (XSCID). In fact XSCID accounts for approximately two thirds of all cases if SCID (Table 2) (Uribe and Weinberg 1998, Fugmann *et al* 1998,

Stephan *et al* 1993).

Table 2: Classification of SCID

Disease	Circulating B cells	Circulating T cells	Inheritance	Chromosome
T^-B^+				
$T^-B^+NK^-$ SCID				
(a)common gamma chain deficiency	Normal or Increased	Markedly decreased	XL	Xq13.1-13.3
(b) Jak3 deficiency	Normal or decreased	Markedly decreased	AR	19p13.1
$T^-B^+NK^+$ SCID				
(a) IL7-Rα deficiency	Normal or Increased	Markedly decreased	AR	5p13
T^-B^-				
T-B-NK+ SCID				
(a) RAG 1 or RAG 2 deficiency	Markedly decreased	Markedly decreased	AR	11p12-13
(b) Omenn Syndrome	Decrease	Decrease	AR	
(c) CD45 deficiency	Normal but affected	Markedly decreased	AR	1q31-32
T-B-NK- SCID				
(a) ADA deficiency	Progressive decrease	Progressive decrease	AR	20q13-ter
(b)Reticular dysgenesis	Markedly decreased	Markedly decreased	AR	-

XSCID is caused by a mutation in the gene encoding the common gamma chain. It is characterized by a profound defect of T and NK cells but with normal to slightly increased numbers of B cells (Ohbo *et al* 1996). This results in arrested T cell development. Mutations in the IL2RG gene may affect the interaction between cytokines an their receptors, prevent dimerisation between γc and other cytokine subunits, or fail to mediate interaction with the intracellular kinase JAK3 and thereby prevent signal transduction (Wengler *et al* 1998).

In 1995 the gene responsible for one autosomal recessive form of $T^-B^+NK^-$ SCID was defined. This proved to be the JAK3 gene, a member of the Janus kinase family, which is associated to the common gamma γc chain of some cytokine receptors. JAK 3 is bound to the intracellular tail of the γc chain and is activated upon cytokine binding to the multi-chain receptor which leads to signal transduction and activation of genes involved in progression of cell division. Present data suggests that JAK3 deficiency accounts for most of the patients affected by non-X-linked ($T^-B^+NK^-$) SCID (Table 2) (Buckley 2000,WHO Scientific group 1997 and 2000).

IL7Rαc deficiency is the only autosomal disorder known to have the $T^-B^+NK^+$ SCID immunophenotype. The α–subunit of the IL7R is essential for IL7/IL7R complex formation, leading to the up-regulation of terminal deoxynulceotidyltransferase (TdT) and cytoplasmic Ig μ heavy chain (cμ) at the early and/or late pro-B cell stages. In 1998 two human mutations were identified in the IL7Rα gene; no other mutations have been reported since (Puel *et al* 1998).

Two closely linked genes, RAG1 and RAG2 (recombination activating genes 1 and 2), encode proteins that promote recombination of the V,D and J genes and allow for the generation of an almost unlimited number of antibodies and T cell receptors. They work together as a transposase (an enzyme) that excises pieces of DNA out of one location and transposes them elsewhere, allowing vertebrates to create millions of different antibodies and T cell receptors from a limited number of genes. Deficiency of either RAG1 or RAG2 causes $T^-B^-NK^+$ SCID (Buckley 2000).

Omenn syndrome (OS) is a rare disorder characterized by diffuse erythrodermia, eosinophilia, hepatosplenomegaly, lymphoadenopathy and elevated serum IgE levels. Unless treated by bone marrow transplantation, this disease is invariably fatal. Omenn's syndrome may arise from different primary defects, the common endpoint being SCID with clinical and pathological features of GvHD caused by an autologous source of self-

reactive T lymphocytes. The immunologic hallmark of this disease is the expansion of an oligoclonal population of T cells. This may be caused by primary thymic defects in some patients or dysplastic thymi in other patients. The identification of partial recombination activating gene (RAG) defects in Omenn's patients confirms that primary defects in VDJ recombination cause the thymic dysplasia and abnormal T cell development in many patients due to abnormal TCR rearrangement and assembly (Buckley 2000, Brooks *et al* 1999). This disease also has a $T^-B^-NK^+$ SCID immunophenotype.

The haematopoietic–specific transmembrane protein tyrosine phosphatase CD45 functions to regulate *Src* kinases required for T- and B cell antigen receptor signal transduction. Kung *et al* (2000) identified a male patient who presented at 2 months of age with a deficiency in CD 45 due to a large deletion at one allele and a point mutation at the other. The patient's peripheral blood T lymphocytes were greatly diminished and unresponsive to mitogen stimulation. Despite normal B-lymphocyte numbers, his serum immunoglobulin levels decreased with age. CD 45 deficiency thus results in a T^-B^- immunophenotype with mild NK cell lymphopena. No other human deficiency had ever been reported in a tyrosine-specific phosphatase (Kung *et al* 2000, Fischer 2000).

Most of the autosomal recessive ($T^-B^-NK^-$) forms of SCID are caused by defects in adenosine deaminase (ADA) (less than 15% of total SCID's) and purine nucleoside phosphorylase (PNP) (about 4% of total SCID's), enzymes involved in purine metabolism. Both T and B cells are absent; as are immunoglobulins, but there may be functional natural killer cells present (Stephan *et al* 1993).

Reticular dysgenesis is defined as T and B cell lymphocytopenia with persistent, profound neutropenia of central origin and thrombocytopenia. This rare hereditary autosomal recessive disease is generally lethal shortly after birth. It results from failure in the maturation of both lymphoid and myeloid precursors. It is characterized not only by striking lymphopenia, but also by severe granulocytopenia and thrombocytopenia, and overwhelming infections with early death. These newborns often exhibit engraftment of maternal cells (Buckley *et al* 1997).

1.7 Genetic and molecular basis of SCID

The five candidate genes that came under close scrutiny during this study were the common gamma chain (γc), interleuken 7 (IL7), interleuken 7 receptor alpha chain (IL7Rαc), the recombination activating genes 1 and 2 (Rag1 and Rag2) (Buckley 2000, WHO Scientific Group 1999). These and other relevant genes, and their protein products are briefly discussed in the following paragraphs.

1.7.1 Common gamma chain (γc)

The gene encoding the IL-2Rγc has been localized to human chromosome Xq13 and was found to be mutated in XSCID. IL-2Rγc is shared by at least five different cytokine receptors: IL-2R, IL-4R, IL-7R, IL-9R and IL-15R. For this reason, the IL-2Rγ is also known as the common γc. The γc gene comprises 8 exons, which roughly correspond to functional domains of the encoded protein (Fig 6) (Uribe and Weinberg 1998, Puck *et al* 1996, Voss *et al* 1994).

Exons 1 to 5 encode the signal sequence and extracellular domain, exon 6 the single transmembrane domain, and exons 7 through 8 the intracellular domain. The mature protein has a relative molecular mass of approximately 65 kilodaltons (kD), and has several glycosylation sites in the extracellular domain (Uribe and Weinberg 1998).

The γc plays a central role in the IL2/IL2R signaling transduction system through activation of its associated tyrosine kinase, JAK3. It is also constitutively expressed by T cells, B cells and NK cells Monitoring and manipulation of this system has important diagnostic and therapeutic implications.

1.7.1.1 IL2/IL2R signaling pathway

The biological activities of IL2 are mediated through the binding of IL2 to a multisubunit cellular receptor. Although three distinct transmembrane glycoprotein subunits

contribute to the formation of the "definitive" high affinity IL-2R, various combinations of receptor subunits are known to occur. The alpha-beta-gamma heterodimer is generally considered to be the high affinity, signal transducing receptor for IL2 (Fig 4) (Leonard 1996). The non-signaling α-chain, which is not a member of the cytokine receptor superfamily, complexes with the β-chain and the γ-chain, both of which are members of the cytokine receptor superfamily (Matthews *et al* 1995, Cao *et al* 1993). The IL2R subunits do not appear to be covalently linked but can be crosslinked in the presence of IL2. Each component of the IL2R has different binding activities. The α-chain has a cytoplasmic tail of only 13 amino acids and has low affinity binding to IL2, thus suggesting a limited role in signal transduction. The β-chain forms an intermediate affinity receptor and contains a large intracytoplasmic region lacking any tyrosine kinase motifs. It has a conserved region of 4 regularly spaced Cys residues and a Trp-Ser (WS) motif in the extracellular domain. The WS motif is essential for the formation of a high affinity receptor (Uribe and Weinberg 1998).

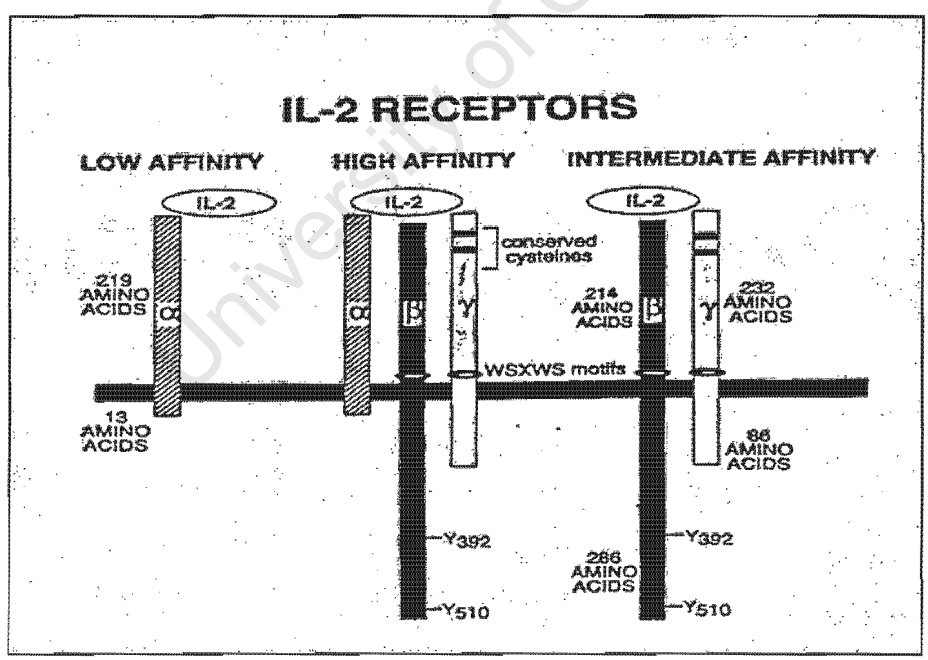


Fig 4 The Interleuken-2 receptor (IL2R) complex (taken from Voss *et al*, 1994)

Expression of the α and β–chain without the γc results in the formation of a pseudo-high affinity receptor, from which IL-2 rapidly dissociates. The receptor phenotype (α+β+γ–) is presumably the same one expressed by the rare T cell precursors that develop in patients with X-SCID. The γc contains the conserved 4 Cys residues and WS motif of the cytokine receptor superfamily. In addition there are 2 motifs in the intracytoplasmic domain that are likely to be involved in signal transduction.

1.7.1.2 γc Mutations

In the IL-2Rγc database, there are currently 220 mutations in unrelated patients with X-SCID, which span all eight exons (Fig5). The mutations causing XSCID include point mutations, deletions, insertions and splicing defects. Several hotspots for mutation have been noted as they account for a disproportionate share of mutations. For example, mutations involving Arg 224 and Arg 226 represent 13% of all XSCID cases. Another mutational hot spot in γc results in a premature stop codon at Arg 289 in the cytoplasmic domain. This truncated protein is expressed at the cell surface normally, binds ligand efficiently, but cannot bind Jak3 or transduce signals. This mutant could theoretically compete for receptor formation with a normal γc protein introduced by gene therapy. Thus, forms of XSCID with truncated protein production may not be as amendable to gene therapy approaches as those forms in which no γc protein is produced. The accumulated data indicates that the most common mutations are single base-pair substitutions, followed by splice-site mutations and deletions or insertions resulting in frameshifts. Insertions account for only a small number of mutations ((URL 4, Puck *et al* 1996).

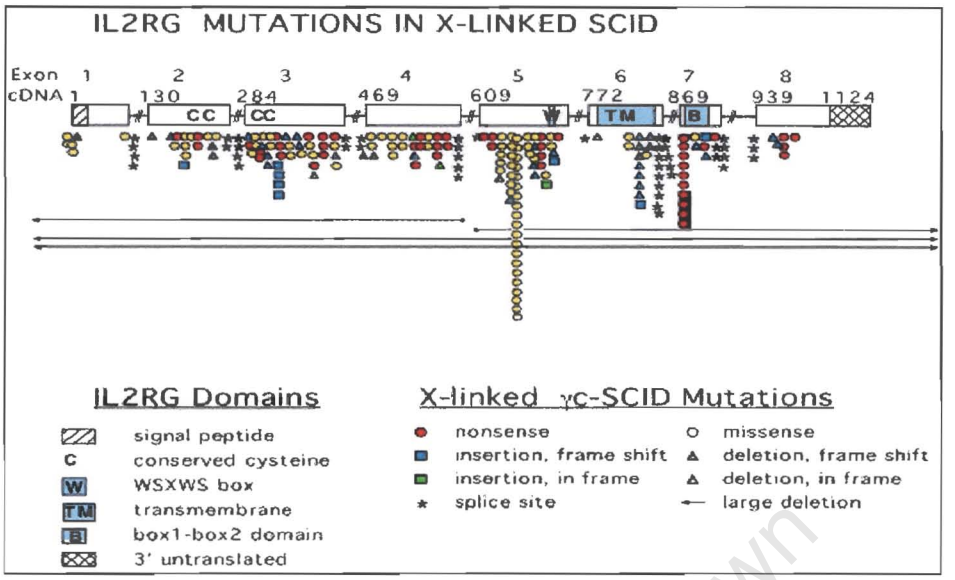


Fig 5 IL-2Rγ chain exons, intervening sequences, and known mutations as of the year 2000 (URL 4).

Analyses of phenotypic data still reveal no clear genotype-phenotype correlation.

1.7.2 IL-7

IL-7 is a 25-kDa soluble protein, is localized to the long arm of chromosome 8 at position 8q12-q13 and is produced primarily by the bone marrow- and the thymic stromal cells. It functions as a growth factor for pro- and pre-B cells, thymocytes, and has a number of effects on lymphocytes. In addition it augments the generation of anti-viral, alloreative, and anti-tumor cytotoxic T cells and the proliferation of NK cells. However, IL-7 is unable to stimulate the proliferation of mature B cells (Wei *et al* 2000, Tang *et al* 1997, Perumal *et al* 1997, Pandrau-Garcia *et al* 1994).

1.7.3 IL7Rαc

The gene encoding the IL7Rαc is localized to chromosome 5p13, and has a predicted molecular mass of approximately 50 kDa. The mRNA contains alternate splicing sequences that could allow for the generation of a soluble form of the receptor. IL7R (CDw127) displays both low- and high affinity for its ligand, the low affinity receptor being the more represented. Binding of IL7 to IL7R, which lacks a protein tyrosine kinase domain, induces tyrosine phosphorylation by recruiting the activity of p59 fyn tyrosine kinase (Pandrau-Garcia *et al* 1994). IL7R consists of 2 subunits, α and β.

1.7.3.1 IL7/IL7R signaling pathway

IL7 stimulates cells through the interaction of a high affinity receptor complex composed of the IL-7Rα and the IL2R γchain, both members of the haemopoietin receptor family (Maraskovsky *et al* 1996). The IL7Rα chain associates with the IL2R γchain, which is mutated in X-linked SCID. The IL7R promotes both proliferation and immunoglobulin heavy-chain rearrangement, by distinct signaling pathways. Studies have shown that IL7Rα-deficient mice lack γδ T cells, but have relatively normal numbers of αβ T cells and NK cells. It was also shown that γδ T cells do not develop in IL7 -/- mice; αβ T cells are normal in the absence of IL7. It is now known that disruption of the IL7Rα chain not only impairs thymocyte survival and expansion, but also results in defective T cell functional maturation (Baird *et al* 1999, Uribe and Weinberg 1998, Maraskovsky *et al* 1996,).

1.7.3.2 IL7Rα mutations

Only two human IL7Rα mutations, with the same immunophenotype, have thus far been identified and reported in the human mutation database.

Gene disruption of human IL7 gene or the IL7Rα–subunit leads to severe perturbation of T and B lymphopoiesis similar to that found in γc-deficient mice (Kumaki *et al* 1999, Baird *et al* 1999).

A potential IL7 deficiency should lead to the same phenotype as IL7Rα , but unlike IL7Rα deficiency, this defect would not be cured by classical bone marrow transplant as IL7 is produced by the stromal cells.

1.7.4 RAG1/RAG2

The RAG1 and RAG2 genes, which are located on chromosome 11p13 in humans, are closely linked and convergently transcribed. A single exon encodes the majority of the RAG1 DNA, and a separate single exon encodes the majority of the RAG2 DNA (Fig 6). The nucleotide sequences of RAG1 and RAG2 are highly conserved among species which carry out V(D)J recombination such as humans, rabbit, dog, etc. Nucleotide sequencing of human and mouse RAG1 cDNA revealed a 90% sequence similarity (URL 5).

RAG1 and RAG2 genes are expressed during lymphocyte developmental stages in which V(D)J rearrangement occurs. These genes are active in early lymphocyte development prior to the divergence of B cells and T cells lineages. RAG1 and RAG2 expression is temporarily suppressed following the first successful V(D)J rearrangement (URL 5).

1.7.4.1 RAG1/RAG2 mutations

A mutation in the RAG1 or RAG2 genes leading to the inability to make functional RAG1 or RAG2 is one of several defects, which can result in SCID in humans. Nucleotide substitutions or deletions in the genes for RAG1 or RAG2 have been linked to the B cell negative form of SCID (in which no functional B cells or T cells are produced).

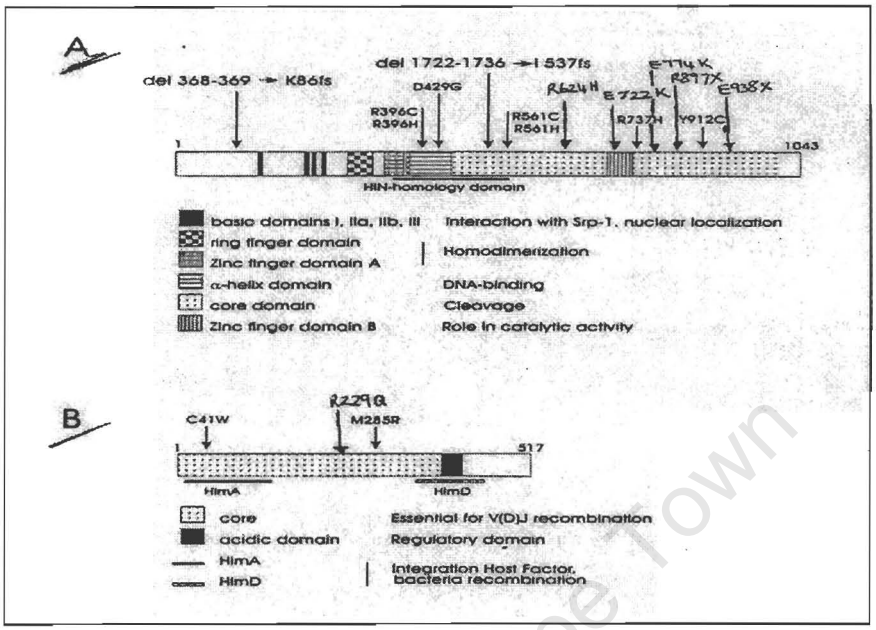


Fig 6 Schematic representations of the full-length human Rag1 and Rag2 proteins are shown in A and B, respectively. The structural and functional domains are also given as well as the gene mutations reported in the scientific literature (taken from Villa *et al* 1999).

Chapter 2: Methods

2.1 Introduction

This chapter details the results of investigations into the genetic basis of SCID in five unrelated patients who presented to the Red Cross Children's Hospital during the period of 1983-1999. All patients were severely immuno-compromised and all had T cell lymphocytopenia. In anticipation of DNA investigations blood samples were collected from these patients and where available, their immediate families. White blood cells were isolated as was genomic DNA, with both samples being stored at -70^{0}C.

Precise genetic diagnosis of XSCID is important for informative genetic counselling and accurate prenatal diagnosis. This can best be achieved by mutation analysis in the gamma chain gene of the affected proband. A search for mutations in the mother of the affected child may be useful but is limited by the negative results in cases of germline mosaicism and de novo mutations in the proband. If possible, maternal gene studies should be preceded by X-inactivation studies where non-random skewing in isolated T-cells will confirm carrier status in the mother. It is very important that blood be processed for DNA studies, before bone marrow transplantation. Peripheral blood shows express donor genotype following successful engraftment and is therefore unsuitable for genetic investigations (Ting *et al* 1999). In our study we carried out sequencing studies on DNA from the probands only. We also limited theses studies to exonic sequence as the alternate approach of cDNA sequencing through reverse transcription polymerase chain reaction (RT-PCR) was not possible as fresh cells were unavailable. The approach thus chosen for mutation detection in these patients was that of PCR amplification of gene exonic regions, followed by SSCP analysis and direct sequencing.

PCR entails the enzymatic amplification of specific DNA sequences using two oligonucleotide primers that flank the DNA segment to be amplified. It involves repeated cycles of denaturation of DNA, annealing of oligo's homologous to sequences flanking the segment of interest, and primer extension by a DNA polymerase, resulting in a theoretical doubling of the amount of the specific DNA fragments with each cycle

(Gyllenstein and Erlich 1988). All 8 exons of the common gamma chain were amplified by PCR using intronic primers, which were located as close to the start and end of each exon as possible. This was done in order to reduce the length of flanking intronic sequence co-amplified with each exon, and thus minimise the possibility of complicating intronic polymorphisms. The exact details of the PCR reactions and the sequences of the primers have been given in appendix 1. PCR products were run on an SSCP gel to detect any deviations from the wild type.

PCR-SSCP exploits the fact that the electrophoretic mobility of single stranded DNA (ssDNA) during nondenaturing polyacrylamide gel electrophoresis (PAGE) is dependent not only on size but also on the folded secondary structure of the DNA strand. Any base alteration can potentially affect the strand's conformation and therefore it's mobility and position in the gel (White *et al* 1992). The absence of a mobility shift does not necessarily exclude the presence of a mutation, some mutations do not cause sufficient conformational change to result in a detectable mobility shift on the gel. As a general rule electrophoresis at room temperature in TBE will detect most mutations, but some are better analysed at 4^0C in glycerol-free gels (Spagnolo *et al* 1994).

SSCP analysis of the exons of a gene involves the sequential running of gel compositions under varying conditions until mobility shifts are observed in the banding patterns of one or more exons from each patient. SSCP was selected as a screening technique of choice in the search for sequence alterations, in the amplified exonic DNA, as it is relatively simple and capable of detecting 80-90% of base alterations in short sections of DNA. Strand separation and electrophoresis under non-denaturing conditions yield two bands on staining as the complimentary strands have differing sequences and thus conformations. The latter feature results in differing mobility's through the gel for the two strands. Single base sequence alterations caused by base substitutions, deletions or insertions most often cause a conformational change in one of the two strands that result in a mobility shift when run on SSCP gels.

Exons showing mobility shifts on SSCP gels were sequenced on an ABI sequencer using a PCR approach and fluorescent labelled nucleotides. Here the primers used in each of the 4 chain extension reactions are each 5' linked to a different fluorescing dye. The 3' terminal base at each fragment is then identified according to its characteristic fluorescence spectrum. The fluorescence detectors used are computer controlled and hence the data acquisition is automated. All sequence alterations detected by this protocol were confirmed by repeat PCR, and restriction digestion.

2.2 Genomic DNA extraction protocols

2.2.1 Whole blood extraction method

Whole blood was collected in a Vacutainer tube containing 100 ul of 15% EDTA (1mg/ml). Blood (5ml) was transferred into a 15ml centrifuge tube containing 5ml of low salt buffer. To this 125ul of Nonidet P-40 (NP-40, Sigma) was added to lyse the cells. Tubes were mixed well by inversion several times to lyse the cells. Samples were then centrifuged for 10 minutes @ 2200RPM at room temp. (Beckman centrifuge). Supernatant was decanted and the nuclear pellet (the small pellet at the very bottom of the tube) was washed in 5ml of TKM1 buffer. Samples were centrifuged as before. The pellet was gently resuspend in 0.8ml of high salt buffer and 50ul of 10% SDS was also added. By pipetting back and forth several times, the whole suspension was mixed thoroughly, incubation was for 10 minutes @ 55^{0}C. To the tubes 0.30ml of 6M NaCl was added and mixed well. Samples were centrifuge for 5min @ 12000RPM, in a microcentrifuge. The supernatant containing the DNA was saved and the precipitated protein pellet at the bottom of the tube was discarded. To the supernatant 2 volumes of 100% ethanol was added at RT. The tubes were inverted several times to precipitate the DNA in the solution. Precipitated DNA strands were put in a microfuge tube containing 1ml of ice-cold 70% ethanol and microfuged for 5min @ 12000RPM at 4^{0}C. Pellet was dried in a Speed-vac and the DNA was resuspend in 0.5ml of TE buffer and incubated @ 65^{0}C for 15min to allow for complete dissolution (Lahiri and Nurnberger, 1991).

2.2.2 Frozen blood extraction method

Frozen blood (5ml) was thawed @ 37^{0}C and mixed with 50 ml of cell lysis buffer (blood: lysis buffer ratio of 1:5) in a Beckman tube and incubated for 15 min. on ice. Samples were centrifuge for 10 min. @ 6800g and 4^{0}C and the supernatant was decanted. The pellet was resuspended in 50 ml of wash buffer and centrifuge for 10 min. @ 6300g and 4^{0}C, and the supernatant was decanted. The remaining pellet (which should be almost white) was mixed with the following reagents in this order: 350 *u*l of 20% sodium sarkosyl, 250 *u*l of 7.5 M-ammonium acetate, 3.5ml of 6 M-guanidine hydrochloride and 125 *u*l of proteinase K (10mg/ml). Beckman tubes were incubated for 15 minutes @ 60^{0}C or until the solution was clear and immediately cooled to 0^{0}C. DNA was

precipitated by the addition of 10 ml ice-cold ethanol. Gelatinous DNA was spooled onto a hooked glass rod and released into 500 *u*l of TE buffer and allowed to dissolve completely in the tube by incubating it for 1 hour @ 37^0C or overnight @ room temperature (Parzer and Mannhalter, 1991).

Generally, under these conditions the DNA is not completely soluble. However the insoluble material can easily be separated and removed by centrifugation (10-15s) in a Microfuge B.

The concentration and purity of the DNA was determined by measuring the OD_{260}/OD_{280} ratio and by viewing the extracted DNA on agarose gel electrophoresis.

2.2.3 Bloodspot extraction method

Whole blood (EDTA or heparinized) was pipetted onto sterile filter paper, allowed to dry overnight, sealed in a plastic sleeve and stored @ -20^0C for future studies.

A 3mm x 4mm square of the bloodspot was cut out with a sterile blade and dropped into a 1.5ml eppendorf. Methanol was added to the tube to cover the square and was left to stand for 5 minutes and then allowed to dry completely. Boiling each piece in 50 *u*l sterile water for 15 minutes liberated the DNA. The mixture was then centrifuged for 10 minutes @ 10 000rpm's and kept on ice. On average 10-20 *u*l of the extract was used for PCR reactions with a final volume of 50 *u*l.

2.3 Determination of DNA concentration

2.3.1 By Spectrophotometric readings A_{260}

A 1: 100 dilution of DNA was prepared in distilled water (10 *u*l in 990 *u*l) and the absorbance readings taken at OD_{260} and OD_{280} on a spectrophotometer. The A_{260}/A_{280} ratio gives an indication of the purity of the DNA preparation. A pure sample would have a ratio of 1.8 - 2.0.

The calculation is as follows:

For double stranded DNA 1 OD = 50 *ug*/ml

For single stranded DNA 1 OD = 33 *ug*/ml

DNA concentration = OD_{260} x 50 *ug*/ml (or 33 *ug*/ml) x dilution factor

2.3.2 Agarose gel electrophoresis

A 1 µl DNA sample was mixed with 3ul of 6 x loading buffer. Sample was made up to 10 *ul* with distilled water. The 0.8% agarose gel was electrophoresed in 1 x TAE buffer @ a constant voltage of 80V for 30 minutes. A high molecular weight biomarker of known concentration was run on the gel for comparison to the genomic DNA.

Ethidium bromide is a florescent dye that intercalates between the DNA strands, allowing visualization of the product under a UV transilluminator. Smearing would indicate degradation of the DNA.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Primer design

All primers were designed using the OLIGO™ computer program. Here sequence length, GC content, melting temperatures and oligo self-complimentarity was easily compared.

2.4.2 PCR amplification

The following quantities were used per reaction mix in a 50 µl sample tube: 4µl 2.5mM dNTP's, 4.5 µl 10 x Promega buffer, 2.0 µl 25mM $MgCl_2$, 1.0 µl 20pM forward primer, 1.0 µl 20pM reverse primer, 31.5 µl distilled water and 1.0 µl 0.5µg/µl DNA. Control samples with no DNA were added as well. PCR mixtures were overlaid with mineral oil (45µl) to prevent evaporation and condensation of the samples. Tubes were placed in a thermocycler and subjected to a "hot start" by the addition of the 5µl enzyme mix after the first 95^0C for 3 minutes step in the PCR cycle. An enzyme mix contained the

following quantities per reaction: 0.5 µl Taq DNA polymerase, 0.5 µl 10 x Promega buffer, 4.0 µl distilled water.

2.4.3 Thermocycling

An appropriate annealing temperature profile was chosen for each set of primers and the individual annealing temperatures were programmed into the thermocycler (Appendix). The following PCR program was used for each primer set: initial step 1:- 95^{0}C for 3 minutes, initial hotstart step 2:-75^{0}C for 1 minute (*Taq* polymerse was added); cycling step 1:-95^{0}C for 30 seconds, cycling step 2:-(__^{0}C)* for 1 minute, cycling step 3:-72^{0}C for 1 minute (10 cycles for cycling steps 1-3), cycling step 4:- 92^{0}C for 30 seconds, cycling step 5:- (__^{0}C)* for 1 minute, cycling step 6:-72^{0}C for 1 minute (23 cycles for cycling steps 4-6); and final step 7:-72^{0}C for 5 minutes.

* annealing temperature specific for different primer pairs (see appendix 1)

2.4.4 Agarose gel electrophoresis

An aliquot (5µl) PCR product was mixed with 2µl of 6 x loading dye and electrophoresed on a 2.0% agarose gel in 1 x TAE buffer @ a constant voltage of 100V for 45 minutes. A high molecular weight marker was prepared and run on the gel for size determination of the PCR product.

2.5 Single stranded conformation polymorphism (SSCP)

2.5.1 Preparation of plates

The surfaces of both plates were thoroughly washed with detergent and 70% ethanol and allowed to dry completely. Siliconizing agent was applied (in a fume cupboard) to one plate and vigorously rubbed onto the surface. The plate was allowed to stand in the fume cupboard for a few minutes. A plate glue mix, containing 90µl 10% acetic acid and 3ml of the plate glue stock, was thoroughly rubbed onto the surface of the other plate which was allowed to stand for 3 minutes. Excess plate glue was rubbed off with 100% ethanol. The ethanol wipe was done twice to ensure the removal of the plate glue, to prevent the plates sticking together after electrophoresis, due to the diffusion of the glue through the gel. The plates were assembled and

secured with the aid of bulldog clips and with spacers (0.4mm) separating the two plates.

2.5.2 Preparation of PAGE

2.5.2.1 Gel type 1

Following volumes were added to make gel type1: 18.75ml 40% Acrylamide stock (39.6:0.4), 9.0ml 0.6 x TBE, 5.625.0g pure Urea, 400µl 10% APS, 40.0ml distilled water and 25µl TEMED. TEMED was added last as this caused polymerisation of the gel mix. The solution was gently mixed, taken up in a 50 ml syringe and poured in between the two plates which were lying on a flat surface. A well-forming comb was inserted at the top of the two plates and bulldog clamps were used to hold the two plates firmly together until polymerisation was completed. This process occurred in about 90 minutes. The gels were then ready for use.

2.5.2.2 Gel type 2

Following volumes were added to make gel type 2: 27.0ml 10% acrylamide (30:0.8), 8.0ml 5 x TBE, 4.0ml glycerol, 12.0g 10%Urea, 35.0ml distilled water, 800µl 10%APS and 80µl TEMED. TEMED was added last as this caused polymerisation of the gel mix. The solution was gently mixed, taken up in a 50 ml syringe and poured in between the two plates that were lying on a flat surface. A well-forming comb was inserted at the top of the two plates and bulldog clamps were used to hold the two plates firmly together until polymerisation was completed. This process occurred in about 90 minutes. The gels were then ready for use.

2.5.3 Sample preparation and electrophoresis

An aliquot (5 µl) of the PCR product was mixed with SSCP loading dye (5 µl). Tubes were incubated @ 95^0C for 3 minutes to denature the DNA and immediately put on ice. Combs were removed from the gel to form the wells. The gel was placed into the electrophoresis tank and filled with TBE buffer. The wells were cleaned of urea with the aid of a syringe. Two different gel conditions were followed. Gel type 1 was run at room temperature @ 6W overnight. Gel type 2 was run in the cold room, which

was at 4^0C, overnight. Gel type 2 had an initial run of 75W for 5 minutes and then the wattage was decreased to 9W for overnight.

2.5.4 Silver Staining

2.5.4.1 Method 1

The silver stain relies on the change in pH and the precipitation reaction of the silver ions onto the DNA in the gels. Ammonia was used for gel impregnation and a dilute acid solution of formalin was used for image development, which was brought about by the change in pH from basic to acidic which caused the silver ions to deposit onto the DNA thereby forming insoluble silver salts.

The two plates were disassembled and the plate containing the gel was put in a gel tray for silver staining. This plate was covered with 2 litres of distilled water and gently agitated for 30 minutes following which, water was discarded and the ammoniacal silver solution was added. The tray was covered to limit evaporation and slowly agitated for 20 minutes. The silver solution was poured off. Distilled water was added to the tray to cover the gel and left to shake for 30 seconds. Water was discarded and the developing solution (Citric acid, Formalin) added to the tray. The reaction was stopped when faint bands became visible on the gel. The developing solution was discarded and water was added to the tray. When the bands were clearly visible a photo of the gel was taken and the plates soaked in 0.5M NaOH to remove the gel from the plate following which the plates were given a quick rinse and stored for the next gel.

The high sensitivity of the silver stain technique renders it susceptible to staining artefacts and impurities. The use of deionised water is critical for the success of the procedure and the conductivity of the distilled water should be measured before use.

2.5.4.2 Method 2

Following electrophoresis and disassembly the plate containing the gel was put in a gel tray for silver staining. The gel was then covered with 2 litres of distilled water and gently agitated for 1 minute. The water was then discarded and the silver nitrate solution was added. The tray was slowly agitated for 10-15 minutes. The silver

nitrate solution was decanted and distilled water was poured into the tray and left to shake for 2 minutes. The water was then discarded and the formaldehyde solution was

then added to the tray. The solution was left in the tray for 10-15 minutes or until the bands were clearly visible. This solution was discarded and water was added to the tray and left to agitate for 1 minute. Thereafter the water was discarded and the sodium carbonate solution was added to the tray to sharpen the bands. The sodium carbonate solution was discarded and the gel was then reimmersed in water. The gel was then viewed under a light box. A photograph of the gel was taken and the plates were soaked in 0.5M NaOH to remove the gel from the plate. The plates were given a quick rinse and then stored for the next gel.

The exons showing a band shift were then amplified through PCR, purified and submitted for sequence analysis.

2.6 DNA sequencing

2.6.1 Purification of PCR product

2.6.1.1 Ammonium acetate precipitation

To the PCR product 1/2 volume of cold 7.5M ammonium acetate was added and the tubes mixed. Cold absolute ethanol (2.5 volume) was added and mixed. The tubes were then placed @ -70^{O}C for 30 minutes to precipitate the amplified DNA. This was recovered by centrifugation for 20-30 minutes in microfuge @ 140000rpm's. The supernatant was discarded and the pellet washed with 70% cold ethanol and centrifuged for 10 minutes @ 140000rpm's. The supernatant was discarded and the pellet was dried for 5 minutes in a speedvac. The amplified DNA pellet was then dissolved in 50 µl TE or distilled water.

2.6.1.2 Gel electrophoresis

The 50µl of amplified DNA was then mixed with 6ul 40%sucrose and pipetted into the wells of the 1% low melting point agarose gel and electrophoresed in 1 x TAE buffer at a constant voltage of 60V for 45 minutes. An appropriate molecular weight marker was run to be able to track the migration of the bands in the gel. The

separated bands were viewed under the UV transilluminator and the appropriate bands were cut out with a sterile blade, cutting as little of the agarose around the band as possible. The excised bands were then weighed and dropped into a 1.5ml eppendorf.

2.6.1.3 Qiagen II gel extraction kit

The bands were then purified with the aid of the Qiagen II Gel Extraction Kit. A volume of 0.3ml QX1 solubilization buffer was added per 100mg of gel. After solubilization 10 µl of Qiaex beads was added to the eppendorf and the suspension was then incubated at 50^0C for 10 minutes, with vortexing at 2minute intervals. The samples were then centrifuged in a microfuge for 30 seconds. Supernatant was carefully removed and the pellet in 0.5ml QX1 buffer to get rid of residual agarose. The pellet was then resuspended in 0.5ml equilibration buffer PE and washed twice to the remove the salt contaminants after which the pellet was left to air dry for 15 minutes or until it became white. DNA was eluted from the beads by the addition of 20 µl TE buffer, vortexing, incubation at room temperature for 5 minutes, which was then followed by centrifugation for 30 seconds. The supernatant, now containing the amplified DNA, was carefully removed. This elution process was repeated twice. The purity and concentration of the PCR product was determined through spectrophotometer readings and 0.8% agarose gel electrophoresis.

2.7 Automated Sequencing

For the sequencing of amplified DNA template a concentration of 180-250ng was required per reaction. The template (250ng) and the primer (20pM/ul per reaction) were supplied in a single tube, which was made up to a total volume of 12 µl with distilled water for each primer. The instrument used was an ABI prism 377 DNA sequencer from Perkin Elmer.

2.8 Mutation validation by restriction digestion

2.8.1 Primers design

Often a mutation can be verified with the aid of restriction enzymes as the sequence alteration in the DNA destroys or creates a cutting site for one of these enzymes. A

mutation not altering a restriction cutting site can often be detected through the introduction of a cutting site using designer PCR primers.

In 2 of the 3 families studied, primers were designed which allowed the introduction of a restriction site in the PCR product from the mutant allele only.

2.8.2 PCR amplification

The PCR amplification protocol was followed as in 2.3.1, 2.3.2 and 2.3.3

2.8.3 PCR digestion

Bst X1 (10U/µl)-10 x Buffer H incubated at 45^0C

Bsi Y1 (10U/µl)- 10 x Buffer M incubated at 55^0C

Taq1(10U/µl) - 10 x Buffer B incubated at 65^0C

The restriction digest contained the following quantities per 20 µl sample: 5 µl PCR product, 2 µl 10 x Buffer, 1 µl Enzyme (10U/µl), 1 µl Casein and 11 µl distilled water. The undigested samples were used as controls and contained no enzyme but instead contained equal volumes of distilled water as replacement. The PCR products were incubated with the correct enzyme and buffer combination at the respective temperatures. Digestion was allowed to occur overnight and the products were run on a 20% PAGE gel.

2.8.4 Electrophoresis and visualisation

The following gel mix for a 20% PAGE gel was added to an Erlenmeyer flask: 33.3ml 30% acrylamide (29:1), 10.0ml 5 x TBE, 6.3ml distilled water, 400 µl 10% APS and 25 µl TEMED.

Two small plates (15cm x 15cm) were a cleaned and assembled. Two spacers of sizes 0.75mm were used to separate the plates. A 0.75mm comb was used to form the wells. After polymerisation the comb was removed and the gel was placed in the tank in 1 x TBE buffer.

10µl of digested product was mixed with 2µl of 6 x loading dye and loaded into the wells. The gel was electrophoresed at 220V for 3 hours. The products were visualised with the aid of a silver stain or an ethidium bromide stain (9µl EtBr mixed with 125ml 5 x TBE).

3.1 Kindred 1

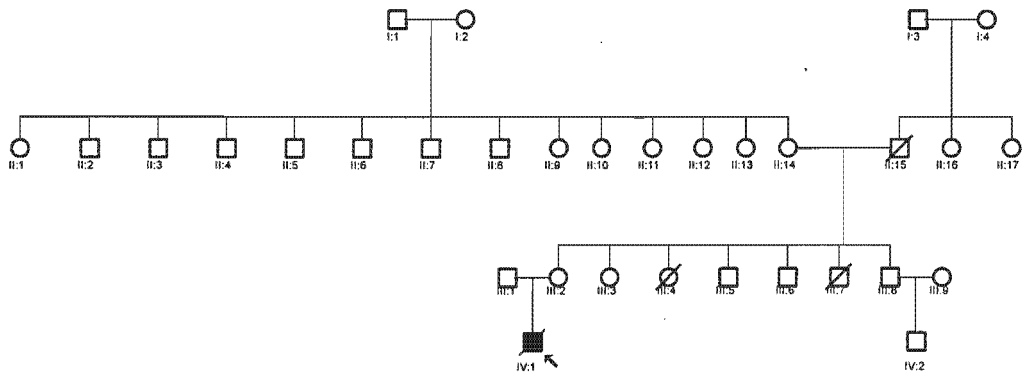


Fig 7. The pedigree of kindred 1 showing the affected boy

3.1.1 Immunophenotype $T^-B^+NK^-$

Candidate genes GAMMA CHAIN (γc) gene, Janus kinase (JAK3)

The proband in this family presented during 1994 at 4.5 months of age with diarrhoeal disease, *Streptococcus feacalis* septicaemia, chronic oral candidiasis and failure to thrive. The patient died one month after diagnosis before transplantation with T-cell depleted haploidentical bone marrow could be carried out. The family pedigree recorded in the patient's folder is given in Fig 7. No information was available to ascertain whether additional relatives in or before generation 1 might have been affected with SCID. Patient 1 (P1) had been investigated clinically and had been found to fulfill the diagnostic criteria set out by the World Health Organization (WHO) committee on immunodeficiency (WHO Report 1999). The fact that there were no functional, circulating T ells and NK cells but normal to increased numbers of circulating B cells ($T^-B^+NK^-$) in the peripheral blood of the proband was suggestive of a genetic defect in the γ chain of the IL2 receptor, further supported by the male gender of the proband as this is an X-linked condition and the most common SCID disorder encountered. JAK3 was also considered as a candidate gene as the JAK3 deficiency phenotype is indistinguishable from that of XSCID, with the only difference being that JAK3 deficiency has an AR mode of inheritance.

In about one third of the cases of typical XSCID, there is no previous family history, implying a recent mutation, so the lack of previous history of XSCID in this family is not unusual. In these families, the suspicion of XSCID is raised by the male gender of the patient and the immunophenotype. The detection of carrier status in the mother via skewing of the T cells or band shifts on SSCP analysis of exons from the γc gene are strong indicators of XSCID in the patients. It should be remembered that environmental factors, including age of diagnosis, infections, and the transplacental transfer of maternal lymphocytes into SCID patients, may be important contributors to lymphocytic phenotypes in these patients.

3.1.2 PCR

All eight exons of the IL2RG chain were successfully amplified using optimised PCR conditions.

3.1.3 SSCP analysis

Each of the amplified exons from the IL2Rγc gene was run on a SSCP gel. Direct sequencing of the PCR product identified the underlying mutation.

Normal migration patterns were seen in all IL2Rγc exons of P1, except for exon 2 (Fig 8: lane 6-SS2), where the lower band showed an accelerated migration rate compared to that of the control DNA (Fig 8: lanes 1-5 and lanes 7-11; SS1 and SS2). This altered SSCP profile was presumed to be due to a mutation in exon 2, which resulted in a conformational change in the coding strand of the proband. The underlying mutation was identified by direct sequencing of the PCR product of the abnormal exon by automated sequencing.

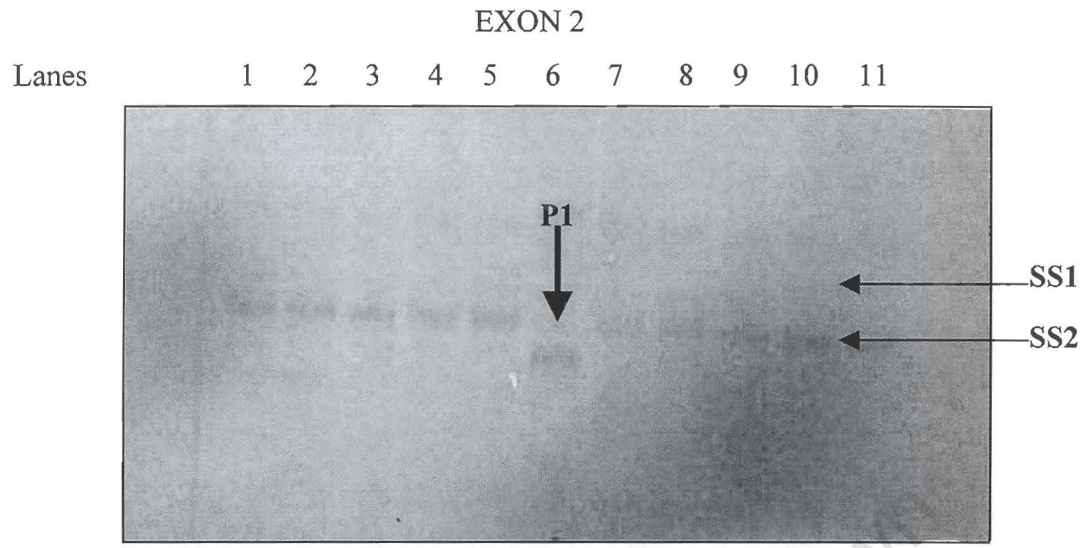


Fig8. SSCP banding pattern of exon 2 of the IL2Rγc. The separated sense and antisense strands from normal controls are shown as SS1 and SS2. Lanes 1-5 and 7-11- normal controls; lane 6- P1. Normal control male or female (N). Arrows indicate mobility shifted bands.

3.1.4 Sequencing results

Sequencing revealed a single base transition of G → A at base position 216 of the cDNA sequence. This base change replaced the normal glutamic acid-encoding codon (GAG) with a lysine-encoding codon (AAG) (Fig 9A and B). Glu216 is located in the extracellular domain of the γc molecule, outside the consensus motifs belonging to the class 1 cytokine receptor family and is conserved in the mouse. Substitution of glutamic acid by lysine would be expected to impinge on the function or stability of the synthesised γc as it entails a charge inversion, where a negatively charged residue has been substituted with a positively charged one (Fig 10). This mutation is not novel and has been previously reported. There are currently 220 mutations in unrelated patients in the common gamma chain database; 147 of these mutations are unique, having only been reported once. At present there are 17 mutations in exon 2, one of these being the Glu216Lys; the rest are caused by nonsense mutations (3), deletions (4), splice site mutations (2), one insertion and missense mutations (6).

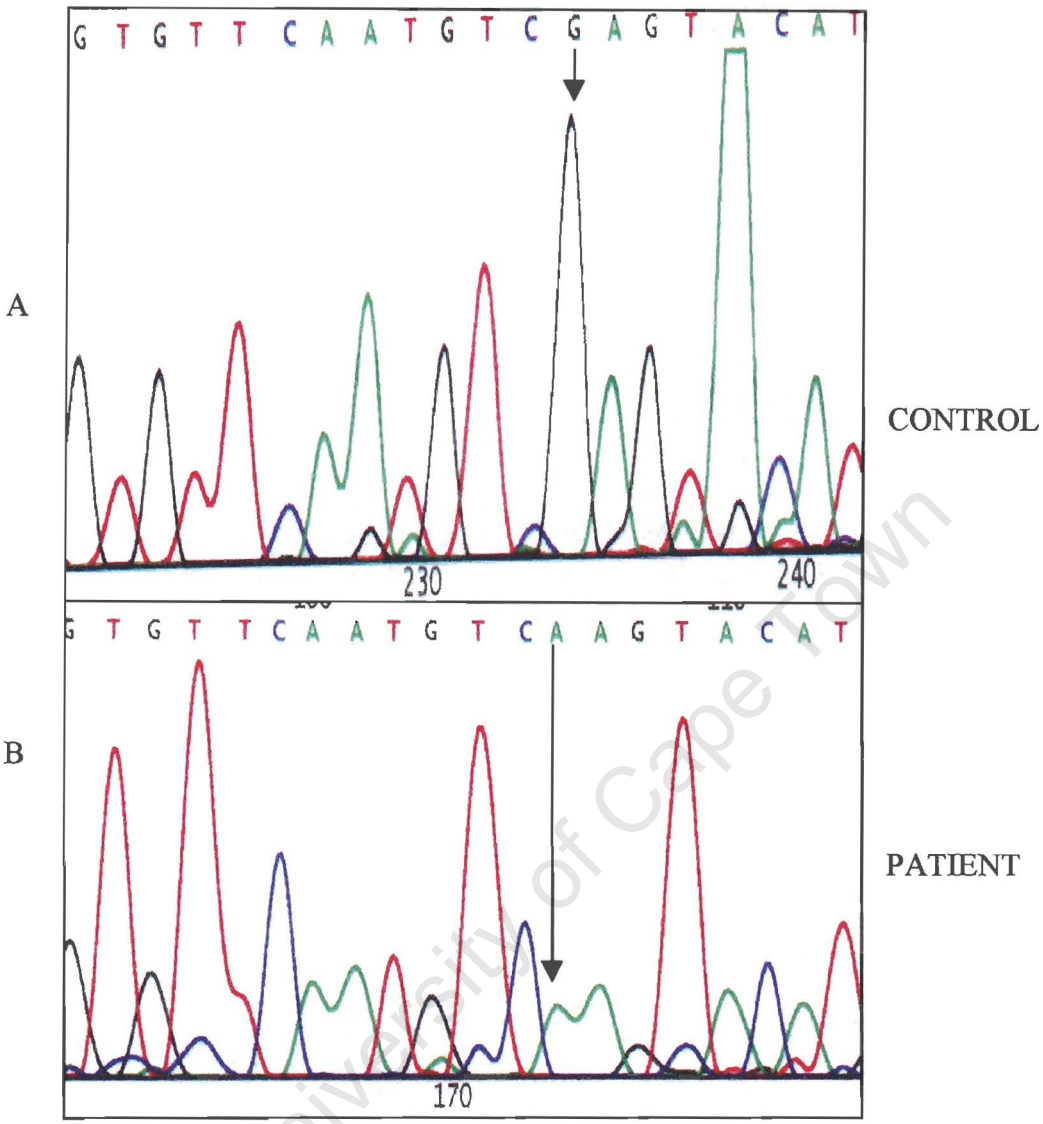


Fig 9 Sequence traces of the sequence strand of exon 2 from the affected male of kindred 1 and a control subject, showing the G to A transition at nucleotide position 216 of the cDNA sequence (GenBank accession number L12176).

	K ↑
abbreviation:	N V E Y M
amino acid sequence:	Asn Val Glu Tyr Met
wild type sequence:	AAT GTC **G**AG TAC ATG
mutated sequence:	AAT GTC **A**AG TAC ATG
codon:	66 67 **68** 69 70
transition:	$G_{216} \rightarrow A_{216}$
change in charge:	(-) ve → (+) ve; glutamic acid → lysine
mutation nomenclature:	E68K

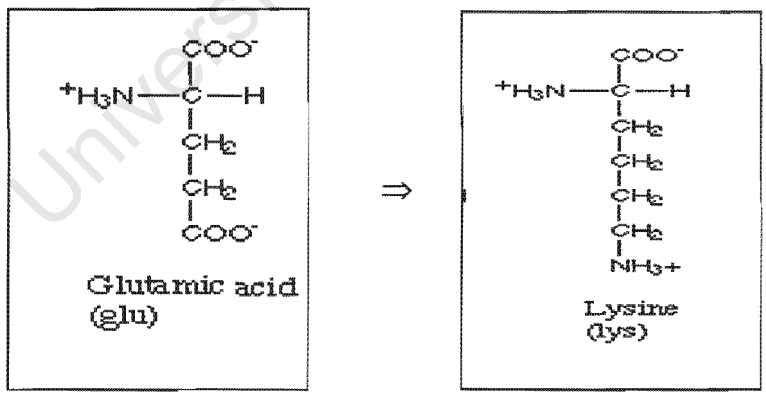


Fig 11 A summary of the nature and characteristics of the IL2Rγc gene defect detected in the proband of kindred 1. The wild type sequence depicted is taken from the cDNA sequence as recorded in GenBank under accession number L12176.

3.1.5 Mutation verification

The E68K mutation (patient 1)

The G→A transition could be confirmed by PCR and restriction analysis as the base change destroyed a cutting site for the endonuclease *Taq* 1. This proved to be the only *Taq* 1 site in exon 2 (Fig 11). Amplified DNA from the patient would thus show one band only and of the original size after digestion with *Taq* 1. To demonstrate suitability of the PCR product for restriction analysis, co-digestion with *Hin* f1 was introduced into the protocol.

*Taq*1 recognition sequence T↓CGA

*Hin*f1 recognition sequence G↓ANTC

		Taq1							
Wild Type sequence	5'-	TTT	GTG	TTC	AAT	GTC	GAG	TAC	ATG -3'
Amino acid sequence		F	V	F	N	V	E	Y	M
Mutated sequence of Patient 1	5'-	TTT	GTG	TTC	AAT	GTC	AAG	TAC	ATG -3'
cDNA position						216			

		Hin f1							
Wild Type sequence	5'-	ACC	ACT	ATG	CCC	ACT	GAC	TCC	CTC -3'
Amino acid sequence		T	T	M	P	T	D	S	L

Fig 11 Sections of the sequence of exon 2 containing the *Taq*1 and *Hin* f1 cutting sites. The cutting sites for these enzymes are underlined while the G→ A transition affecting the *Taq* 1 site is given in bold type.

Theoretically the double digestion of the PCR product of control exon 2 generates 3 fragments of 125bp, 69bp, and 59bp (Fig 12). The patient and carriers, in this family, could thus be identified by the loss of the *Taq* 1 cutting site, which generated fragments of 184 and 69bp (Fig 12).

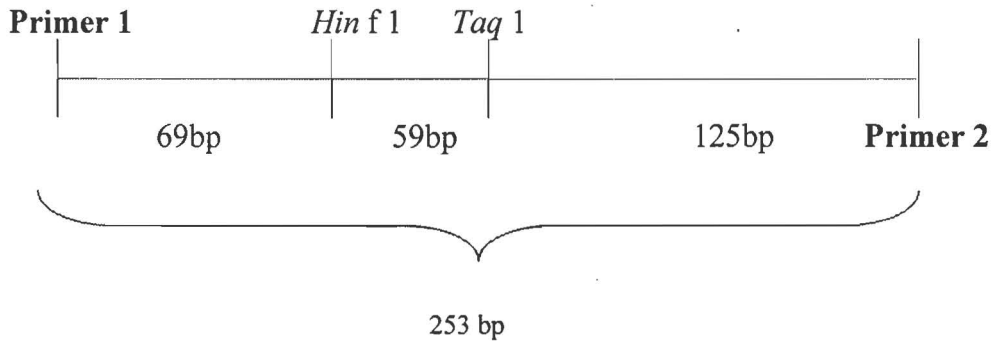


Fig 12 Schematic diagram of the PCR product of exon 2 digested with *Taq*1 and *Hin*f1 and the sizes of the fragments generated.

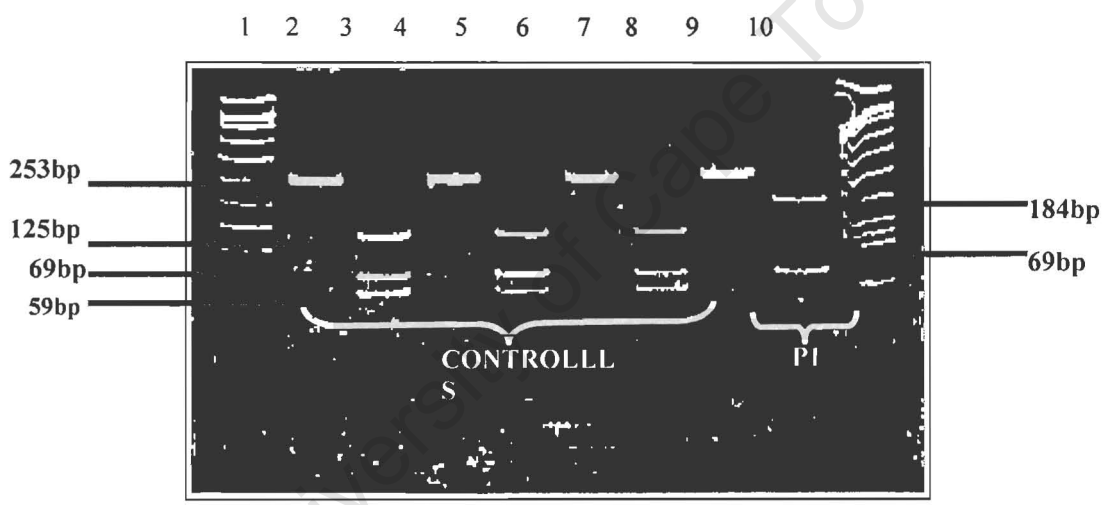


Fig 13 PAGE of exon 2 of the IL2R γc gene of patient 1, co-digested with *Taq* 1 and *Hin* f1. Lanes 2,4 and 6 - undigested control DNA. Lanes 3,5 and 7- digested control DNA. Lane 8-undigested DNA of P1 and lane 9- digested DNA of P1. Lanes 1 and 10-molecular weight marker VIII from Roche Biochemicals (appendix 3).

The PCR product was amplified, digested with *Taq* 1 and *Hin* f1 and run on a 20% polyacrylamide gel (Fig 13). The undigested DNA of the control samples and P1 was 253bp (Fig 13: lanes2, 4, 6 and 8); three fragments (125bp, 69bp and 59bp) can

clearly be seen in the control DNA (Fig 13 lanes 3, 5 and 7) while two (184bp and 69bp) are given by the mutant DNA (Fig 13: lane9); this is explained by the fact that only one of the enzymes (*Hin* f1) had cut the PCR product even though the product had been double digested with both enzymes. This gel further verifies the loss of the *Taq* 1 cut site in exon 2 of the IL2Rγc of P1. At the time of this study there were no female siblings of the proband. However, should the situation change, carrier detection (through restriction digestion) and genetic counselling could be offered to this family. Carrier detection for the patient's mother could not be effected, as DNA from her was not available. Restriction digestion would now be a much quicker method for carrier screening in this family than SSCP or X-inactivation.

3.2 Kindred 2

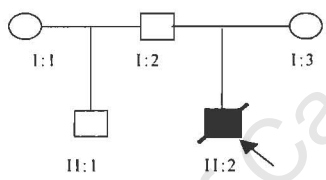


Fig 14 The family tree of kindred 2 showing the affected male and his carrier mother.

3.2.1 **Immunophenotype:** $T^-B^+NK^-$

Candidate genes: GAMMA CHAIN (γc) gene, JAK3

The proband in kindred 2 presented to Red Cross Children's Hospital during 1998 at 3.5 months of age with failure to thrive, combined with *Escherichia coli* and *Klebsiella pneumoniae* septicaemia and persistent thrush. He died one month later due to recurrent *Escherichia coli* septicaemia before treatment with T-cell depleted haploidentical bone marrow could be carried out. Only the immediate relatives are shown in the family tree in Fig 14. Despite the absence of a relevant family history for the proband, the $T^-B^+NK^-$ immunophenotype again suggested X-linked SCID. With the proband being male, it was felt that XSCID was a far stronger possibility than JAK3 deficiency.

3.2.2 PCR

All eight exons of the IL2Rγc for patient 2 were successfully amplified.

3.2.3 SSCP analysis

Two different SSCP gel running conditions were used. Exons 1, 2, 4, 5, 6, 7 and 8 were separated by running the SSCP gel at 4^0C; as exon 3 did not separate very well at 4^0C it required running the gels at room temperature to separate the two bands. The size of the PCR product of exon 8 necessitated the running of the SSCP gel for an extra 2 hours to allow for separation and was thus not included in the gel shown in Fig 15. Also no band alteration was evident. A normal SSCP migration pattern was seen for all exons of the γ chain, except for exon 5 (Fig 15: exon5, lane2-SS1). Expecting a base change in this exon, it was PCR amplified and sequenced to determine the nature of the mutation.

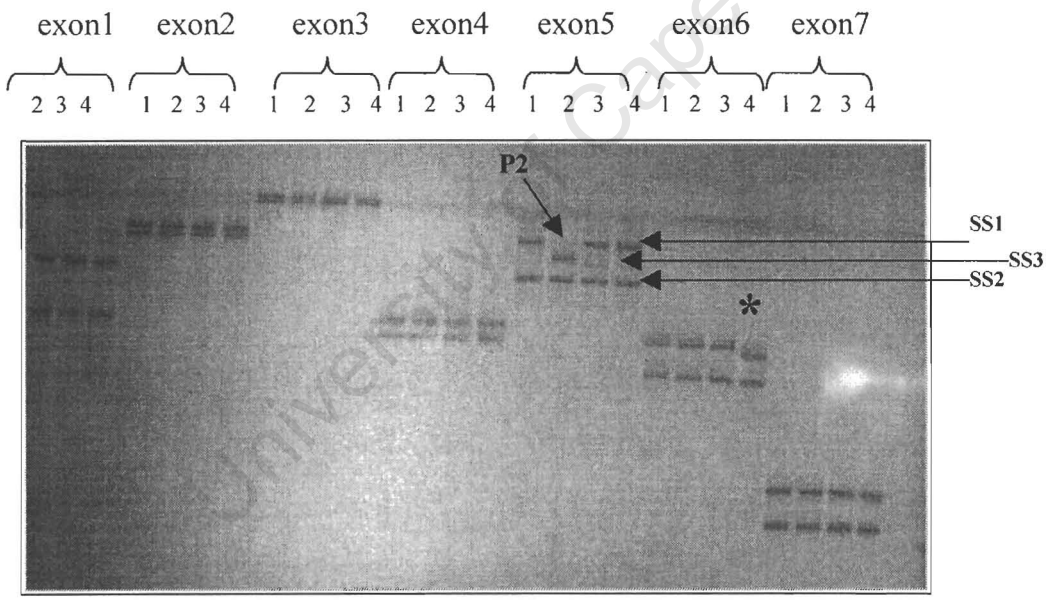


Fig 15 SSCP screening of exons 1-7 of the common gamma chain gene. DNA bands were visualised by silver staining. The lanes for each exon are labelled as 1, 2, 3 and 4. Single strands are indicated as SS1, SS2. The mobility shifted band in exon 5 of P2 is indicated by the black arrow (SS3). Lane 1,3 – control DNA, lane 2 –patient 2 DNA, lane 4- patient 3 DNA.

The band shift in exon 6 indicated with an asterisk (*) in Fig 15 will be dealt with later in the text. The extra band apparent in the SSCP gel photo (Fig 15: exon 5- lane 3, SS3) is non specific and is most likely explained by a single strand assuming more than one conformation, thereby yielding two or more bands. As the ratio of the intensity between the strands remains constant from sample to sample, these different "conformers" are usually readily identified.

3.2.4 Sequencing results

A C→T transition (Fig 17A and B) at cDNA nucleotide position 684 was identified in P2, which altered the CGG codon for arginine at position 224 to that of TGG which codes for tryptophan. This missense mutation resulted in a non-conserved amino acid substitution in that a basic residue has been substituted for a bulky hydrophobic one (Fig 18). This substitution would be expected to impact on the conformational stability of the IL2Rγc. Residue 224 is located in the extracellular domain of the γc. The region of cDNA 666-691 which spans the C→T transition contains 5 cytosine-guanine (CpG) dinucleotides (Fig 16). The last of these CpG dinucleotides is the site of 21 independent missense mutations, which make it the most prominent hot spot for mutations in the γc gene (Puck *et al* 1997). This CpG-rich region is of major import as it lies just 5' to the highly conserved WSXWS motif in exon 5. The R224W mutation is found just six nucleotides 5' to this hot spot. The R224 position is absolutely conserved in the human receptors for erythropoietin, IL12 receptor, IL2Rβ and the prolactin receptor. Currently there are 56 mutations reported in exon 5 in the SCID database, of these the R224W mutation was found to have occurred 7 times in unrelated patients and can be regarded as another CpG hot spot.

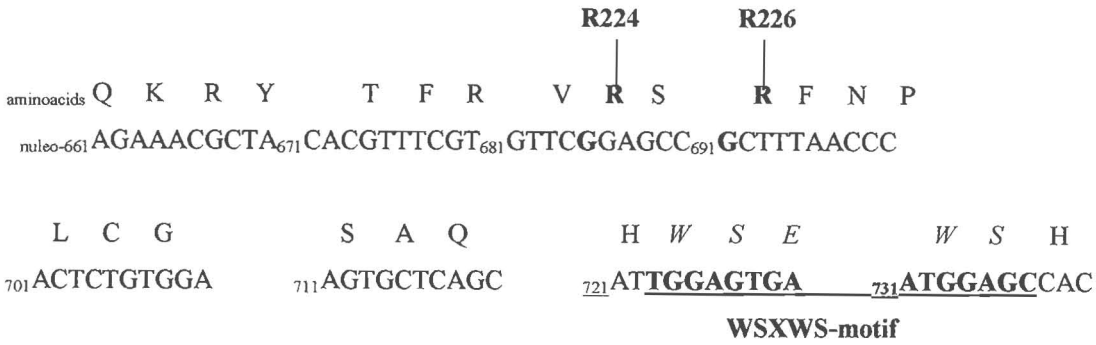


FIG 16 Schematic presentation of the transmembrane region of the IL2Rγc containing the WSEWS motif as well as the 2 CpG hotspots in exon 5.

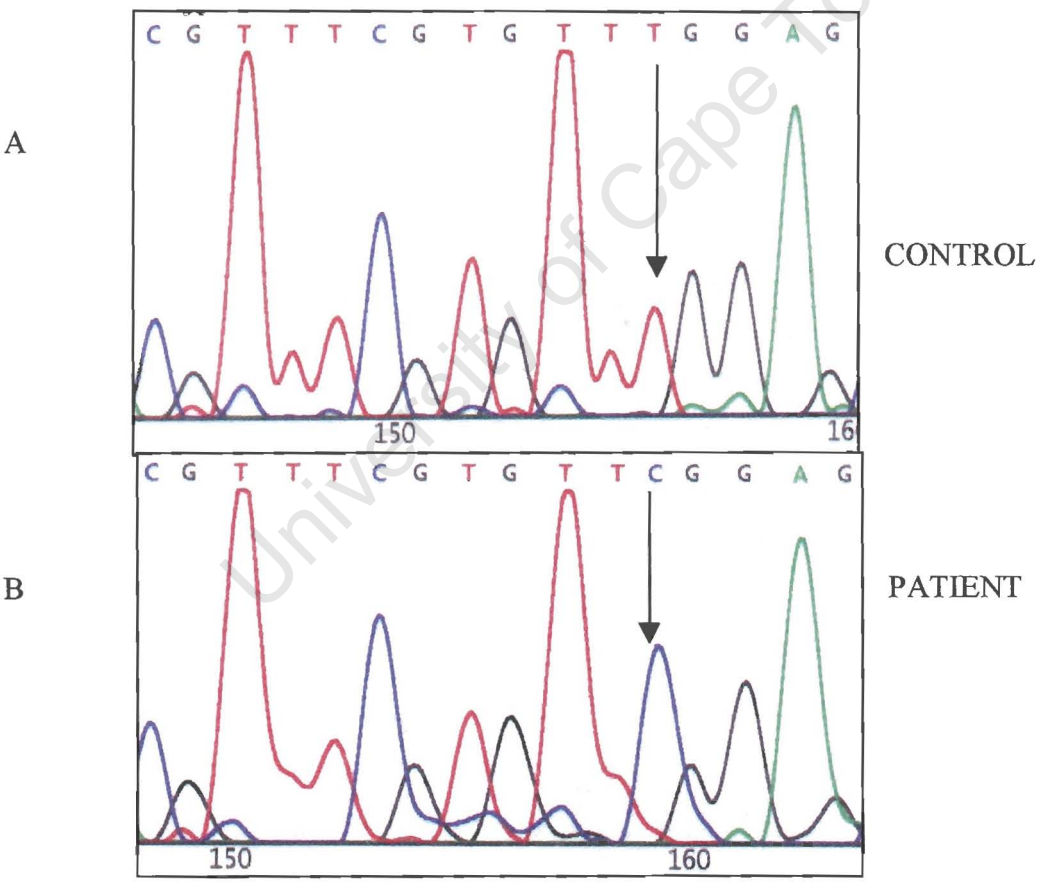


Fig 17 Sections of the sequencing traces from exon 5 showing the C to T transition at nucleotide position 684 of the IL2Rγc cDNA of patient 2.

	W ↑
amino acid sequence:	R V **R** S R
amino acid sequence:	Arg Val Arg Ser Arg
wild type sequence:	CGT GTT **C**GG AGC CGC
mutated sequence:	CGT GTT **T**GG AGC CGC
codon:	222 223 **224** 225 226
transition:	$C_{684} \rightarrow T_{684}$
change in charge:	basic → hydrophobic (loss of positive charge)
mutation nomenclature:	R224W

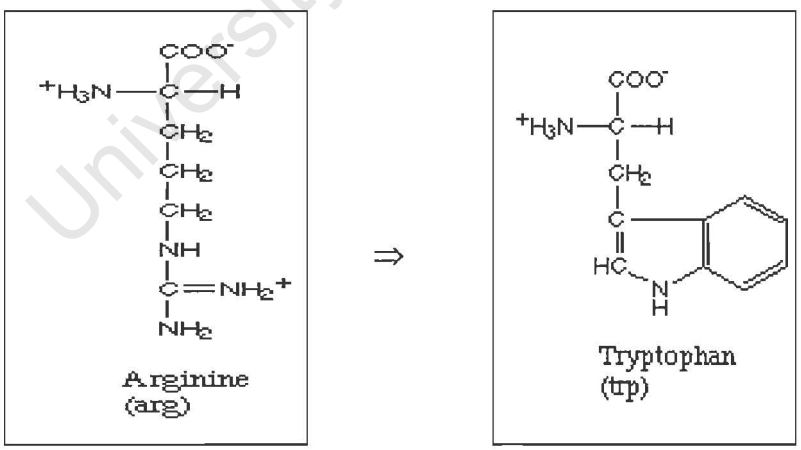


Fig18 A summary of the nature and characteristics of the IL2Rγc gene defect detected in the proband of kindred 2. The wild-type sequence depicted is taken from the cDNA sequence as recorded in GenBank under accession number L12180

3.2.5 Mutation verification

R224W mutation (patient 2)

The C→T transition underlying the R224W mutation could not be confirmed by restriction analysis as it neither created nor abolished a restriction enzyme cutting site. A PCR primer was therefore designed to artificially generate a new *Bst* X1 cutting site at the site of the mutation. As a naturally occurring *Bst* X1 cut site is found in exon 5, the introduction of an additional *Bst* X1 site in the PCR product from the mutant allele, generates 3 fragments of sizes 22bp, 64bp and 62bp (Fig 19). In contrast the PCR product from the normal allele lacks the additional *Bst* X1 site and gives two fragments only (86bp and 62bp) (Fig 20). The assay was used to confirm carrier status in the mother of the proband and is available for carrier testing should other female relatives request genetic counselling.

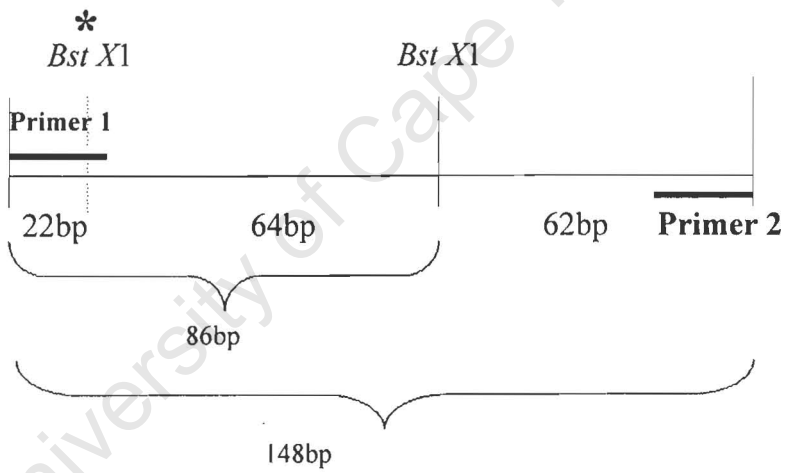


Fig 19 Schematic diagram of the PCR product from exon 5 digested with *Bst* X1 and the respective fragment sizes (*, introduced cutting site).

*Bst X*1 recognition sequence $CCAN_5 \downarrow NTGG$

Designed primer **AG AAA CGC TAC ACG CCA CGT GTT**

CCA

Mutated sequence of patient 3: 5' AG AAA CGC TAC ACG **TTT** CGT GTT **T**GG AGC CGC-3'

Wild type sequence: 5' AG AAA CGC TAC ACG TTT CGT GTT **C**GG AGC CGC-3'

Amino acid sequence: Q K R Y T F R V R

Codon position 224

Fig 20 Sections of the sequence of exon 5 containing the *Bst* X1 cutting sites. The cutting sites for these enzymes are underlined while the C→ T transition affecting the *Bst* X1 site is given in bold type.

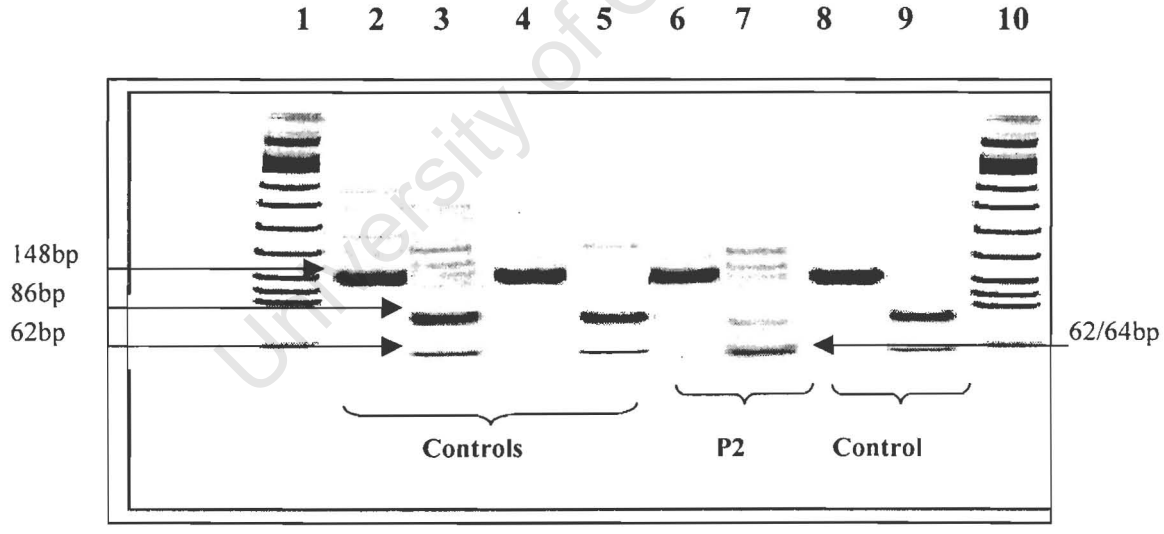


Fig 21 Restriction digest analysis of exon 5 with *Bst* X1 run on 20% PAGE. Lane 2,4 and 8-undigested control DNA. Lane 3, 5 and 9- digested control DNA. Lane 6 and 7- undigested and digested DNA of P2. Lanes 1 and 10 contain molecular weight marker VIII from Roche Biochemicals (appendix 3).

This kindred differed significantly from the previous kindreds investigated in that there was a family history of affected male siblings; with early male deaths occurring in 3 generations, this being strongly suggestive of an X-linked condition (Fig 22). The $T^-B^+NK^-$ immunophenotype of the proband led geneticists and immunologists to suggest the γc gene as the possible cause of the disorder (Fig 22). P3 had a successful HLA-identical bone marrow transplant 3 months after diagnosis which resulted in the full reconstitution of his immune system and is effectively cured. P3 has a phenotypically normal, unaffected older sister and younger brother.

3.3.2 PCR

All eight exons of the IL2Rγc of patient 3 were successfully amplified by PCR and were run on a SSCP gel under the specified conditions.

3.3.3 SSCP analysis

SSCP revealed a mobility shift in exon 6 (Fig 23: exon 6, lane 4). The exon was PCR amplified and sequenced. The PCR product of the mother of P3 was run alongside that of the proband (Fig 23: exon 6, lane 3); no altered migration pattern was observed. This was unexpected but subsequently showed to be due to a sample mix up. Carrier status was confirmed initially by X-inactivation studies and eventually by mutational analysis.

3.3.4 Sequencing results

Sequencing revealed a G→A transition (Fig 24A and B) at cDNA position 869, which caused a substitution of arginine for glutamine. This missense mutation resulted in a charge shift with replacement of a basic residue with a neutral one in the transmembrane domain of exon 6 (Fig 25). The R285Q mutation has been previously reported by Puck *et al* 1997 and Clark *et al* 1995. This codon is considered a mutational hot spot as 9 unrelated patients have been reported to carry the G→A transition.

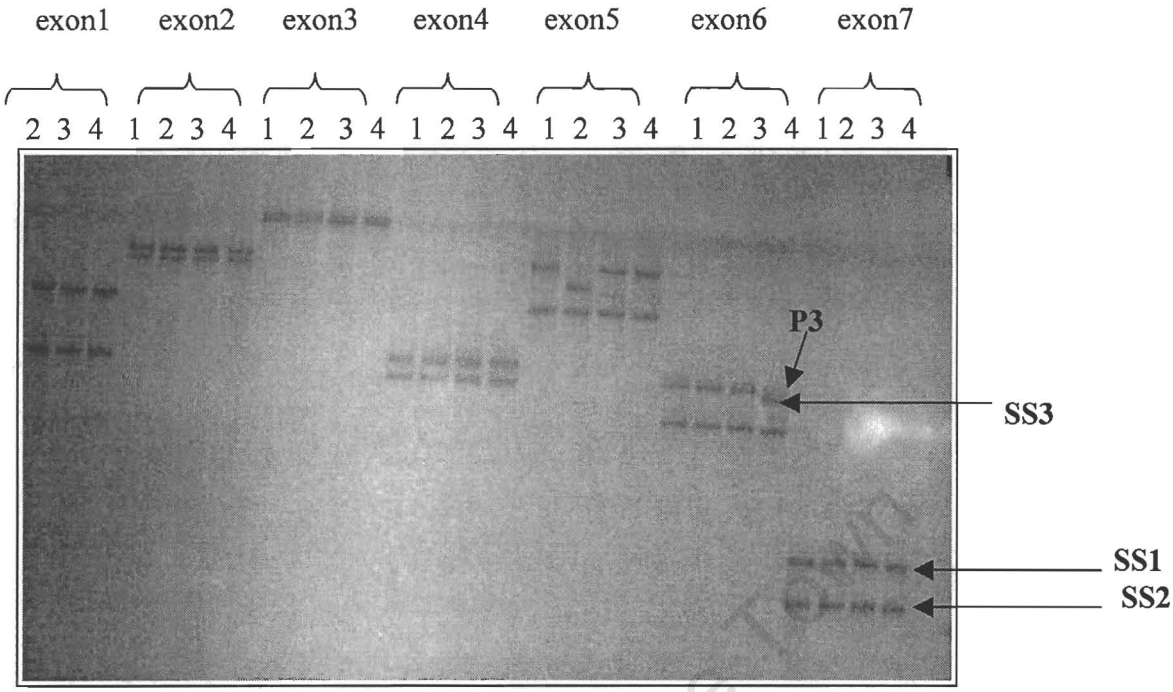


Fig 23 SSCP screening of exons 1-7 of the common gamma chain. DNA strands were visualised by silver staining. The lanes for each exon were labelled 1, 2, 3 and 4. Single strands are indicated by SS1, SS2. Mother of P3 - exon 6, lane 3. The mobility shifted band in exon 6 of P3 is indicated by the black arrow (SS3).

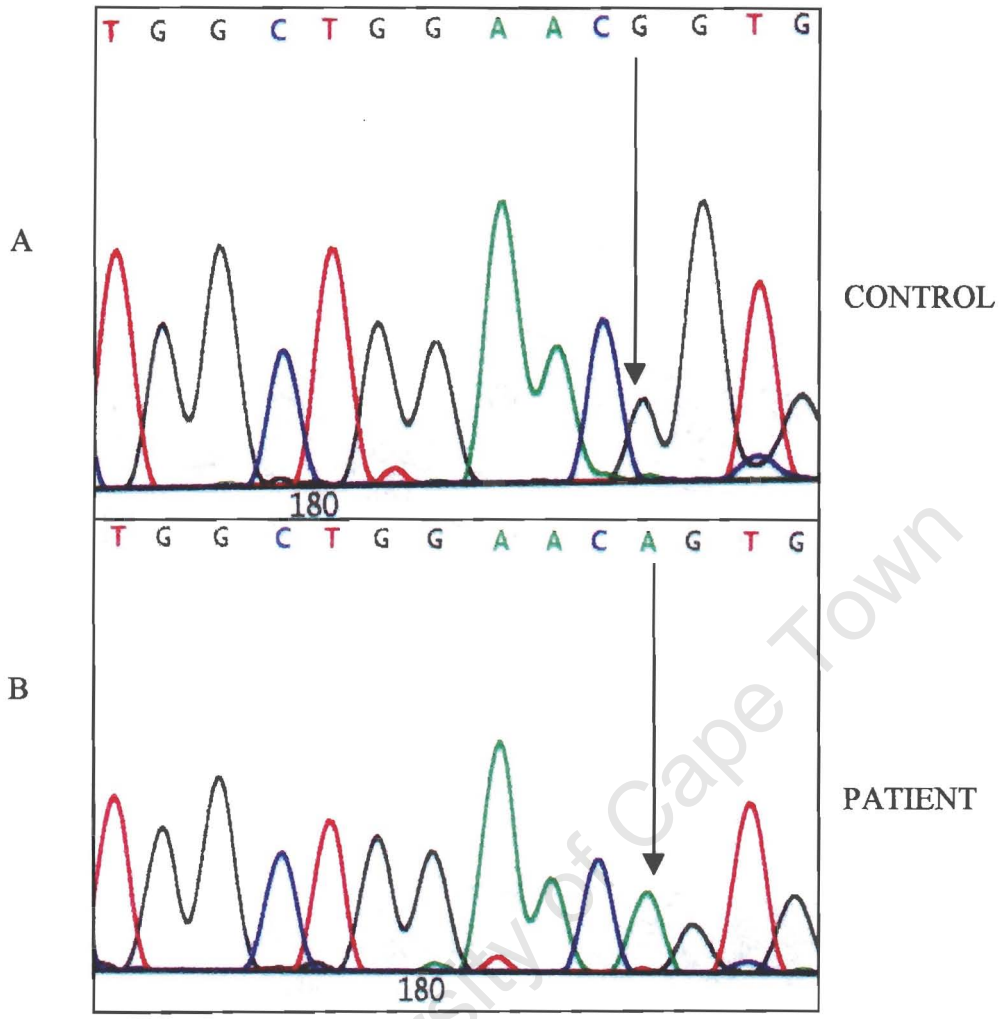


Fig 24 Sequencing traces of the sense strand of exon 6 from a control and the affected male of kindred 3, showing a G to A transition in the proband.

					Q
					↑
amino acid sequence:	F	W	L	E	**R**
amino acid sequence:	Phe	Trp	Leu	Glu	Arg
wild type sequence:	TTC	TGG	CTG	GAA	C<u>G</u>G
mutated sequence:	TTC	TGG	CTG	GAA	C<u>A</u>G
codon:	281	282	283	284	**285**
transition:	$G_{869} \rightarrow A_{869}$				
change in charge:	basic → neutral				
mutation nomenclature:	R285Q				

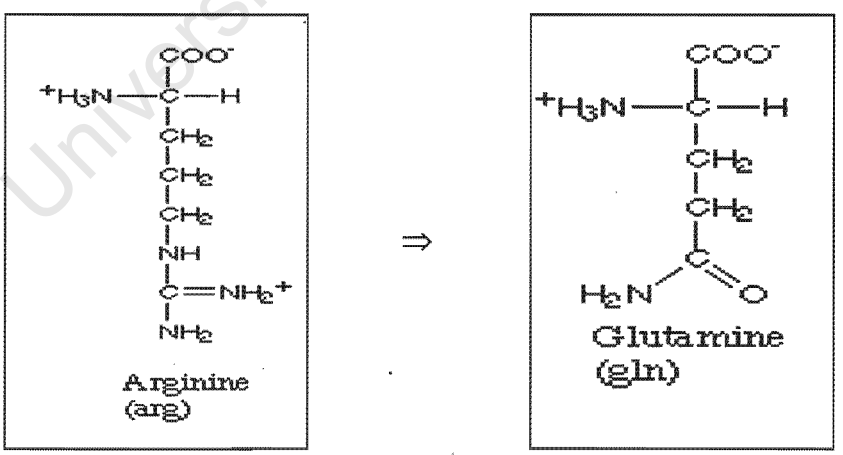


Fig 25 A summary of the nature and characteristics of the IL2Rγc gene defect detected in the proband of kindred 3. The wild type sequence depicted is taken from the cDNA sequence as recorded in GenBank under accession number L12181.

3.3.5 Mutation verification

R285Q mutation (patient 3)

The G to A transition at cDNA position 869 in P3 neither created nor abolished a restriction enzyme cutting site. A PCR primer was therefore designed to incorporate the *Bsi* Y1 cutting sequence at the site of the mutation by exploiting the G to A substitution. This primer introduced the *Bsi* Y1 cutting site into the PCR product from the normal allele. Thus, theoretically, cutting of exon 6 of control DNA with the enzyme *Bsi* Y1 should result in two fragments (60bp and 21bp). As the mutation in patient 3 resulted in the abolishment of this *Bsi* Y1 cutting site, we expected the generation of single fragment (81bp) (Fig 26). Carrier status of the mother and daughter was determined, with both proving to be carriers as both showed a loss of the *Bsi* Y1 site (Fig 27).

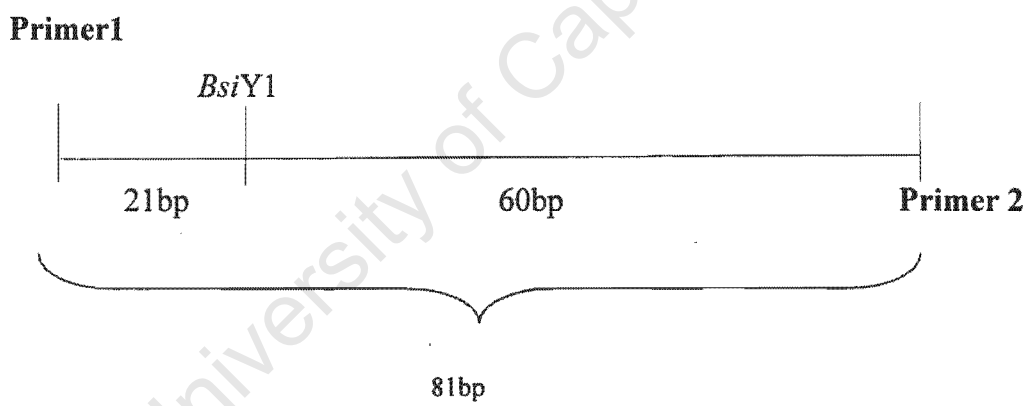


Fig 26 Schematic diagram of the PCR product digested with *Bsi* Y1 and the respective sizes of the fragments.

*Bsi Y*1 recognition sequence $CCN_5 \downarrow N_2GG$

Designed primer **C TGT GTG TAT TTCTCC CTG GAA C**

CC

Designed primer 5' ——————————/\——————

Mutated sequence of patient 3: 5' CTC TGT GTG TAT TTC TGG CTG GAA CAG -3'

Wild type sequence: 5' CTC TGT GTG TAT TTC TGG CTG GAA CGG -3'

Amino acid sequence: L C V Y F W L E **R**

cDNA position 869

Fig 27 Sections of the sequence of exon 6 containing the *Bsi* Y1 cutting site. The cutting site for this enzyme is underlined while the G→ A transition within the *Bsi* Y1 site is given in bold type.

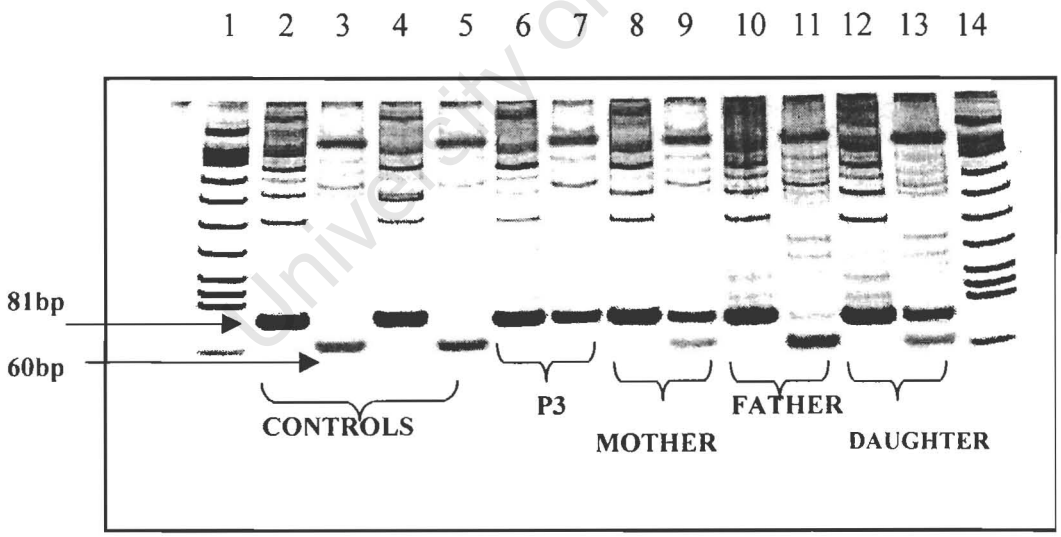


Fig 28 PAGE of PCR amplified DNA from exon 6, digested with *Bsi* Y1. Lane 2, 4 and 10- undigested control DNA; lanes 3, 5 and 11-digested control DNA; lane 6,7- undigested and digested DNA from patient 3; lanes 8, 9 and 12, 13- undigested and digested DNA from the mother and daughter respectively. Lanes 1 and 14 -molecular weight marker VIII from Roche Biochemicals (appendix 3).

Exon 6 amplified from the DNA of patient and control subjects were digested with *Bsi* Y1 and run on a 20% polyacrylamide gel (Fig 28). All control samples (Fig 28: lanes 3, 5 and 11) showed 2 fragments of sizes 60bp and 21bp, in contrast, amplified DNA of the proband generated a single fragment of size 81bp (Fig 28: lane7); only the 60bp fragment is seen as the 21bp fragment had already run off the gel. The digested DNA samples of the mother and daughter generated 2 fragments of sizes 81bp and 60bp. The low intensity 81bp band in Fig 28 lane 11 carries two possible explanations: it likely represents minimal undigested product or the sensitivity of the silver stain is such that it stains numerous extraneous bands, whether single stranded or double stranded, running at the same position as the band of interest. Many of these extraneous bands can be seen in all the lanes. The unequal intensity of the bands in the various lanes could be explained by heteroduplex formation during PCR. Heteroduplexes are not cleaved by *Bsi* Y1 due to the fact that the cutting sequence is not recognised by the enzyme. PCR restriction analysis thereby confirmed the A→G transition in the proband and offers a convenient test for carrier status should female relatives require screening in the future.

3.4 Kindred 4

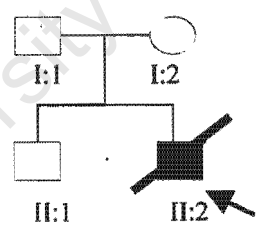


Fig 29 The pedigree of family 4

3.4.1 **Immunophenotype:** $T^-B^+NK^+$

Candidate genes: GAMMA CHAIN (γc) gene /IL7 and IL7R genes

Patient 4 (P4) presented at RXH during 1998 at 23.5 months of age with a long history of failure to thrive and a variety of infections: recurrent otitis media, recurrent thrush, recurrent pneumonia and recurrent herpes labialis. He died one month after diagnosis before a BMT could have been performed. The patient had a normal,

unaffected brother. No data is available on the pedigree and family history of this proband. The $T^{-}B^{+}NK^{+}$ immunophenotype suggested that the IL7 and IL7R genes were candidates for a defect within the family. However, the fact that the proband was male led to the inclusion of the γc gene as a possible candidate in this study.

3.4.2 PCR

All eight exons of the IL2Rγc were successfully amplified and run on an SSCP gel under various gel conditions

3.4.3 SSCP analysis

Three different SSCP gels were run for all exons of γc of the patient; no abnormal migration bands were seen. This negative SSCP result was expected as a IL2Rγc defect was considered to be a rare possibility.

3.4.4 Sequencing results

To further exclude an IL2Rγc defect all exons of this gene were sequenced to cover the possibility of non-detection by SSCP. The sequence trace results were normal for all exons of the gamma chain gene thus further ruling against the possibility of a mutation in the γc. The possibility also remains that a mutation could have been present in the promoter region; this is a rare possibility as no mutations have been reported in the promoter region of the γc in the IL2RG database.

3.5 Kindred 5

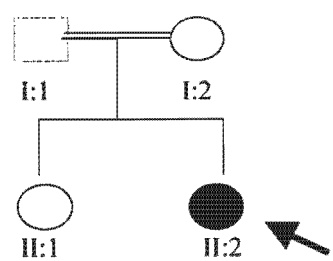


Fig 30 The family tree of kindred 5

The affected female (P5) presented to Red Cross children's hospital during 1999 at 3 months of age with failure to thrive, interstitial pneumonia, chronic otitis media, persistent diarrhoea, generalised lymphadenopathy and generalised eczema. She died at 5 months of age of persistent pneumonia and respiratory failure. The parents of the proband are first cousins and the fact that the patient was female with a $T^-B^-NK^+$ immunonophenotype suggested an AR-SCID disorder. The proband has a normal, unaffected sister. By taking together all the facts that were known about the case two genes immediately immerged as strong candidates for the defect, RAG1 and RAG2. They are the only genes known thus far to cause this type of AR-SCID (Fig 30).

Microsatellite analysis on the DNA of P5 was carried out to strengthen the hypothesis that the mutation in P5 was caused by one of the rag genes. Two microsatellites were used, with both being informative and giving inheritance patterns compatible with a Rag1/Rag2 defect (Chapter 4). Later, due to time constraints and the fact that the *rag* genes were such large genes it was decided to send the DNA of P5 for mutation analysis to a laboratory in Italy, well known for their studies on AR-SCID. Sequencing analysis confirmed homozygosity for a single base deletion (Adenine) in the RAG1 gene, corresponding to position 859 of the cDNA of the published sequence.

The RAG1mutation database lists 40 mutation entries showing 59 independent mutations and 44 unique molecular events. The most common mutation type is missense following (16%) followed by frameshift deletions (20%), nonsense mutations (17%) and a frameshift insertion (2%) (URL 11).

This 859delA mutation is assumed to be novel and does not appear within the RAG1/2 mutation database (as April 2001).

Chapter 4 Microsatellite analysis

The objective of this aspect of the study was to find microsatellite inheritance patterns that would (1) strengthen the hypothesis that the defect in P4 was due either to an IL7Rαc or IL7mutation, (2) exclude the gamma chain as a third genetic possibility; and (3) strengthen the hypothesis that the defect in P5 was the result of a mutation in either the Rag1 or the Rag2 genes; this would then validate the sending of the P5 DNA to another laboratory to be sequenced.

4.1 Introduction

All 8 exons of the gamma chain had been screened through SSCP in chapter 3 without finding any abnormal allelic migration patterns; these results were not suggestive of a gamma chain defect for family 4. As the blood samples for the unaffected brother had also been collected and stored at the beginning of the study it was possible to use microsatellite analysis as a technical tool to strengthen the hypothesis of a defect in the IL7/IL7R system in this family.

Human DNA contains many sequences of nucleotides that occur repeatedly from a few to many thousand times within the genome. These vary in complexity and can be widely dispersed copies of a single sequence unit or they can occur in tandem arrays of units. The latter sequences, called Variable Number Tandem Repeats (VNTR's), are unique to each person and are, most often, the bases for the DNA fingerprinting used in forensics.

Most nucleotide sequence differences occur in the 97% of the genome that does not code for proteins. These non-coding regions of DNA may be located within genes (where they are called "introns") or in between genes. Variations within this region are most likely functionally inconsequential and hence well tolerated during evolution. This has allowed tremendous genetic diversity to develop in these regions. Much of this non-coding DNA consists of highly repetitive segments consisting of several iterations of a specific sequence known as "DNA repeats" (URL 6).

One such class of sequences in humans consists of simple tandem repeats (STR's), often a dinucleotide (sometimes tri- or tetra-) repeat consisting for example, of CA (adenosine and cytosine) on one DNA strand and GT (guanine and thymine) on the other. This repeated element (CA repeat) is the commonest type of STR and occurs in an estimated 50,000 to 100,000 locations within the genome. Such repeats of 2-5 nucleotide segments are known as microsatellite DNA. Frequently, the number of copies of the repeating unit of the STR differs in the two chromosomes, thus producing heterozygosity for the repeat copy number. A single pair of PCR oligonucleotide primers that surround such sequences produce variably-sized DNA fragments depending upon the number of repeats (Fig 31) (URL 6).

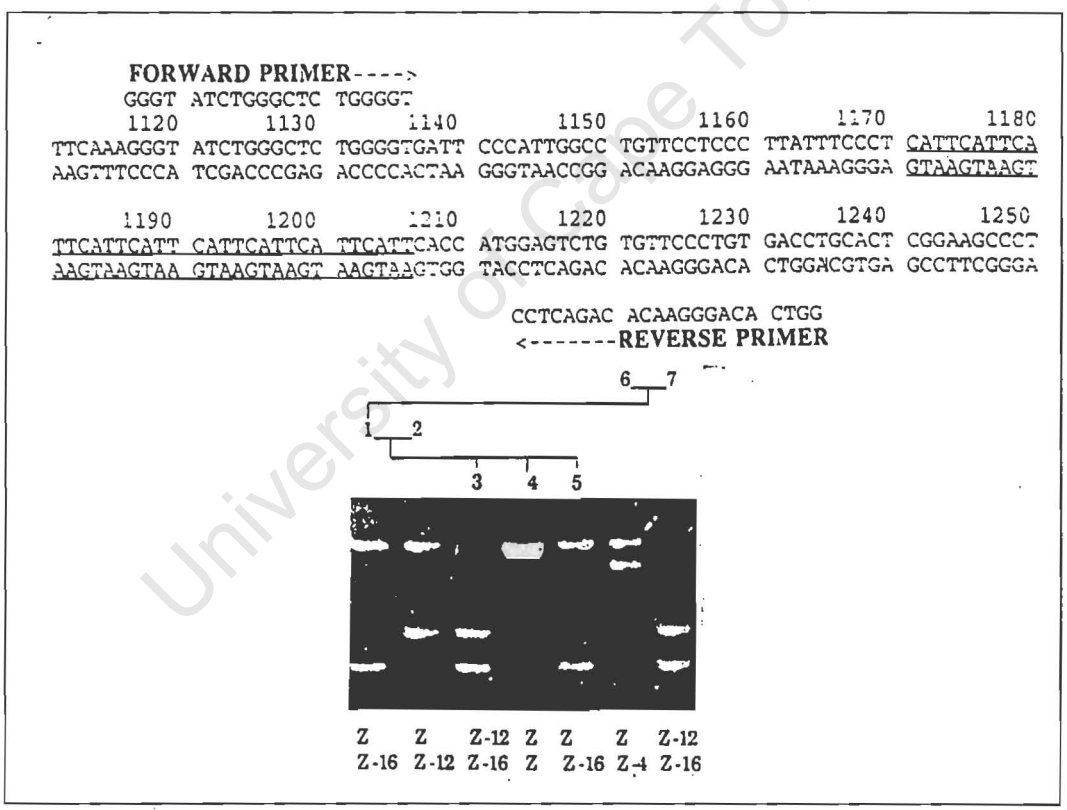


Fig 31 An example of a microsatellite showing a CATT tetranucleotide repeat in the tyrosine hydroxylase (TH) gene on human chromosome 11p15 (Hearne *et al*, 1992).

Microsatellites have increasingly been used as gene markers of choice. They are found in large numbers and are relatively evenly spaced throughout the genome. Technically microsatellites are more desirable than the larger VNTR loci because they can be analyzed via PCR and the alleles can be unambiguously sized on polyacrylamide gels (URL 7).

Microsatellites are useful for a number of analyses. They were originally utilized for genetic mapping and have been extensively used for linkage analyses in the association with disease susceptibility genes. In addition they have proven useful in the analyses of paternity and kinship. Due to their high level of heterozygosity, they are informative markers that can be used for many population genetic purposes, ranging from the individual level (e.g. clone and strain identification) to closely related species. They can be used to estimate effective population size and to gain insight into the degree of population substructure including both the amount of migration between subpopulations and genetic relationships among the various subpopulations (URL 7). Due to the small amount of DNA required they are also suitable in identifying people for forensic purposes.

The enormous variation conveyed by microsatellite repeats results from the fact that the repeats are especially prone to DNA-replication errors, often through what is called slipped-strand mispairing due or unequal crossing over due to misalignment of sister chromatids. During replication the ladder splits down the middle, separating the base pairs. As the DNA polymerases copy each strand a new strand is made which then pairs with its template. Slipped-strand mispairing can occur when either the old, template strand or the newly forming complimentary strand slips and pairs with the wrong repeat on the other strand (Fig 32). This then leads to modest increases or decreases in size. Repeat length and base composition also affect the mutation rate, e.g. dinucleotides (CA) mutate faster than trinucleotide repeats (CAG), and sequences with a high AT content mutate faster than those with a high GC content. The mutation rate of microsatellites is estimated to be around 5×10^{-5} (, Moxon and Wills 1999).

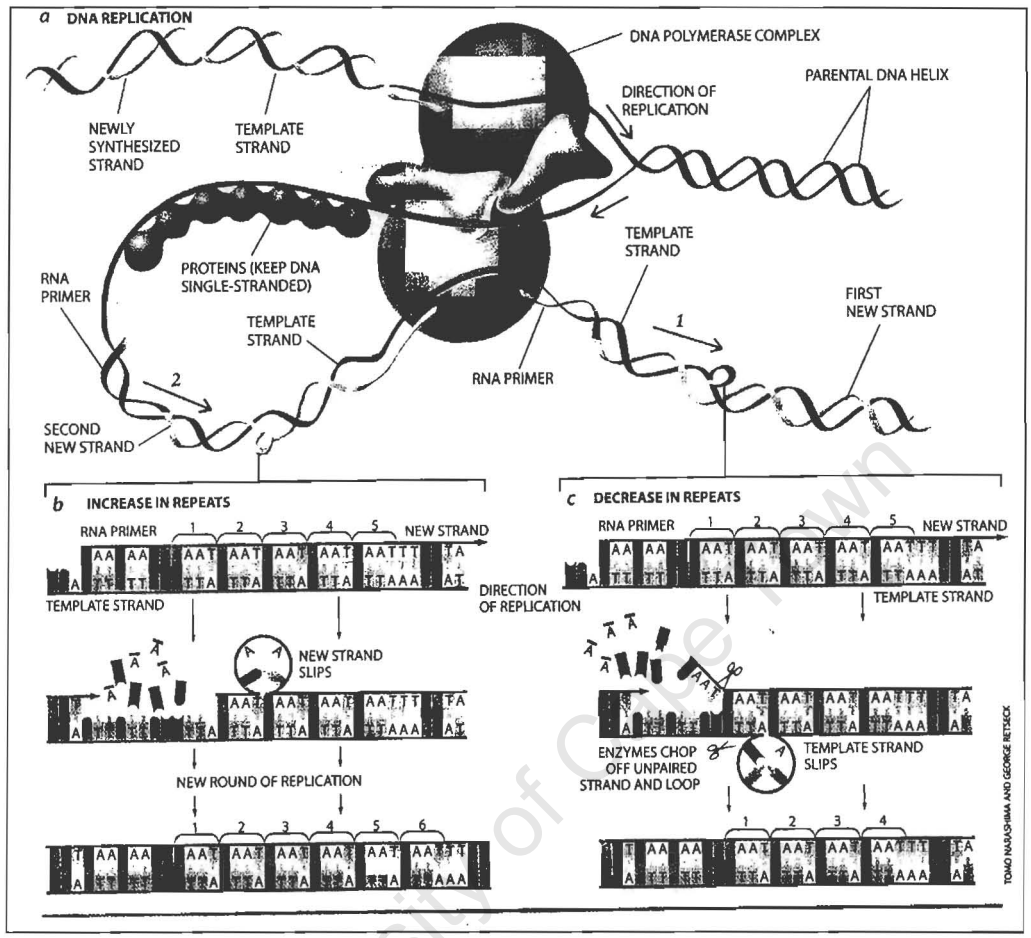


Fig 32 The mechanism of slipped-strand mispairing. (a) DNA helicase unzips the parental DNA helix while the DNA polymerase complex copies both strands. (1) Lagging strand- synthesis of a short fragment beginning with an RNA primer (2) skips ahead and generates a second short fragment. (b) Increase in the number of repeats when the new strand slips down one repeat in its binding to the old, template strand resulting in an extra repeat in the new strand. (c) The old, template strand slips and the enzyme deletes a repeat unit with the result that a shorter fragment is produced (Moxon and Willis 1999).

Because of their small size microsatellite loci can be amplified by PCR and the products analysed by electrophoresis on native or denaturing gels to separate the alleles according to size. Alleles can be visualised using radioactive endlabelling or incorporation,

silverstaining, ethidium bromide staining or florescent dye labelling. The alleles are then scored according to their position on the gel. Sometimes, though, allele scoring or determination can be a problem due to a phenomenon called "stutter bands" which can be explained by slipped-strand mispairing during extension by *Taq* polymerase (Hearne *et al* 1992).

There are some disadvantages to using microsatellites, these being high development costs involved in cases where the primers are not yet available and the classification of heterozygotes as homozygotes when null-alleles occur or when there is a mutation affecting the primer annealing sites. Further, stutter bands occurring on the gels may complicate accurate scoring of polymorphisms. However, the advantages to using microsatellites far outweigh these disadvantages (URL8).

4.2 Methods

4.2.1 Radioactive end-labelling for a ladder or a microsatellite

4.2.1.1 Protocol

The reaction mix for a ladder of 10µl volume comprised the following: 1.0µl 10 x PNK buffer, 0.8µl -40 Primer (5µM), 4.2µl distilled water, 3.0µl [γ^{32}P] ATP, 1.0µl diluted T4 PNK (30U/µl).

The reaction mix for a microsatellite of 20µl volume was as follows: 2.0µl 10 x PNK buffer, 5.0µl Primer Forward (25pM/µl), 9.5µl distilled water, 1.5µl [γ^{32}P] ATP, 2.0µl diluted T4 PNK (30U/µl).

The samples were incubated at 37^{0}C overnight and heat inactivated at 95^{0}C for 2 minutes thereafter. Samples were given a brief spin and stored at -20^{0}C until use.

4.2.2 Protocol for ladder synthesis

4.2.2.1 PCR preparation of ladder using cycle sequencing

A PCR reaction mixture with a 35.6µl volume was prepared as follows: 5.0µl M13 DNA single stranded, 4.47µl 10 x Taq buffer, 2.83µl radiolabelled -40 Primer, 18.53µl distilled water, 0.3µl Taq (5U/µl), 4.47µl $MgCl_2$.

Four microlitres (4 µl) of ddATP + dNTP and ddTTP + dNTP mixes were aliquoted into a clean tube (this mix contained the dNTP's and the specific ddNTP).

Aliquotes of 500µl were made up as follows: tube1 (ddATP+dNTP)- 25µl dNTP + 65µl ddNTP and 410µl distilled water; tube 2 (ddTTP+dNTP) - 25µl dNTP + 75µl ddNTP and 400µl distilled water.

Into each ddATP and ddTTP tube, 16µl of the above reaction mix was aliquoted and overlaid with mineral oil.

4.2.2.2 Thermocycling

The following program was utilised: initial step 1:- 94^{0}C for 2 minutes; cycling step 2:-94^{0}C for 30 seconds, cycling step 3:-55^{0}C for 30seconds, cycling step 4:-72^{0}C for 1 minute (28 cycles from steps 2-4).

The reactions were stopped with the addition of 8µl formamide stop mix. All reactions were mixed together. The samples were denatured for 2-5 minutes @ 95^{0}C before loading. The total volume now was 32+16=48µl. A 4µl aliquot of the ladder was loaded per run..

4.2.2.3 Preparation of ladder using the Sequenase KIT

A PCR reaction mix with a 35.6µl volume was prepared as follows: 10.0µl M13 DNA single stranded, 4.0µl Reaction buffer, 10.0µl radiolabelled -40 Primer.

The sample was incubated at 65^{0}C for 2 minutes, slowly allowed to cool to room temperature for 15-30 minutes, centrifuged and cooled on ice. To the above tube the following was added: 7.0µl distilled water, 2.0µl DTT and 1.0µl diluted sequenase. The 34µl mix was divided equally (16µl) into the 2 termination tubes.

The termination tubes had been prepared in advance, had been preheated to 37^{0}C and now contained the following: tube 1 - 4µl ddATP; tube 2- 4µl ddTTP. The two tubes were gently mixed and incubated at 37^{0}C for 5 minutes followed by 70^{0}C for 7 minutes and then 14µl of stop solution was added to each reaction tube. The two mixes were combined, mixed, spun and stored at -20^{0}C. The ladder was heated to 95^{0}C for 5 minutes before loading onto the gel.

4.2.3 Radioactive PCR reaction

4.2.3.1 PCR protocol

A reaction mix containing a 9.0µl volume was prepared as follows: 0.8µl 2.5mM dNTP's, 0.9µl 10 x Buffer, 0.4µl 1mM $MgCl_2$, 1.33µl end-labelled Forward primer (20pM), 0.3µl Reverse primer (20pM), 4.77µl distilled water, 0.5µl DNA (0.5µg/µl), 1.0µl diluted Taq enzyme.

The 9.0µl reaction sample was overlaid with 10µl light mineral oil (Sigma) and briefly centrifuged. The tubes were placed in a thermocycler and subjected to a "hot start" by the addition of the 1.0ul enzyme mix after the first 95^{0}C for 5 minutes step in the PCR cycle.

4.2.3.2 Thermocycling

An appropriate annealing temperature profile was chosen for each set of primers and the individual annealing temperatures were programmed into the thermocycler (Appendix 1).

The following program was used: initial step 1:-95^{0}C for 5 minutes; cycling step 2:-94^{0}C for 30 seconds, cycling step 3:-__^{0}C for 30seconds, cycling step 4:-72^{0}C for 1 minute (33 cycles for steps 2-4) and final step 5:-72^{0}C for 7 minutes.

To each sample 4µl of stop solution was added and the samples were stored until ready for use.

4.2.4 PAGE

4.2.4.1 Preparation of plates

The plates were prepared as in section 2.4.2 with a few slight modifications. No plate glue was used on either plate. Shark-tooth combs of size 1mm were used for the formation of wells.

4.2.4.2 Preparation of PAGE

The reagents were used to make up the polyacrylamide gel: 80.0ml 6% cold Polyacrylamide working stock, 280µl APS and 64µl TEMED.

4.2.4.3 Sample preparation and electrophoresis

Polyacrylamide gels were pre-electrophoresed for 30 minutes at 65W in 1 x TBE buffer. The PCR samples and the ladder were heated for 3 minutes @ 95^{0}C and immediately put on ice, ready for use. The gel was run for 3 hours @ 65W depending on the size of the fragments. Plates were disassembled and carefully removed. Filter

paper (Whatmanns 3mm) was cut to the size of the gel and gently pressed onto the plate to which the gel was adhering. Whatmann was carefully pealed from the top down with the gel sticking to the paper. The exposed part of the gel was wrapped with cling wrap and placed in a prewarmed gel dryer for 1 hour @ 80^0C to dry. The gel was then placed in a cassette with an autorad in the darkroom. Exposure time was 12-24 hours depending on the intensity of the bands.

4.2.5 Visualisation of the microsatellites

4.2.5.1 Protocol for developing of the autorad

In the darkroom (using the red light only) the cassette was opened, the autorad removed and placed in developer solution for 90 seconds, in stop solution for 30 seconds and in the fixer solution for 2 minutes. The autorad was then placed in running tap water for 5 minutes and left to dry. The alleles were then scored as seen on the autorad.

4.3. Results and Discussion

Microsatellite analysis was used to lend technical strength to the hypothesis that the mutation in P4 was due to either an IL7 or IL7Rα mutation and (2) to eliminate the common gamma chin gene from the study as a possible candidate and (3) to strengthen the hypothesis that the defect in P5 was due to a mutation in either the Rag1 or Rag2 genes.

4.3.1 Gamma chain (γc) microsatellites

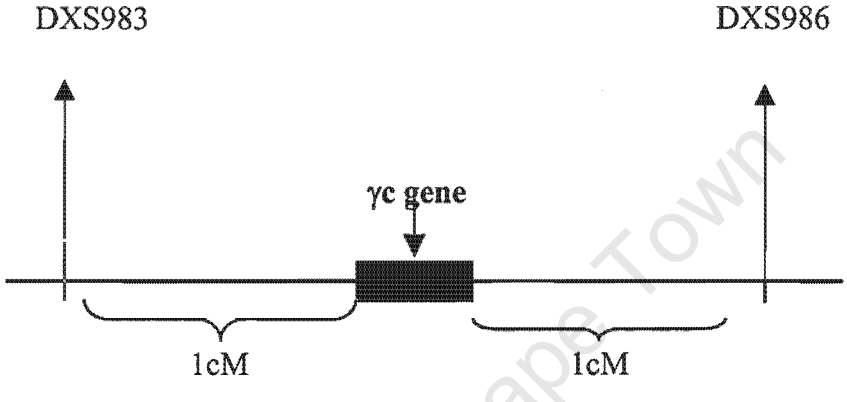


Fig 33 Schematic diagram of the position of markers DXS983 and DXS986 in relation to the γc gene on the X chromosome.

Two of the closest microsatellite markers, DXS983 and DXS986, were chosen for study. These were selected from published polymorphic markers in the Genome Database (GDB), the Gènèthon linkage map, with additional information taken from Centre d'Etude du Polymorphisme Humaine (CEPH) and the Human Genetics Organisation (HUGO). These microsatellites are found within 1cM of the gene and have a recombination probability of less than 1% (Fig 33).

To exclude the γc gene, markers DXS983 and DXS986 for family 4, a linkage pattern should emerge where both brothers, the normal and the affected, had inherited the same allele from their mother. As one son is affected and the other is not, both could not have inherited the same allele from their mother. The proband should have inherited the defective allele while the unaffected son should have inherited the normal allele. Had both boys inherited the same allele form their mother; it would have validated the exclusion of gamma chain gene as a candidate in P4.

As the mutation in family 3 had been confirmed through PCR-SSCP, sequencing and restriction digestion it was decided to use this family as a control for the microsatellite analysis study. Theoretically, to be informative, markers DXS983 and DXS986 for family 3 should reveal a linkage pattern where the affected son inherited the defective allele from his mother; his sister, being a carrier, should have inherited the normal from her father and the defective allele from her mother.

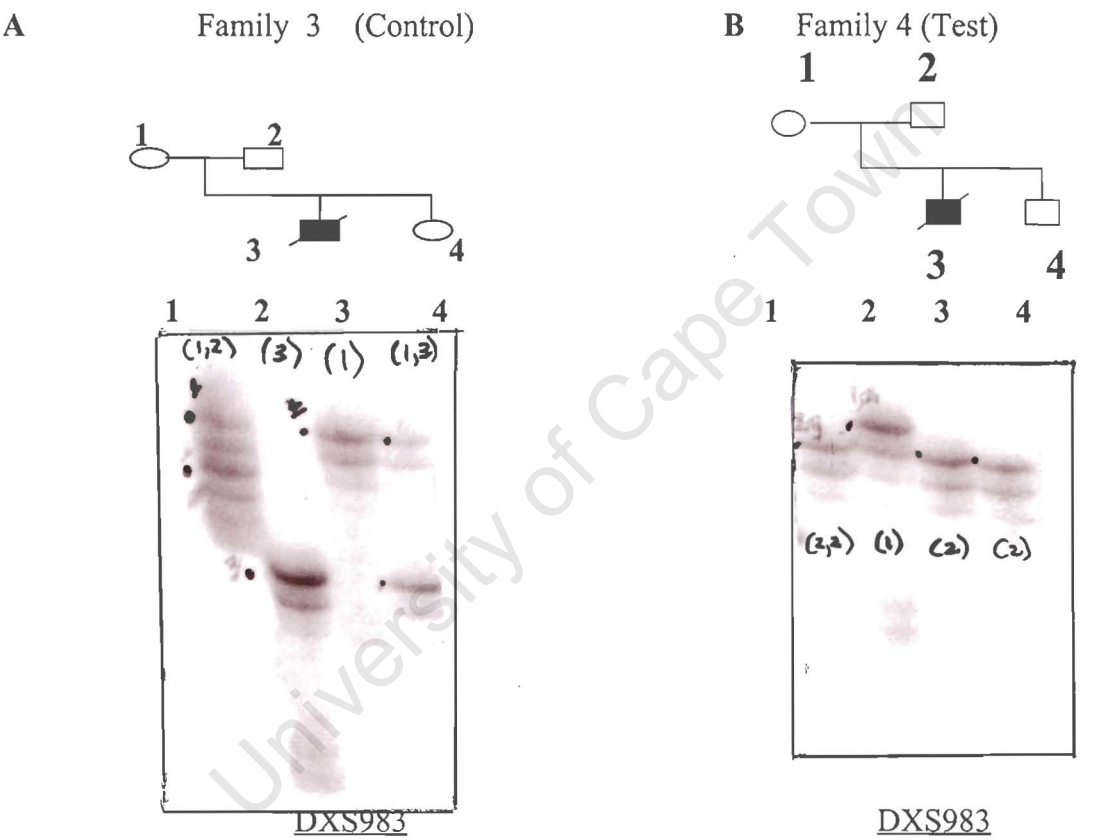


Fig 34. Autoradiographs showing γc gene microsatellite marker DXS983 for family 3 **(A)** lane 1-mother, lane 2-father, lane3-proband and lane4-unaffected sister. Family 4 **(B)** lane 1-mother, lane 2-father, lane 3-proband and lane 4- unaffected brother.

Microsatellite DXS983 proved to be fully informative for family 3. Both alleles of the mother were clearly discernible and distinct from that of her husband, enabling accurate scoring of the alleles for each family member. The resultant genetic linkage pattern with marker DXS983 revealed that patient 3 (Fig34A: lane 3) had inherited allele 1 from the mother (Fig 34A: lane 1). The sister of patient 3 (Fig 34: lane 4) had also inherited allele 1 from her mother and allele 3 from her father (Fig 34A: lane 3). These results are compatible with the IL2Rγc gene being the cause of the mutation in patient3 and reveal that allele 1 of the mother carries the γc mutation. The autoradiograph for this marker confirmed what had been proven in chapter 3; both the proband and his sister had inherited the same defective allele from their mother.

For marker DXS983 in kindred 4, it was not possible to distinguish between the maternal alleles (Fig 34B: lane 1). Thus no clear segregation pattern emerged for either of the two boys (Fig 34B: lanes 3 and 4) as the defective allele could not be determined. This marker was thus uninformative for this family; with the result that the γc gene could not be excluded as the cause of this disease.

Family 3 was again used as a control for marker DXS986. The two alleles of the mother had separated clearly. It was clear that the affected son (Fig 35A: lane3) had inherited allele 2 from his mother (Fig 35A: lane 1) while the daughter (Fig 35A: lane 4) had inherited allele 1 from her father (Fig 35A: lane 2) and allele 2 from her mother. This marker was very informative for this family as allele separation enabled correct scoring of the alleles. This autoradiograph was again, compatible with a mutation in the γc gene, underlying the disorder in P3 and the positive carrier status in the sister. Again the patient and his sister had inherited the same defective allele from their mother.

Analysis with marker DXS986 for family 4 revealed that the affected male (Fig 35B: lane3) had inherited allele 3 from his mother (Fig 35B: lane 1) while his brother (Fig 35B: lane4) had inherited allele 1 from his mother. This marker was not very helpful at exclusion in this family as each boy had inherited a different allele. This finding would however, be compatible with X-linked SCID.

DNA sequencing, where all 8 exons were shown to comprise normal sequences, finally excluded the γc gene.

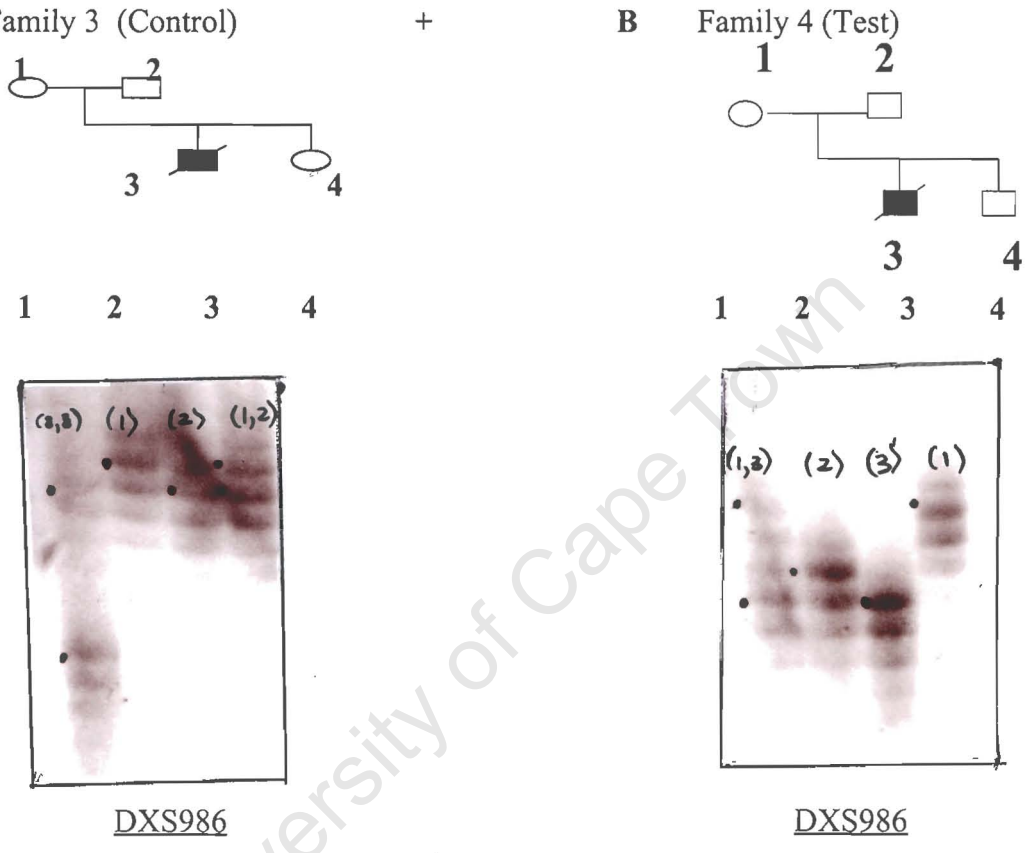


Fig 35. Autoradiographs showing γc gene microsatellite marker DXS986 for family 3 **(A)** lane 1-mother, lane 2-father, lane3-proband and lane4-unaffected sister. Family 4 **(B)** lane 1-mother, lane 2-father, lane 3-proband and lane 4- unaffected brother.

4.3.2 Interleuken 7 (IL7) microsatellites

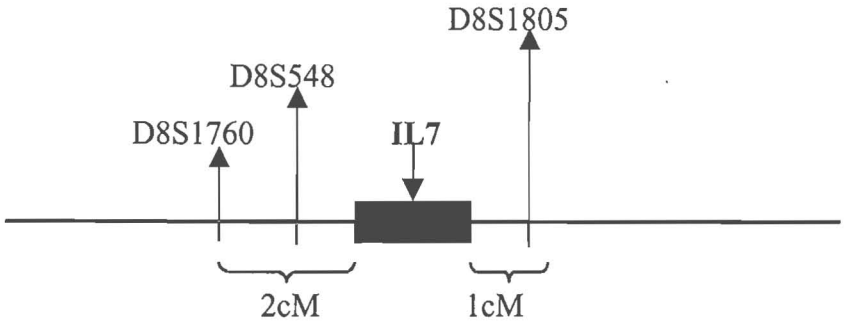
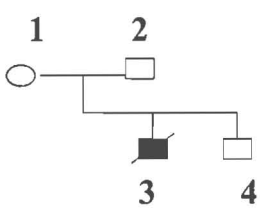
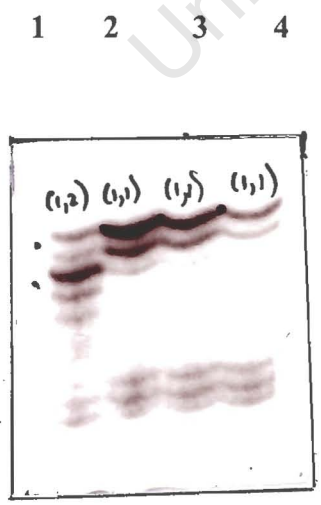


Fig 36. Schematic diagram of the position of markers D8S1760, D8S548 and D8S1805 in relation to the IL7 gene on chromosome 8.

Three markers, D8S1760, D8S548 and D8S1805, in the region of the IL7 were selected from published polymorphic markers in the GDB and the Gènèthon linkage map, with additional information taken from CEPH. They are located on chromosome 8 on either side of the IL7 gene, have an average separation size of 3 cM and a recombination probability of less than 2% (Fig 36).

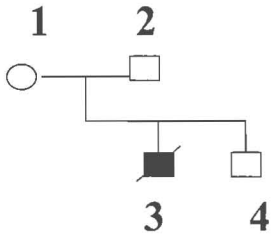
A Family 4 (Test)

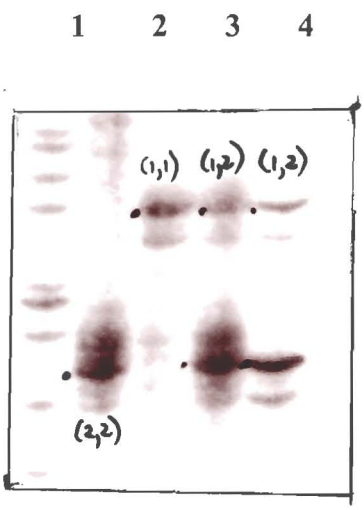




D8S1805

B Family 4 (Test)





D8S1760

C Family 4 (Test)

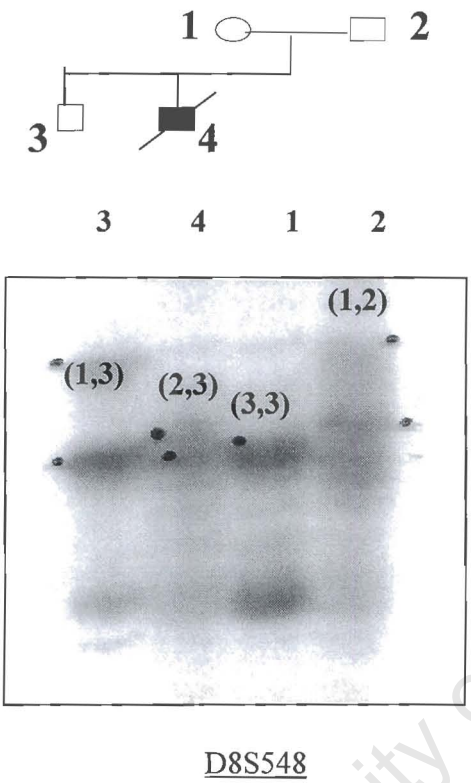


Fig 37. Autoradiographs showing microsatellite markers **(A)** D8S1805 **(B)** D8S1760 **(C)** D8S548 for family 4; **(A+B)** lane 1-mother, lane 2-father, lane3-proband and lane4-unaffected brother. **(C)** lane 1-unaffected brother, lane 2-proband, lane 3-mother and lane 4- father.

Theoretically we expected a linkage pattern where both boys had either (1) inherited the same alleles from their parents thereby leading to the exclusion of the IL7 gene or (2) inherited different alleles, thereby leading to the retention of the IL7 gene as a candidate for the defect in this family. These scenarios applied to all three markers.

Unfortunately, all three microsatellites in Fig 37 were uninformative in this family. In each case one of the parents was found to be homozygous, precluding the identification of allele segregation in the two boys. Exclusion of the IL7 gene was thus not possible and it remained a candidate gene for the defect in this family.

4.3.3 Interleuken 7 receptor alpha (IL7Rα) microsatellites

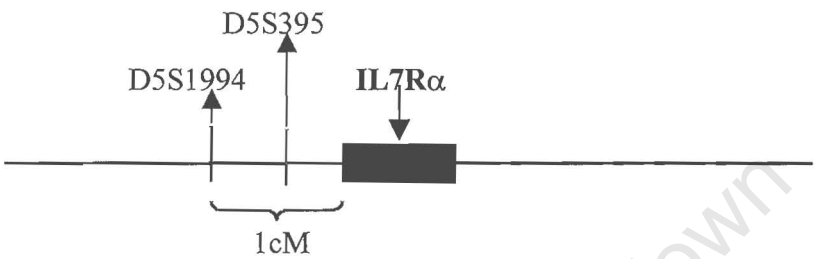


Fig 38 Schematic diagram of the position of markers D5S1994 and D5S395 in relation to the IL7Rα gene on chromosome 5.

In the search for exclusion/ inclusion of the IL7Rαc gene two markers, D5S1994 and D5S394 were selected from published polymorphic markers in the GDB, the Gènèthon linkage map, with additional information taken from CEPH and HUGO. These markers are located 5' to the IL7αc gene, lie within 1 cM and thus have a recombination probability of less than 1% (Fig 38).

Segregation analysis of marker D5S1994 for family 4 revealed that the affected boy (Fig 39A:lane 3) had inherited allele 2 from the father (Fig 39A:lane 2) and allele 3 from the mother (Fig 39A: lane 1). The brother (Fig 39A: lane 4) had inherited allele 1 from the father and allele 3 from the mother. Marker D5S1994 was informative for family 4 as both alleles of the mother and the father were separated well on the gel. The inheritance of different alleles at the IL7Rαc locus in the two brothers precludes exclusion of the gene and thus is compatible with the postulate of a possible IL7Rα chain defect in this family.

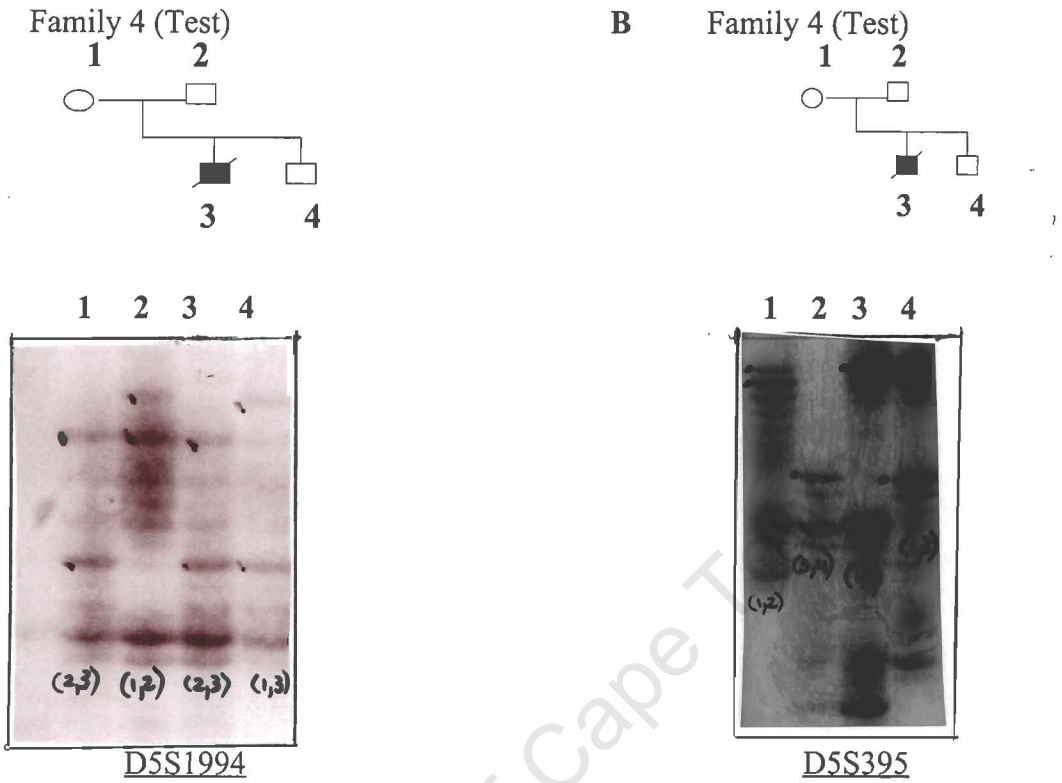


Fig 39 Autoradiographs showing microsatellite markers for IL7Rαc in family 4. **(A)** D5S1994; lane 1-mother, lane 2-father, lane3-proband and lane4-unaffected brother. **(B)** D5S395; lane 1-mother, lane 2-father, lane 3-proband and lane 4- unaffected brother.

For marker D5S395 the autoradiograph revealed that the affected male (Fig 39B: lane 3) had inherited allele 1 from his mother (Fig 39B: lane 1) and allele 4 from his father, (Fig 39B: lane 2) while his normal brother (Fig 39B: lane 4) had inherited allele 1 from his mother and allele 3 from his father. This microsatellite marker was fully informative in this family but was unable to exclude the IL7Rαc as a candidate.

4.3.4 Rag1/Rag2 microsatellites

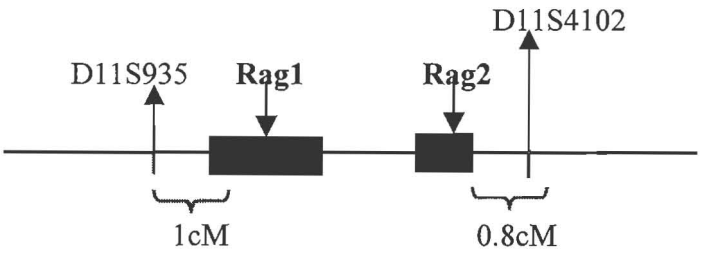


Fig 40 Schematic diagram of the position of markers D11S935 and D11S4102 in relation to the Rag1 and Rag 2 genes on chromosome. 11.

The markers in the region of the Rag1/Rag2 genes were selected from published polymorphic markers in the GDB, the Gènèthon linkage map, with additional information taken from CEPH and HUGO. The two markers, D11S4102 and D11S395, flanking the Rag1/Rag2 genes are approximately 2cM apart and thus have a recombination probability of 2% (Fig 40).

Theoretically we expected P5 to be homozygous for the mutation. As the proband's parents are consangious and the disease is AR. We anticipated the proband to have inherited the same defective allele from each parent and would thus be homozygous for a gene mutation, and homozygous for markers flanking the defective gene. At least one of these markers would also be different from those inherited by the unaffected daughter.

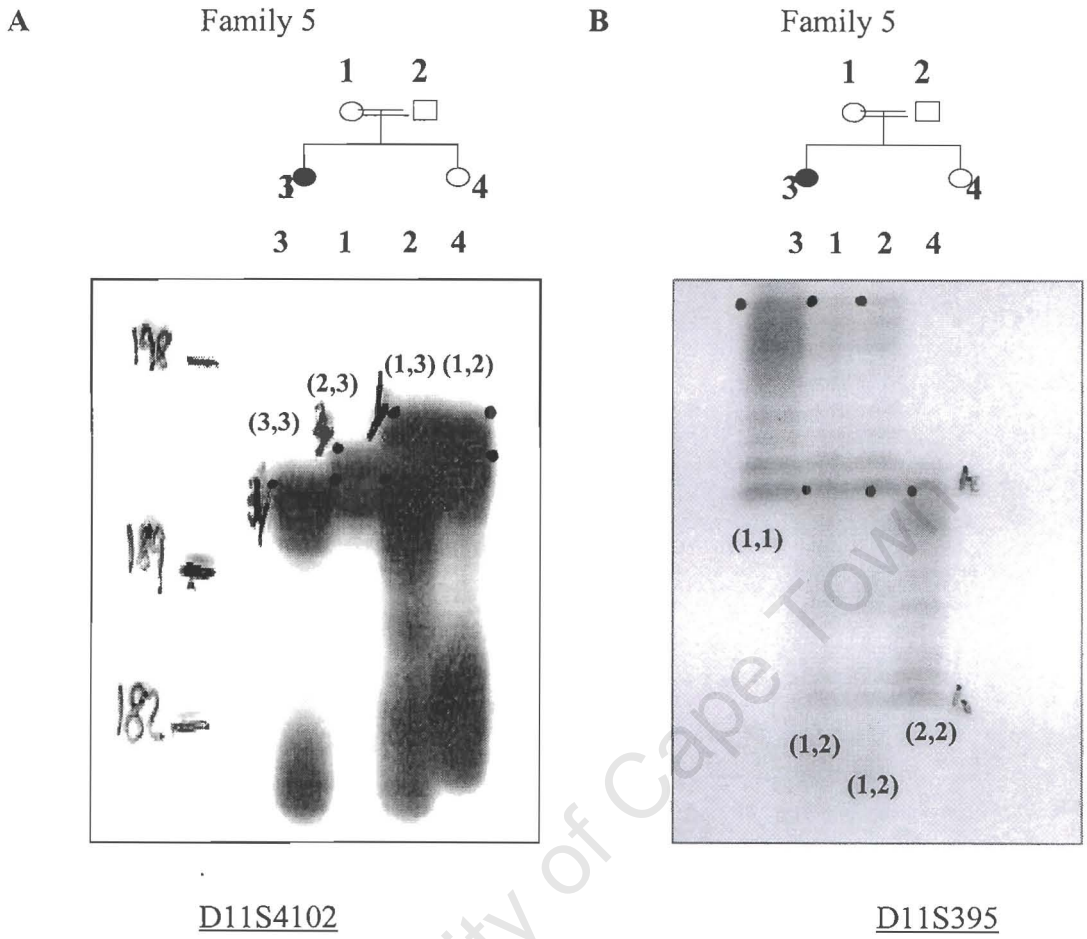


Fig 41 Autoradiographs showing microsatellite markers **(A)** D11S4102 :lane 1 proband, lane 2-mother, lane3-father and lane4-unaffected sister. **(B)** D11S395:lane 1-proband, lane 2-mother, lane 3-father and lane 4-unaffected sister

Marker D11S4102 revealed that the affected daughter (Fig 41A: lane 1) had inherited allele 3 from her mother (Fig 41A: lane 2) and the other allele 3 from her father (Fig 41A: lane 3). The unaffected sister (Fig 41A: lane 4) had inherited allele 1 from her father and allele 2 from her mother. If positive for a Rag1/Rag2 mutation this result indicate that the unaffected daughter would not be a carrier of this disease, as she has not inherited the defective allele (allele 3) from either of her parents. For marker D11S395 the autoradiograph revealed that the patient (Fig 41B: lane 1) had inherited allele 1 from her mother (Fig 41B: lane 2) and the other allele 1 from her father (Fig

41B: lane 3). The unaffected sister (Fig 41B: lane 4) had inherited allele 2 from both parents.

Segregation analysis of the microsatellite markers surrounding the Rag1/Rag2 loci (Fig 40) strengthened the hypothesis that the affected female in kindred 5 has a mutation caused by either of the two Rag genes. Genotypic fingerprinting revealed that markers D11S4102 and D11S395 were able to show an association between the disease and the gene. For both markers different combinations were found in the affected and unaffected daughters.

The DNA of P5 was sent to Italy for mutational analysis (Dr Anna Villa, Istituto di Tecnologie Biomediche Avazante). The results revealed and insertion of an A at codon 249 of the cDNA of the Rag1 gene of both alleles. This resulted in a frame shift, which in turn caused a prematurely truncated protein upstream of the original stop codon. A PCR based assay to detect this insertion has not been devised. Analyses of the restriction enzymes surrounding the mutated sequence indicate that these results could be confirmed by the introduction of a *Bsp* W1 cutting site at the site of the mutation at cDNA codon 249.

Chapter 5 X-inactivation

The object of this investigation was to explore the utility of X inactivation studies to carrier detection in XSCID families. This has proved to be a valuable diagnostic aid in singleton families or in situations where mutational analysis is not possible.

Since the X-linked nature of SCID cannot be assumed from the male gender, the identification of a mother, daughter, or sister as a carrier would be of diagnostic significance. Carrier status would be confirmed by demonstration of skewed X-inactivation in T cells with random inactivation in total white cells. This diagnostic modality is particularly useful in singleton families where the affected child is a boy. Kindreds were selected for scrutiny using this method. Carrier status of the mother and/ or sister was then proven through restriction digestion analysis.

This approach was followed for kindreds 3 and 4, as X-linked immune deficiency was likely. Kindred 3 was used a control for this experiment with kindred 4 being the test family.

5.1 Introduction

In humans, as in the majority of animals, the feature that differentiates females from males is that they possess two X chromosomes, whereas males possess one X and one Y chromosome. Dosage equivalence of the number of X linked genes is achieved due to a phenomenon called X chromosome inactivation, which ensures that the number of X chromosomal RNA transcripts are kept approximately equal in both sexes (Kubota *et al* 1999, Li *et al* 1998, Puck and Willard 1998, Plenge *et al* 1997, Puck *et al* 1995).

The inactive X chromosome is condensed and remains inactivated in all progeny of the initial cell. Before implantation, both X chromosomes are active in XX female embryos, but one must be transcriptionally silenced at the time pluripotent cells differentiate along increasingly restricted embryonic lineages. The precise time in development that inactivation occurs is not known, although it is speculated to occur before the 64-cell stage (Lee and Lu 1999, Li *et al* 1998, Puck *et al* 1997, Marahrens *et al* 1998,Voss *et al*

1994).

Skewing of X inactivation can be seen in females carrying mutations in genes critical to cell survival or differentiation, which aids significantly in the determination of carrier status. Examples here would be skewing in B cells in carriers of X-linked agammaglobulinaemia or T cells in X-linked SCID (Kubota *et al* 1999, Goodship *et al* 1991).

Previous studies of unrelated females have shown that X-inactivation patterns follow a gaussian distribution, with most females demonstrating inactivation patterns ranging from 50:50 to 80:20; only about 1% of females exhibit patterns as skewed as 95:5 or greater (Fig 42) (Puck and Willard 1998, URL 16, Plenge *et al* 1997).

One of the factors, which cause difficulty in ascribing to particular cases an X-linked pattern of inheritance, is the occurrence of new mutations either in the mothers or in the affected boys. Potentially therefore, boys from singleton families may have either an X-linked or AR disorder whether or not the classical phenotype is apparent.

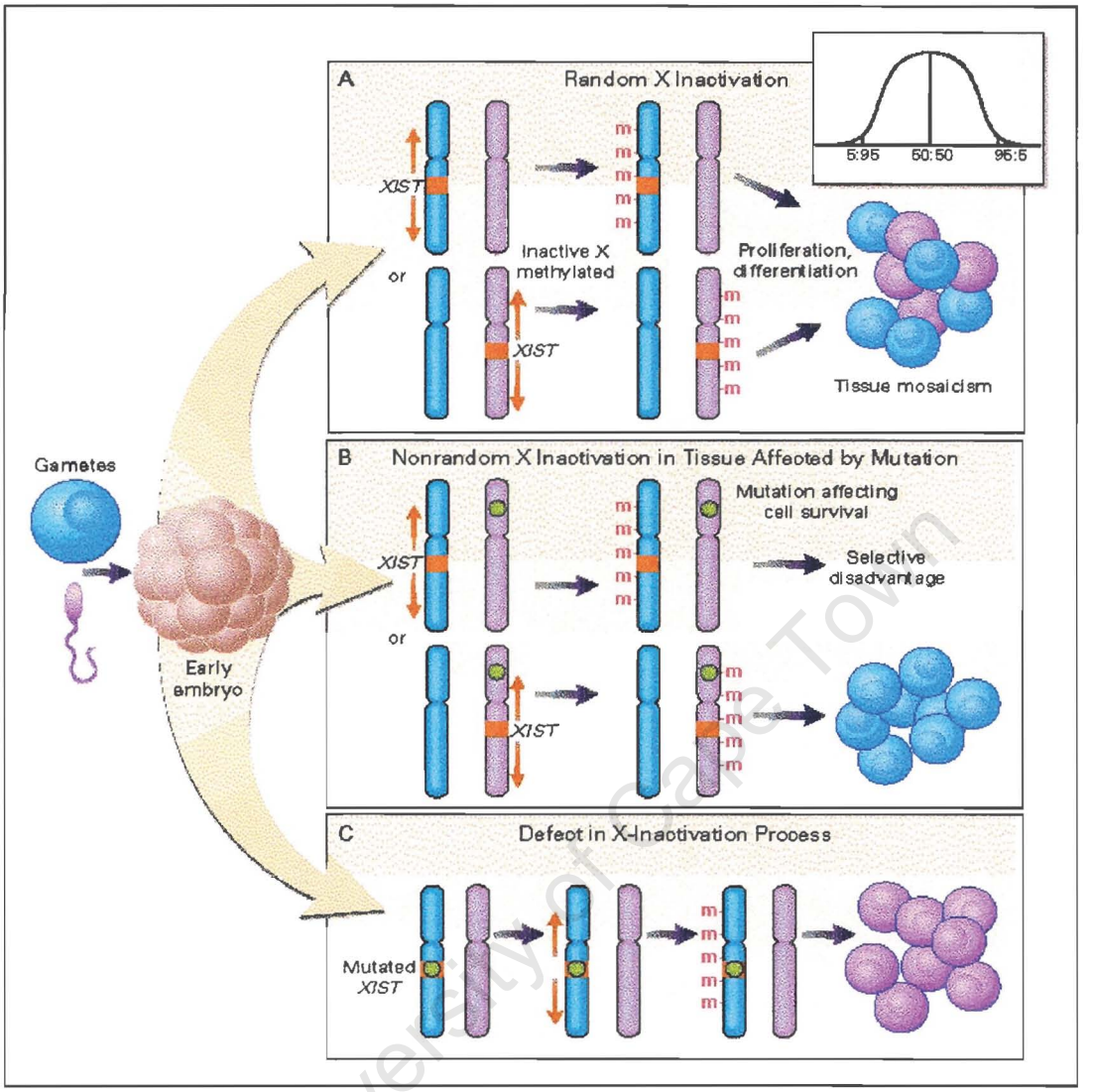


Fig 42 Mechanisms leading to skewed X-inactivation in females. Panel A shows normal, random X-inactivation. X chromosomes are inactivated at random and there is a 50:50 distribution of cells. Panel B shows the mutation bearing X chromosome hindering survival of the cells with the result that this disadvantage leads to a skewed pattern of X-inactivation in mature tissues. In panel C a mutation in *Xist* results in non random selection of the X chromosome that is to be inactivated (Puck and Willard, 1998).

Several methods have been established to examine X inactivation patterns. One such method is by replication timing between the active and inactive X chromosomes. There is one stipulation with this method though; in that one X chromosome has to be distinguishable from the other. Another quantitative technique is the production of a human/rodent hybrid. This technique is very expensive and takes up to several weeks to generate results. The technique most commonly used relies on the differential methylation that exists between the active and the inactive X chromosome. One such locus showing the differential methylation is the human androgen receptor (HUMARA) locus (Kubota et al 1999). Following PCR amplification, methylation-sensitive enzymes such as *Hpa* II are used for the selective digestion of the unmethylated, active X chromosome and the unmethylated HUMARA locus. Cutting sites for the enzymes occur within the first exon of the HUMARA locus, which is in close proximity to a highly polymorphic short tandem repeat (90% heterozygosity) (Fig43)

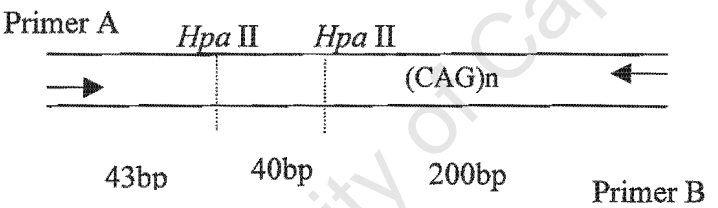


Fig. 43 A schematic diagram of the region amplified in the first exon of the human androgen receptor gene: two Hpa II sites are within 100bp of the polymorphic CAG repeat. Methylation of these sites correlates with X- inactivation (taken from Li *et al* 1998).

5.2 Methods

Peripheral blood was taken from both sets of parents with the fathers' blood used as a positive control. Peripheral blood mononuclear cells (PBMC's) were separated from whole blood using the ficoll method. The PBMC's were pooled and subjected to sheep e-rosetting to allow the T cells to attach to the sheep red blood cells, thereby allowing their separation them from the rest of the PBMC's. The resultant T cell were then coated with rabbit monoclonal antibodies (MoAb's) and scanned using the flowcytometer.

The use of PCR amplification followed by polyacrylamide gel electrophoresis enables identification of the inactivated and active X chromosomes. Amplification of the HUMARA locus will only occur when the X chromosome is inactive, and thus resistant to *Hpa* II digestion as the cutting site for the enzyme contain a methylated base. The assay requires that the restriction digest of the unmethylated DNA is complete as any uncleaved active X chromosomal DNA will be amplified by PCR and may give an inappropriate inactivation pattern. In a female with random X-inactivation both X chromosomes will be amplified equally with the result that the PCR products, when visualised on a gel, will be of similar intensity. In a female with non-random X inactivation, the more frequently inactivated X chromosome will give relatively more PCR product and gel bands of unequal intensity (Fig 44) (Kubota *et al* 1999).

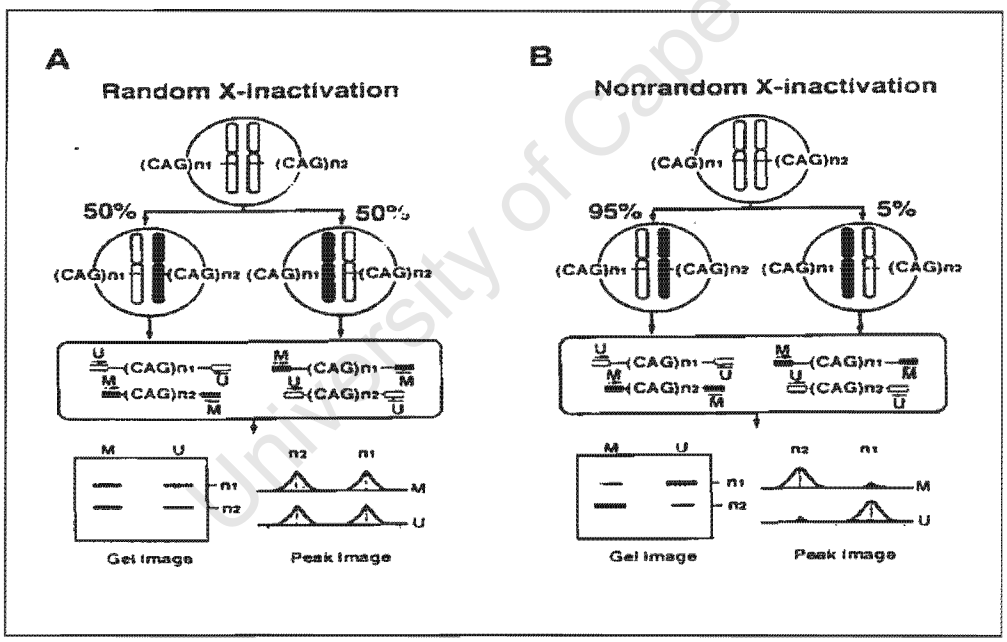


Fig 44 A schematic presentation in a female with random and non-random X inactivation. (A) Random X inactivation. (B) Non-random X inactivation. The white chromosome represents an active X chromosome with an unmethylated allele; the black chromosome represents an inactive X chromosome with a methylated allele (Kubota *et al* 1999).

5.2.1 Peripheral blood mononuclear cells (PBMC's) isolation

5.2.1.1 Protocol for PBMC isolation

Heparinized blood (20ml) was diluted 1:2 with 20ml RPMI in a 50ml Falcon tube The 40ml volume was then gently mixed by inversion several times. Four 15ml falcon tubes, each containing 5ml Ficoll Isopaque, were prepared and 10 ml of the diluted blood was gently layered over the ficoll solution. The tubes were then centrifuged @2200rpm for 15 minutes. The interface layers, containing the lymphocytes or PBMC's, were carefully sucked off and pipetted into clean tubes. These tubes were then filled with RPMI and centrifuged @ 1361rpm for 10 minutes. This wash step was repeated a second time. The supernatant was then decanted gently and the pellet was resuspended in 1ml of RMPI. A 20 µl volume of thus solution was removed to do a white blood cell count. The PBMC's were frozen if they were not to be used immediately and this was accomplished by adding 900µl FCS and 100µl DMSO to the PBMC solution. The cryotubes were then put in a styrofoam container, covered with paper towels, allowed to freeze at -75^{0}C and then placed in liquid nitrogen until they were ready for use. However, if the PBMC's were used immediately they were stored at 4^{0}C.

5.2.2 T cell rosetting with sheep red blood cells

5.2.2.1 Protocol for 5% AET cells

Two black top tubes (#2037) containing 15ml of 1 week old sheep red blood cells were centrifuged @ 2500rpm for 10 minutes. The supernatant was discarded and tubes were filled with cold 1 x PBS. The cells were centrifuged again @ 2500rpm for 10 minutes. The wash step was repeated two more times and the supernatants removed. Of the packed cells, 0.5ml was removed, and pipetted into a clean black top tube that was filled with 9.5ml cold 1x PBS. Tubes were then centrifuged @ 2500rpm @4^{0}C for 10 minutes. The supernatant was then discarded, 2ml of AET added to the pellet, which was then gently mixed. The tubes were then incubated at 37^{0}C for 15 minutes. The cells were washed (3x) with cold PBS and centrifuged for 10 minutes @ 1500rpm at 4^{0}C. The cells were washed in R-10 solution and again centrifuged for 10 minutes @ 1500rpm at 4^{0}C. Of the packed cells, 0.5ml was removed and pipetted into a tube already containing 9.5ml of the

R-10 solution. The tube was gently inverted to mix the cells and stored at 4^0C. The 5% AET cells could only be used for 1 week.

5.2.2.2 Protocol for rosetting

Equal volumes of the separated lymphocytes or PBMC's (10 x10^6/ml) and 5% AET cells were mixed. Cells were centrifuged @970 rpm for 5 minutes and allowed to stand at 4^0C for 90minutes. Black top tubes containing 5ml of Ficoll Isopaque were prepared and the rosetted cells were layered over the ficoll. The tubes were centrifuged @ 1361 rpm for 40 minutes. Cells at the interface were removed as they contained the non-rosetted B cells. Supernatant was removed as the pellet at the bottom of the tube now contained the rosetted cells. The sheep red blood cells, which were rosetted to the T cells, were lysed by the addition of 10ml NH_4CL/Tris and the solution gently resuspended. Tubes were allowed to stand for 10 minutes with inversions every 2 minutes. T cells were washed with 1 x PBS and centrifuged for 10 minutes @ 1500rpm at 4^0C. The pellet was then resuspended in 1ml 1 x PBS and stored at 4^0C for immediate use.

5.3 Flow cytometry

5.3.1 Protocol for flow cytometry

A T cell concentration of 2 x 10^5 cells/ml per tube was used for the flow cytometry readings. Cell concentrations were diluted accordingly in 1 x PBS after cell counts had been taken. The calculated T cell volumes were added to each of three tubes. Antibodies were added as follows: tube 1- 10µl CD45/CD14 mix; tube 2- 10µl of IgG and 10µl of IgG-RD1; tube 3- 10µl CD3 and 10µl CD19. Tubes were vortexed and allowed to stand for 10 minutes. An aliquot (500µl) of Optilyse solution was added to each of the three tubes, vortexed and allowed to stand for 10 minutes. An aliquot (500µl) of 1 x PBS was added to each of the three tubes and vortexed. Samples were now ready to be analysed by flow cytometry.

5.4 DNA extraction and quantitation

5.4.1 DNA extraction

T cell DNA was extracted as per section 2.1.4 (from the wash buffer step onwards).

5.4.2 DNA quantitation

The DNA purity and concentrations were checked as per section 2.1.6 and 2.1.7

5.5 Restriction digestion

5.5.1 Protocol for digestion

The following quantities were added to the reaction mixes per 20µl volume: 2.0µl 10 x Buffer L, 2.0µl Casein, 0.2µl BSA, 9.8µl distilled water, 2.0µl Hpa II (10U/µl) enzyme, 4.0µl DNA (2µg).

Samples were incubated @ 37^0C overnight. Another 1µl Hpa II was added and again the samples were digested @ 37^0C and incubated for another 2 hours. The mixtures were then heat-inactivated @ 95^0C for 10 minutes. Samples were now ready for use.

5.6 PCR

5.6.1 PCR amplification

The reaction mixtures per 1 sample of 50 µl contained the following volumes: 4.0 µl 2.5mM dNTP mix, 4.5 µl 10 x PCR buffer, 1.0 µl 25mM $MgCl_2$, 1.0 µl 20pM ANDR-f, 1.0 µl 20pM ANDR-r, 28.5 µl distilled water, 4.0 µl digested DNA. Control samples with no DNA were added as well. PCR mixtures were overlaid with mineral oil (45µl) to prevent evaporation and condensation of the samples. Tubes were placed in a thermocycler and subjected to a "hot start" by the addition of the 5µl enzyme mix after the first 95^0C for 5 minutes step in the PCR cycle. An enzyme mix contained the following quantities per reaction: 0.5 µl Taq DNA polymerase, 0.5 µl 10 x Promega buffer, 4.0 µl distilled water.

5.6.2 PCR thermocycling

The following PCR program was used for amplification of DNA: initial step 1:-95^{0}C for 5 minutes; cycling step 2:-94^{0}C for 30 seconds, cycling step 3:-58^{0}C for 30seconds, cycling step 4:-72^{0}C for 40 seconds (28 cycles for steps 2-4); and final step 5:-72^{0}C for 7 minutes.

5.7 Visualisation

5.7.1 Preparation of plates

Plates were prepared as in section 2.4.1 and 2.4.2 with a few modifications. Two small plates (150mm x 150mm) were used instead of the 2 large (300mm x 400mm) plates; 0.75mm spacers and comb were used instead of the 1mm spacers and comb.

5.7.2 Preparation of PAGE

Products were visualised using the following gel mixture: 12.0ml 20%Acrylamide (19:1), 4.8ml 5 x TBE, 4.8g Urea, 240µl 10%APS, 7.2ml distilled water and 15µl TEMED. A well-forming comb was inserted at the top of the two plates and side-clamps were used which held the plates firmly together. The solution was gently mixed, taken up in a 20ml syringe and poured in between the two plates that were lying on a flat surface. Polymerisation occurred in about 90 minutes. The gels were then ready for use.

5.7.3 Sample preparation and electrophoresis

An aliquot (5 µl) of the PCR product was mixed with 2 µl of the 6 x loading dye. Combs were removed from the gel to form the wells. The gel was placed into the electrophoresis tank and filled with 1 x TBE buffer. Wells were cleaned of urea with the aid of a syringe. The gel was run at room temperature @ 65W overnight.

5.7.4 Silver stain

The gel was silver stained as per sections 2.63 and 2.6.4

5.7.5 Ethidium bromide stain

9µl of EtBr was mixed in 125ml of 5 x TBE

The gel was gently lifted off the plate and soaked in the EtBr solution for 30 minutes and then visualised under the UV transilluminator.

5.8 Results and discussion

T-cells were isolated from peripheral blood by the E-rosetting technique, which is very specific for T-cells. The T-cell isolates were subjected to flow cytometry using labelled antibodies to T-cell (CD3) and B-cell (CD19) surface antigens. B –cell contamination was found to be less than 5%, while T cell numbers ranged from 50-72%. The balance was presumed to be due to cell debris as the X-inactivation gel for family 3 indicated much higher predominance of T cells.

The A_{260}/A_{280} ratio in the T cell DNA samples ranged from 0.63 to 1.5 most likely indicative of protein contamination in some of the isolates, low spectrophotometer readings could also have been a problem here as very small amounts of DNA were isolated. Further purification was discounted, as this was not required for the PCR applications. T cell DNA was used to prove/disprove X-inactivation and T cell skewing.

5.8.4 X-inactivation pattern on PAGE gels

Determination of X-inactivation in woman, through analysis of the HUMARA locus can only be carried out when they are heterozygous for repeat lengths at this locus. This allows for separation of the PCR amplified allele products and an assessment of the intensity of staining. A random pattern of X-inactivation would thus show no change in the relative intensities of the two PCR products after digestion with the methylation sensitive enzyme, *Hpa* II. Conversely, non-randomly X-inactivation is indicated by a decrease in the relative intensity of one of the allele products after restriction digestion.

Given these scenarios, it is clear that the mother of patient 3 shows non-random and total skewing of her X chromosomes and must be a carrier for X-linked SCID (Fig 45). She shows two distinct PCR products of fairly equivalent staining intensities, before *Hpa* II digestion and a complete loss of the upper band following restriction digestion. It is clear from the gel that the X-chromosome carrying the smaller HUMARA repeat size also carries the mutant IL2 gamma chain. One cannot comment on the X-inactivation status of the daughter (Fig45 lanes 6 and 7) in this family as she is not informative at the HUMARA locus, as both alleles have repeats of the same size. It is clear however, that she has inherited the mutant X-chromosome

she has inherited the mutant X-chromosome from her mother (Fig45 lane9) and must be a carrier. Both the father (Fig45 lanes 2 and 3) and son (Fig45 lane 4 and 5) in this family show the absence of PCR product following *Hpa* II digestion. This is to be expected as their X chromosomes are fully active and being unmethylated will have the HUMARA repeat template destroyed by *Hpa* II digestion.

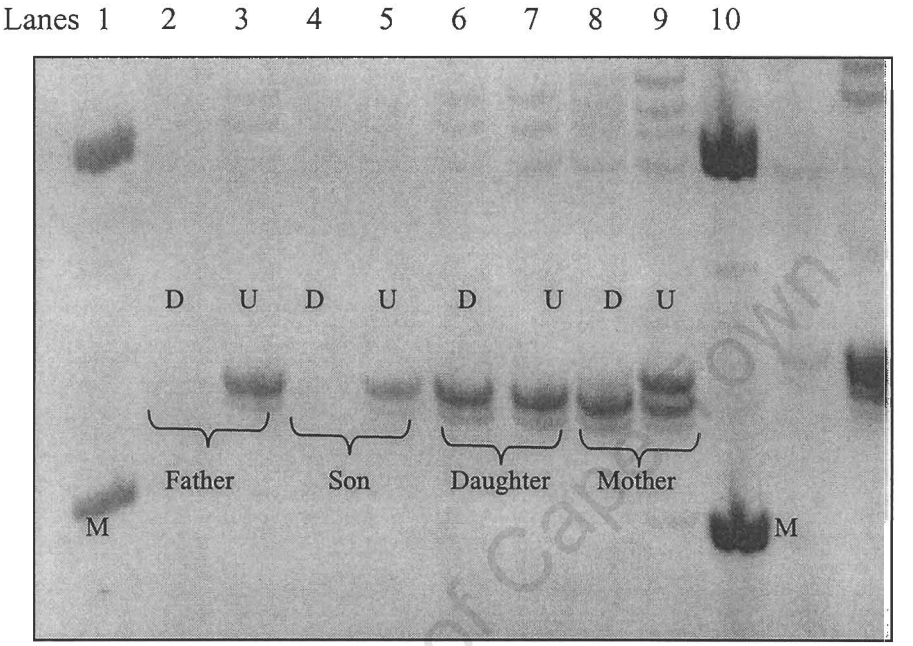


Fig 45 X-inactivation PAGE gel of family 3 using genomic DNA.The genomic DNA of P3 and his father were used as control samples (lanes 2,3,4 and 5). The DNA sample of the daughter was run in lanes 6 and 7. The genomic DNA of the mother of P3 was run in lanes 8 and 9. (D-digested, U-undigsted, M- marker)

Different results were obtained for the mother of patient 4 (Fig 46 Lanes 2 and 3)where this person shows slight partial skewing in her X-inactivation pattern, which is often encountered in females. This result was expected as X-linked SCID was an unlikely candidate disorder in this family, where the phenotype was more suggestive of a defect in the IL-7 signalling system. T-cell DNA from two control females was also included . The first of these, control 1 (Fig46 lanes 6 and 7), on visual inspection shows slight partial skewing, while the other (Fig46 lanes 8 and 9) is not informative and one cannot comment of her X-inactivation status.

Lanes 1 2 3 4 5 6 7 8 9 10

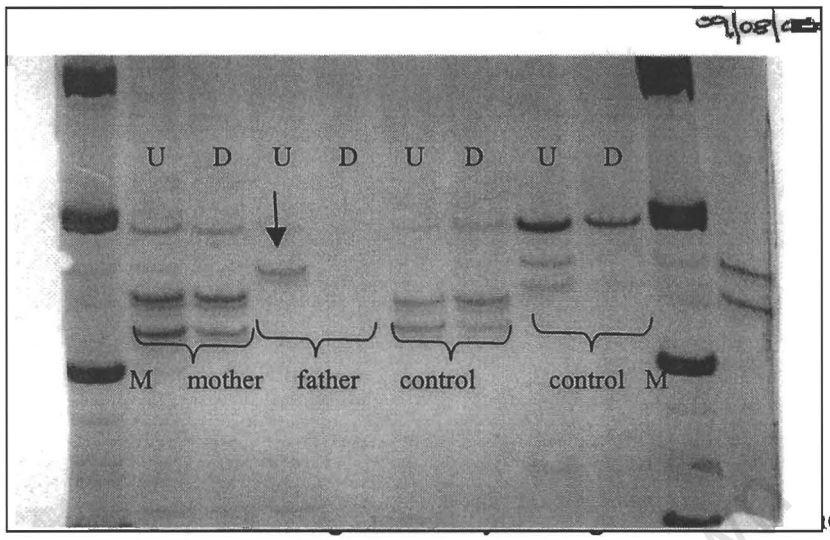


Fig 46 ...e DNA of the father was used as a male control sample (lanes 4 and 5). The DNA sample of the other female controls were run as well (lanes 6, 7, 8 and 9). The T cell DNA of the mother of P4 was run in lanes 2 and 3. (D-digested, U-undigested, M- marker)

5.9 Conclusion

The DNA investigations detailed in this study were carried out on samples derived from 5 patients seen at Red Cross Children's Hospital during the period of 1985-1999. All had the classical severe infantile SCID phenotype. Four of these subjects were male and were thus screened for mutations in the gene for the common γc of the IL2 receptor as this is gene most often implicated in X-linked SCID. An indirect assessment of possible γc defects was sought in the mothers of the affected boys, by looking for skewing of X-inactivation.

Three of the 4 affected boys were found to carry mutations in the gamma chain of the IL2 receptor; these being E68K in exon 2, R224W in exon 5 and R285Q in exon 6. None of these mutations are novel as they already appear in the SCID mutation database. PCR assays for each of these mutations have been developed and it is now possible to do carrier screening and offer genetic counseling to the affected families. In the absence of these mutation data, prenatal testing in these families would have been limited to gender selection or immunological assays of foetal blood lymphocytes obtained by cord-centesis.

The SCID phenotypes in the two remaining patients differed from that of the X-linked cases, in that one was a girl and the other, although being male, had B-cells. The most likely candidate genes in these subjects were those for RAG1/2, or either of the IL7 and IL7α receptor genes, respectively. Support for the candidature of these genes was sought in each case by looking at the inheritance patterns of microsatellite markers. In each case the patterns obtained were compatible with defects in the suspected genes ie. none of these genes could be excluded as candidates by the microsatellite data.

DNA from the female patient with a suspected RAG1/2 defect was submitted to a research laboratory in Italy, where a single base insertion (A) was shown to occur in the RAG1 gene at position 859 of the cDNA and within codon 259. Frameshift mutations are known to introduce premature termination sites, and our patient was almost certainly either, deficient in the RAG1 gene product or manifest only an inactive truncated gene product.

The final patient in this series has the $T^-B^+NK^+$ phenotype and is likely to have a defect in either of the IL-7 or IL-7 receptor genes. Two patients with this phenotype have recently been shown to carry mutations in the gene for the alpha chain of the IL-7 receptor (Puel *et al* in 1998). Unfortunately time constraints precluded a search for mutations in these genes.

The studies reported in this thesis are unable to comment on the genotype / phenotype relationship in X-linked SCID as the number of patients investigated is small and all presented with the severe infantile form. Milder or "leaky" phenotypes have been described but none of the SCID patients presenting to Red Cross Hospital have fallen into this category. What this study has done is to set in place the DNA methodologies required for mutation detection and assay. Hopefully the difficulty of diagnosis and prenatal diagnosis in these rare familial conditions will have been lessoned by an approach, which now allows for the identification of the underlying mutation and the accurate detection of carriers. This is not a trivial approach to diagnosis and success is not guaranteed, but for those families in whom mutations are identified, it offers certainty of diagnosis and the chance to have unaffected children.

5.10 Recent ethical development and DNA testing

The success of the Human Genome Project in revealing the genetic blueprint for human life has raised many ethical questions eg, who has the right to access each person's genetic information and can our own genetic information be kept private, while also benefiting from medical advances?(URL10)

The most worrisome ethical issues will stem from diagnostic tests for diseases that have no cure. Not only will patients be faced with the emotional impact of such knowledge, but they may also worry that insurance companies or employers will find them less desirable (Baker 1999).

Information about these "problem" genes can be more of a burden to some people as many have been refused health insurance or dropped from their health plans. In other cases, they have been told that medical expenses for their genetic condition will not be covered. In still others, they have been told that their children will not be covered because they are at risk for inheriting genetic diseases. The number of such cases may increase as genetic testing becomes more common

The fear of being discriminated against may lead people to reject genetic testing even when it could help diagnose, prevent or treat a health problem. They may be fearful that the information would be used against them. If that happens, then all the benefits of genetic research could come to nothing.

One of the first-ever successes in gene therapy was achieved by researchers in France who have treated four boys with severe combined immunodeficiency syndrome for more than a year. Treating such a disease is a positive use of genetic engineering, but what worries some is the prospect of genetic engineering to achieve a specific eye or hair colour, height, attractiveness, or intelligence.

Knowing your genetic profile could help to suggest what health-related behaviours you should follow. It could tip you off to have frequent checkups for genetic conditions for which you are at risk. It could help you plan your life so that you avoid behaviours and substances that trigger diseases.

It is possible that people who learn their genetic profile will limit the choices they make based on such fears. People may choose not to marry or build a career because they believe that they are doomed by their "problem" genes. The expectation of disease may ruin their enjoyment of life.

A big question is whether children should be told information about the genes they carry or, if so, at what age they should be told. It can be difficult for children to understand some of the important facts about genetics, such as the difference between a risk and a sure thing. There is the possibility that they will misunderstand what they are told.

The maturity of the child also plays a role in whether he/she will be mature enough to cope with the information. However, many parents want to know if their children are at risk for genetic disorders. That way, the parents can be prepared and get treatment for the children in time. They also could make lifestyle choices to avoid triggering the disorder in their child. Parents will have to decide whether and how to share genetic information with their children. Society also may have some say in how genetic information is shared with children, through standards, laws, and regulations that are developed.

Quite obviously genetic research doesn't have all the answers, but it does open up some interesting questions.

Informed consent was obtained from all subjects. The following documents were presented to the relevant individuals.

RED CROSS CHILDREN'S HOSPITAL

REQUEST FOR DNA TESTING

NA Diagnostic Laboratory

Dept. Chemical Pathology
ICH Building,
Rondebosch, 7700

Tel: (021) 6585223 Fax: (021) 6891287

Blood volumes : Children 2-3ml in and EDTA tube
Adults 5-10 ml in an EDTA tube

Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB
Keep blood in fridge at 4°C until able to send to laboratory

Please DO NOT send specimens on ice or frozen.

Please fill in all the information requested:

Surname:_____ First Name(s):_____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name: _____

Medical Aid: _____ Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Day:_____ Month:_____ Year: _____

Number of children: _____

Ethnic Origin : (please indicate ancestry of both your mother and father)_____

Contact Address: _____ Town: _____ Fax: Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax: Tel: _____

Hospital or Address: _____ Town: _____ Fax: Tel: _____

Reason for Referral (Clinical diagnosis):.....

.....

Testing required: ☐ Galactosemia ☐ Spinal Muscular Atrophy ☐ Cystic Fibrosis (☐F508)

☐ Lipoprotein Lipase ☐ McArdles ☐ MCAD ☐ Other.....

Pedigree Drawing

CONSENT FOR DNA ANALYSIS AND STORAGE

1 I, _____ , request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (*Delete where not applicable*) might have inherited a disease-causing mutation in the gene for:_____

2 I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (*Delete where not applicable*) :

3 I request that no portion of the sample be stored for later use. ☐ (*Mark if applicable*)

Or

I request that a portion of the sample be stored indefinitely for (*Delete where not applicable*):

(a) possible re-analysis

(b) analysis for the benefit of members of my immediate family

(c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. The results of the analysis carried out on this sample of stored biological material will be made known to me,

via my doctor, in accordance with the relevant protocol, if and when available.

In addition, I authorise that they may be made known to: (*Delete where not applicable*) :

- other doctors involved in my care
- the following family members:

  _____
- other:

  _____

  _____

5 I authorise/ do not authorise my doctor(s) (delete where applicable) to provide relevant clinical details to the genetic Clinic, Red Cross Children's
6. Hospital

I have been informed that:

(a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.

(b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
(c) the genetics laboratory is under an obligation to respect medical confidentiality .
(d) genetic analysis may not be informative for some families or family members.
(e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
(f) where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

_____ DATE: _____

Patient signature _____ **Witnessed consent** _____

For Laboratory use only:

DNA number: _____ *Vol.Blood:* _____*(ml)* *Other:* _____

Date Received: Year: _____ *Month:* _____ *Day:* _____ *Computer Index No:*_____

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URL1:
http://rex.nci.nih.gov/PATIENTS/INFO_TEACHER/bookshelf/NIH_immune/html/imm01.html
URL 2: http://www.ipopi.org/idf/pfhandbk/page25.html
URL 3: **http://www.netpage.org/macey/who02.html**
URL 4: **http://www.nhgri.nih.gov/DIR/GMBB/SCID/IL2RGbase.html**
URL 5:
http://www.bio.davidson.edu/Biology/Cours...tudents/spring2000/starr/yourprotein. html
URL6: http://www.pds.med.umich.edu/users/frank/MIN.html
URL7: http://helix.biology.mcmaster.ca/brent/node1.html
URL8: http://www.cpro.dlo.nl/cgn/molgen/mcrosats.html
URL9: http://www.yale.edu/opa/ybc/v27.n5.news.08.html
URL 10: http://www.bio.umass.edu/biochem/mydna/links.html
URL 11: http://bioinf.uta.fi/RAG1base/index.html

5.12 Appendix 1

Mutational Analysis

Gamma chain primers

Exon	Annealing Temp (^{0}C)	Size (bp)	Primers	5' → 3'
1	55	209	Forward	CAGGTTCCTGACACAGACAGAC
			Reverse	CCACAGCCACCCTTCTCAC
2	55	253	Forward	CATTTCTCTTTCCCTCCCTGC
			Reverse	GGTCTCTGATCCAACCCACCT
3	55	259	Forward	TTCCTCTTCCTTCCAACCTTT
			Reverse	TCCCACAGTATCCCTGGTCTC
4	55	216	Forward	CAGGGGAATGACCACATATGC
			Reverse	TCTTGGCCTTAGCTGCTACATTC
5	55	237	Forward	AGACACCCACTTTCCCTCATCC
			Reverse	GTTGGGCTCATGGATTGGGT
6	55	202	Forward	AGGGGCACAACAAATATAAGGTC
			Reverse	CCTCTGCTATTGTCAGCTACCG
7	55	164	Forward	GCTTTAACCTATGTGCTCCTGC
			Reverse	CAGGCAGTTGGCAGTTGATAG
8	55	299	Forward	CTAATTGACCCCTGACCTGGAC
			Reverse	AGGTGGGTTGAATGAAGGAAA

Appendix2

Microsatellite Primers

Marker	Annealing Temp (^{0}C)	Size (bp)	Primer	5' → 3'
DXS983	57	173-185	Forward	CACACTGCATTAAATCCTCG
			Reverse	CAAGTTACCCTACCTGCCTC
DXS986	57	149-173	Forward	CCTAAGTGCTCATCATCCCA
			Reverse	AGCTCAATCCAAGTTGCTGA
D8S1760	57	139-159	Forward	CCTCTAACACACTCGGGCA
			Reverse	CCCAACTAGCCTGGCTCTG
D8S1805	57	191-203	Forward	ACCTCTACTCTTGTATTGGGGTT
			Reverse	TGGCTCTCACAGGTCTCC
D8S541	55	194-228	Forward	CTGCCTGTATTACTCAGGGT
			Reverse	CCTCAGCCTGTAGGTGG
D8S548	55	228-248	Forward	CTACCTCATGCAAGGCCA
			Reverse	CATTGAGCACACCCTCAGT
D5S1994	55	109-127	Forward	CTCAAAGAGTGCATAACCCA
			Reverse	TTAAAGATGAAACCCA
D5S395	57	189-215	Forward	GCAGATGGAAAACACCACTT
			Reverse	ACCTGCTGCTGGAAGATTAC
D11S4102	55	142-174	Forward	CACCACTGGGTACTGCCATC
			Reverse	GCTAAATCCTGGAAAGCCCTG
D11S935	55	196-208	Forward	TACTAACCAAAAGAGTTGGGG
			Reverse	CTATCATTCAGAAAATGTTGGC

Mutational Analysis

Low salt buffer (TKM1)

10mM TrisHCl pH 7.6

10mM KCl

10mM $MgCl_2$

2mM EDTA (disodium salt)

0.121g Tris, 0.075g KCl, 0.203g $MgCl_2$ and 0.075g EDTA were dissolved in 100ml distilled water with the pH adjusted to 7.6 with HCl.

High salt buffer (TKM2)

10mM TrisHCl pH 7.6

10mM KCl

10mM $MgCl_2$

0.4M NaCl

2mM EDTA (disodium salt)

0.121g Tris, 0.075g KCl, 0.203g $MgCl_2$, 2.338g NaCl and 0.075g EDTA were dissolved in 100ml distilled water with the pH adjusted to 7.6 with HCl.

6.0M Sodium Chloride

35.06g NaCl was dissolved in 100ml distilled water. This is a saturated solution.

10% Sodium dodecyl sulphate

10g SDS was dissolved in 100ml distilled water.

10 x TAE buffer

48.4g Tris base, 11.42ml glacial acetic acid and 20ml 0.5M EDTA pH 8.0 were dissolved in a total volume of 1000ml distilled water.

6 x DNA loading buffer

0.25% Bromophenol blue

30% Glycerol

0.25g Bromophenol blue and 30ml glycerol was dissolved in a total volume of 100ml distilled water and stored at 4^0C.

TE Buffer

10mM TrisHCl pH 7.6

1mM EDTA pH 8.0 (disodium salt)

1ml 1M Tris pH 7.6 and 200 µl 0.5M EDTA pH 8.0 were dissolved in 100ml distilled water.

7.5M Ammonium acetate

57.81g Ammonium acetate was dissolved in 100ml distilled water, aliquoted and stored at -20^0C.

Cell lysis buffer pH 8.0

0.32M Sucrose

5mM $MgCl_2$

10mM TrisHCl pH 7.6

1% Triton X-100

109.5g Sucrose, 5ml of 1M $MgCl_2$ and 10ml of 1M TrisHCl was dissolved in 990ml of distilled water, autoclaved and while the buffer was warm, 10ml Triton X-100 was added and the mix was stored at 4^0C.

Ethidium Bromide

10mg Ethidium Bromide was dissolved in 10ml distilled water.

6.0M Guanidinium chloride

57.32g Guanidinium chloride was dissolved in 100ml of distilled water and stored at 4^0C.

10mg/ml Proteinase K

100mg Proteinase K (from *Tritirachium album*) (Rouche) was dissolved in 10ml distilled water, aliquoted and stored at 4^0C.

20% Sarkosyl solution

20g N-Lauroysarkosine (sodium salt) (Sigma) was dissolved in 100ml distilled water, aliquoted and stored at -20^0C.

1ml 1M Tris pH 7.6 and 200µl 0.5M EDTA pH 8.0 was dissolved in 100ml distilled water.

Wash buffer

10mM NaCl

10mM EDTA (disodium salt) pH 8.0

0.58g NaCl and 3.72g EDTA were dissolved in 1000ml distilled water and stored at 4^0C.

0.8% Agarose gel

0.8g (w/v) low melting point agarose (Whitehead Scientific)

100 ml 1 x TAE

2.5 *ul* Ethidium Bromide

Preparation of high molecular weight marker V111

2 µl marker V111

2 µl 6 x loading buffer

6 µl distilled water

6 x Loading dye for agarose gel electrophoresis

0.25% Bromophenol blue

30% Glycerol

0.25g Bromophenol blue and 30ml glycerol was dissolved in a total volume of 100ml distilled water and stored at 4^0C.

Deoxynucleotidetriphosphates (dNTP's)

The Promega dNTP set (dATP, dGTP, dCTP, dTTP) was used, each with a concentration of 100mM, pH 7.5. Each dNTP was diluted to 10mM in water and stored at -20^0C.

$MgCl_2$ (Sigma)

A 1.00M $MgCl_2$ was diluted to 25mM in distilled water.

Primer/DNA dilution buffer

10mM Tris pH 7.6

1mM EDTA

1ml 1M Tris pH 7.6 and 200 µl 0.5M EDTA pH 8.0 were dissolved in 100ml distilled water. The solution was filtered, aliquoted and stored at -20^0C.

10 x PCR buffer

The Promega stock solution was used.

Taq DNA polymerase

The Promega Taq was used (10U/ul)

Primers

All primers were diluted in Primer dilution buffer. Stock solutions of 500pM was made of each primer and stored at -75^0C. Working stocks of 20pM was used in the PCR reactions and stored @ -20^0C.

DNA template

Working stocks (0.1µg/µl) of the isolated DNA was made using DNA dilution buffer.

Stock plate glue solution

50µl γ-Methacryloxypropyl trimethoxysilane (Sigma) added to 100ml absolute ethanol

70% ethanol

70ml absolute ethanol and 30ml distilled water

Siliconizing agent

Dimethyl dichlorosilane solution (BDH)

5 x TBE buffer

54.0g Tris base, 27.5g Boric acid and 20ml 0.5M EDTA (pH8.0) was dissolved in a total of 1000ml distilled water.

SSCP loading dye

10mM NaOH

95% Formamide

0.05% Bromophenol blue

0.05% Xylene Cyanol

9.5ml Formamide, 10µl 10M NaOH, 250µl 2% Bromophenol blue and 250 µl 2% Xylene cyanol were all combined.

40% Acrylamide stock (39.6: 0.4)

39.6g Acrylamide (BDH Laboratory Supplies) and 0.4g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on a magnetic stirrer in 100ml of distilled water.

30.8% Acrylamide stock (30 : 0.8)

30g Acrylamide (BDH Laboratory Supplies) and 0.8g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on the magnetic stirrer in 100ml of distilled water.

Ultra pure urea (Promega)

10% APS (Sigma)

0.1g Ammonium Persulphate (APS) dissolved in 1ml distilled water.

N,N,N',N'-Tetramethylethylenediamine (TEMED) (Promega)

Ammoniacal silver solution

0.1% $AgNO_3$

0.1% NaOH

0.1% NH_4OH

2g NaOH was dissolved in just less than 2 litres of distilled water, then 8ml of NH_4OH was added and covered while stirring gently to avoid evaporation of the NH_3 Immediately before use 2g $AgNO_3$ was added while stirring.

Developing solution

0.005% Citric acid

0.02% Formalin (34% stock)

0.1g of the citric acid and 1.176ml of the formalin was dissolved in 2 litres of distilled water

Silver nitrate solution

2g $AgNO_3$ was dissolved in 2 litres of distilled water

Formaldehyde solution

30g of NaOH, 0.2g NaBH4 and 8ml formaldehyde was dissolved in 2 litres of distilled water.

Sodium carbonate solution

15g Na_2CO_3 was dissolved into 2 litres of distilled water.

Sequence Analysis

40% Sucrose (w/v)

10g of sucrose was dissolved in 25ml of distilled water, filtered, aliquoted and stored at -20^{0}C.

1.0% Agarose gel

1.0g (w/v) low melting point agarose (Whitehead Scientific)

100 ml 1 x TAE

2.5µl Ethidium Bromide

30% Acrylamide (29:1)

29g of Acrylamide and 1g of NN'-methylenebis-acrylamide dissolved on the magnetic stirrer in 100ml of distilled water.

X-inactivation

Heparin (1000U/ml)

Preservative free Heparin 1000U/ml (Sigma) was diluted 1:10 in sterile distilled water before use and 100µl (1000 U) used per 10ml blood.

Sterile Heparin tubes

100µl heparin was pipetted into each tube to prevent clotting of the blood samples.

RPMI medium

10.44g RPMI-1640 medium, 4.766g Hepes (20mM) and 2.0g $NaHCO_3$ were dissolved in 1000ml distilled water with the pH adjusted to 7.3 with 1N NaOH or 1N HCl, filter sterilised through 0.22 and 1.2 μM size filters and aliquoted in 100ml volumes in sterile bottles. Sterility was checked by incubation at 37^0C overnight. A colour change was indicative of bacterial contamination. The stocks were stored at -20^0C and the bottles in use were kept at -4^0C.

Antibiotics

Penicillin 600mg (1×10^6 U) (Novo Nordisk) reconstituted with 5ml distilled water

Streptomycin 1g/3ml (Novo Nordisk)

0.1ml Penicillin and 0.3ml Streptomycin were added to 9.6ml RMPI and was only allowed to be stored at 4^0C for 1 week. 1ml of this solution was added to approximately 90-100ml of RPMI, giving an approximate 1:100 dilution and final concentrations of 100μg/ml for each antibiotic.

Ficoll Isopaque (formula of Boyum, 1974)

Ficoll 400 (Pharmacia)

Sodium merizoate solution (75% Isopaque w/v in 20ml vials) or Conray 420

56.47g Ficoll was dissolved in 750ml distilled water, covered with a black plastic bag and 128.64ml Isopaque or 152ml Conray 420 added in a total volume of 1000ml distilled water. The S.G. of this solution should be 1.077 on testing with a hydrometer and was adjusted with the appropriate reagents where necessary.

Dimethyl sulfoxide (DMSO)

Feacal calf serum (FCS)

A 100ml volume of feacal calf serum (Highveld Biological) was heat inactivated at 56^0C for 30 minutes, aliquoted into 10ml sterile tubes and stored at -20^0C.

5N NaOH

20g NaOH dissolved in 100ml of distilled water

4% Aminoethylisothiuranium bromide hydrobromide (AET)

0.4g AET dissolved in 10ml distilled water adjusted to a pH of 9 with 5N NaOH

RMPI-HEPES-BICARB with 10% FCS (R-10)

No antibiotics was added to the working stock.

90ml RPMI was mixed with 10 ml FCS

CD45/CD14

Supplied by Coulter (Mo2-RD1/KC56-FITC) as a liquid murine monoclonal antibody

IgG

Supplied by Coulter (MsIgG1-FITC) as a liquid murine monoclonal antibody

IgG-RD1

Supplied by Coulter (MsIgG-RD1) as a liquid murine monoclonal antibody

CD3

Supplied by Coulter (IgG1-FITC) as a liquid murine monoclonal antibody

CD19

Supplied by Coulter (B4-RD1/B4-FITC) as a liquid murine monoclonal antibody

Optilyse ® C

Supplied by Immunotech

1 x PBS

40g $NaCl_2$, 1g KCl, 5.75g Na_2HPO_4 and 1g KH_2PO_4 were dissolved in 4.8litres of distilled water (pH 7.2 - 7.5) and made up to 5litres. The solution was then filtered through a 0.22µ filter.

10 x Buffer L

Supplied by Promega in a 1.5ml volume

Hpa II enzyme

Supplied by Promega with a concentration of 10U/µl in a 20ul volume

Casein

0.0076g Casein (Sigma) diluted in 7.6ml 10mM Tris pH 8.0 and had a concentration of 1mg/ml. The reagent was then aliquoted and stored @ -20^{0}C.

Bovine Serum Albumin (BSA)

Supplied by Promega to a final concentration of 0.1mg/ml

Androgen primer forward (ANDR-f)

Manufactured by Whitehead Scientific. Primers were diluted with primer dilution buffer to a final working concentration of 20pM/µl and stored at -20^{0}C.

5' TGC GCG AAG TGA TCC AGA ACC-3'

Androgen primer reverse (ANDR-r)

Manufactured by Whitehead Scientific. Primers were diluted with primer dilution buffer to a final working concentration of 20pM/µl and stored at -20^{0}C.

5' TGG GCT TGG GGA GAA CCA TCC -3'

DNA template

Working stocks (0.5µg/µl) of the isolated DNA was made using DNA dilution buffer.

20% Acrylamide (19:1)

19g Acrylamide (BDH Laboratory Supplies) and 1.0g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on the magnetic stirrer in 100ml of distilled water.

10 x PNK buffer

Supplied by the USB Corporation

-40 Primer

The primer had a stock concentration of 5μM

5' -3'

T4 PNK

The enzyme was supplied by the USB Corporation and had a stock concentration of 30U/μl. A working stock of T4 PNK was used at a concentration of 5U/μl.

[γ^{32}P] ATP

The radioactive [γ^{32}P] ATP stock was 150mCi/ml > 5000Ci/mmol and was supplied by Amersham PB15068.

M13 DNA single stranded

Supplied by USB Corporation with a stock concentration of 0.2μg/μl

Sequenase KIT

A 1:2 dilution of the sequenase was made before with pyrophosphate and briefly spun.

40% Acrylamide stock

250.0g Acrylamide (BDH Laboratory Supplies) and 13.1g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on a magnetic stirrer in 657ml of warm distilled water, stored in a brown bottle @ 4^0C.

6% Polyacrylamide working solution

A volume of 15.0ml 40% Polyacrylamide stock solution, 42.0g Urea, 10.0ml 10 x TBE and 33ml of water were mixed to give a final volume of 100ml of the working solution. This mix could only be stored for 2 weeks @ 4^0C.

10 x TBE

108g Tris base, 55.0g Boric acid and 40ml of 0.5M EDTA (pH 8.0) dissolved in 1000ml of distilled water.

Developer solution

Stop solution

2% acetic acid

Fixer solution

A 1:3 dilution was made and the pH and silver content was checked.

Appendix 3

Molecular Weight Marker VIII (Roche Biochemicals)