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THE SCID/SCID MOUSE MUTATION: A MODEL FOR THE STUDY OF THE ROLE OF THE MICROFLORA IN THE ONTOGENY OF THE IMMUNE RESPONSE

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INTRODUCTION

The SCID mouse mutation, first described by *Bosma* and his colleagues (1983), was derived from a breeding pair of inbred C.B-17 Icr (C.B-17) mice and is a valuable mouse model for studying the ontogeny of the immune system. The C.B-17 mouse is a congenic partner of the BALB/cAnIcr strain, differing from it only by a portion of chromosome 12, that was derived from the C57Ka strain, which carries a recessive gene for diabetes. The Ig heavy chain, found on the same chromosome, and TCR genes have been shown to be present by northern blot analysis, yet, southern blot analysis of SCID/SCID mouse bone marrow and foetal liver hybridomas showed no Ig heavy chain rearrangement (*Schuler* and *Bosma*, 1989; *Schuler* et al., 1990). The mutant locus implicated in immunodeficiency followed autosomal recessive gene control, was mapped close to mahoganoid and centromeric to the Ig lambda light chain locus on chromosome 16 and had low frequency of Ig gene and T-cell receptor gene rearrangements (*Bosma*, 1989; *Bosma* et al., 1983, 1989; *Schuler* and *Bosma*, 1989). This new strain was deficient in all major immunoglobulin classes and T-cell activity, with the ability to accept allografts and/or xenografts. Myeloid and erythroid lineages are apparently unaffected by the mutation, with near normal numbers of spleen colony forming units (CFU-S) and granulocyte-macrophage colony forming units (GM-CRU) (*Phillips* and *Fulop*, 1989). Macrophage and natural killer (NK) spleen cell activity are normal (*Kumar* et al., 1989). Macrophage activation can occur in a T cell-independent manner and may constitute an important model to unravel the mechanism of 'natural' resistance to infection (*Ansell* and *Bancroft*, 1989; *McCune* et al., 1988).

Penetrance of this mutation is not complete (*Caroll* et al., 1989) with 41% of SCID/SCID mice older than 9 months and 15% of younger SCID/SCID mice producing a limited clonal diversity of serum Ig as shown by isoelectric focusing (*Gibson* et al., 1989). These mice are termed 'leaky' mice; the degree of 'leakiness' is directly related to the number and kind of contaminants in the microflora, therefore, this animal is a good model for demonstrating the role of the microflora in the ontogeny of the immune response.

Nishikawa and colleagues (1989) have shown *in vitro* that the pluripotent stem cells are committed to produce lymphoid cells; their defect appears prior to expression of cytoplasmic or surface immunoglobulin (supporting chromosomal defect) with arrest in the pre-pre-B stage, thus, Ig-cells bearing Thy-1 and low levels of the leukocyte common antigen B220 can be detected in the spleen and bone marrow, although they are considerably reduced in number (*Hardy* et al., 1989; *Nishikawa*

et al., 1989).

While pre-B cells are undetectable in SCID/SCID tissues, they can be generated from SCID/SCID bone marrow by long-term Witlock-Witte/Dexter culture methods in the presence of IL-7 (*Lee* et al., 1989; *Nishikawa* et al., 1989). The frequency of responding cells and the expansion potential of pre-B colonies produced by these methods is severely limited, and they have limited survival (*Nishikawa* et al., 1989). Since SCID/SCID mouse stromal cells are able to support bone marrow and foetal liver transplants, a micro-environmental defect that suppresses B-cell differentiation is ruled out (*Nishikawa* et al., 1989).

T-cell development in SCID/SCID mice arrests at a point equivalent to 14-15 days of gestational age (C.B-17 control) with the majority of SCID/SCID thymocytes expressing double negative CD4 and CD8 with positive Thy-1 (*Habu* et al., 1989). Forty to sixty percent express interleukin 2 (IL-2) receptors and will divide in response to recombinant IL-2 (*Hardy* et al., 1989; *Nishikawa* et al., 1989). *Shores* et al. (1990) introduced normal bone marrow cells into TcR^- SCID mice and these gave rise to TcR^+ cells within the SCID thymus and promoted the differentiation of SCID thymocytes into $CD4^-CD8^+$ and $CD4^+CD8^+$ TcR^- cells.

Kumar and colleagues (1989) demonstrated that natural killer cell differentiation in the SCID/SCID mouse spleen cell population is unaffected by the mutation with normal numbers of NK progenitors in the marrow giving rise to functional $NK2.1^+$ $ASG1^+$ cytotoxic cells which do not express T-cell markers. Mature NK cells, but not their progenitors have been detected in SCID/SCID spleen. It was concluded that either NK cells were derived from T cells or they diverged from a common progenitor in the marrow prior to the expression of the SCID/SCID phenotype (*Kumar* et al., 1989).

Garni-Wagner et al. (1990) investigated the relationship between NK cell and T-cell progenitors using the thymus of SCID/SCID mice. Two populations of cells have been identified in the hypocellular SCID/SCID mouse thymus. Eighty percent of the cells are $Thy\text{-}1^+$, $IL\text{-}2R(7D4)^+$, $J11d^+$ (T progenitors), $CD3^-$, $CD4^-$, $CD8^-$, and twenty percent of the cells are $IL\text{-}2R^-$, $J11d^-$, $CD3^-$, $CD4^-$, and $CD8^-$; NK activity is found in the second population, which is phenotypically similar to splenic NK cells. Cultured $J11d^+$ thymocytes acquired non-MHC-restricted cytotoxicity, but differed from mature NK cells by containing mRNA for the γ, δ, and ε-chains of CD3. This suggests that $J11d^+$ cells are early T cells that can acquire cytotoxic potential for non-MHC-restricted cells, but they do not give rise to NK cells *in vitro*. *Garni-Wagner* et al. (1990) suggest that mature NK cells reside in the SCID/SCID mouse thymus, but they are not derived from a common NK/T progenitor.

SCID/SCID genotype

Normal murine germline rearrangement results in the generation of immune diversity with antigen specific antibody (Ig), thymocyte cell receptors (TCR), and major histocompatibility complexes (MHC). Antigen is recognised by the variable domains of the Ig molecule (*Ward* et al., 1989); diversity in the variable domain of the heavy chain is achieved somatically by the joining of three gene segments, VH (variable), DH (diversity) and JH (joining) (*Hozumi* et al., 1976; *Kurosawa* et al., 1981). The VH segment consists of two exons, one that encodes most of the leader peptide and which is not pre-

Table 1: Genome of mouse and man (*Lewin*, 1985)

Family	Located on chromosome		Number of V genes		Number of C genes		% chain type	
	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse
Lambda	2	16	~300	2	>6	3	40	5
Kappa	22	6	~300	~300	1	1	60	95
Heavy	14	12	~300	>100	9	8	100	100

the leader peptide and the first 95 amino acids of the variable domain.DH is a small gene segment encoding about 3-8 amino acids of the third hypervariable region and all of framework 4. In addition N sequences, i.e., nucleotides that can be added to the boundaries of the gene segments during V(D)J joining, can be present (*Wu* et al., 1990). VH gene usage by mouse and human has an early bias at the 3' end of the array of VH segments; the closer a gene segment is to the (D)J structure, the more likely it is to recombine (*Yancopoulos* et al., 1984); this bias may reflect the functioning of these gene products early in ontogeny (*Wu* et a., 1990). This biased usage is strain dependent, notably in the BALB/c strain from which the C.B-17 mouse strain was derived.

Variable region genes are assembled during the antigen-independent phase of B cell differentiation (*Malynn* et al., 1990). This occurs in primary B cell differentiation organs, e.g., foetal and neonatal liver and bone marrow. Pre-B lymphocytes assemble and express heavy and light chain genes to become surface Ig+ B cells; this is the "newly generated" antibody repertoire unselected by external antigens (*Malynn* et al., 1990).

The mouse immunoglobulin protein is composed of two identical heavy chains and two identical light chains. All heavy chain genes are found on germline chromosome 12 and are arranged linearly of greater than 100 variable genes, approximately 10 diversity genes, 4 joining segment genes, and 8 constant genes (*Lewin*, 1985). In contrast, light chain production may be either kappa (κ), found on germline chromosome 6, or lambda (λ), found on germline chromosome 16. Only 5% of light chain production is of lambda origin (Table 1), with the majority being kappa (95%). The kappa germline gene linearly is composed of approximately 300 variable genes, 5 joining segment genes, and 1 constant gene; the lambda germline gene is composed of 2 variable genes, 1 joining gene, and 3 constant genes (*Lewin*, 1985).

All murine somatic cells contain the above germline genes, which, undergo somatic recombination in lymphocytes to produce immature B cells with specific antibody diversity. Upon antigenic stimulation, one specific antibody-presenting immature B cell will proliferate to secrete antibody or remain dormant as a memory cell (*Lewin*, 1985).

Recombinase activity can be conferred to 3T3 cells, via transfection, using SCID/SCID DNA as a source of the recombinase-activating gene (Rag-1 element). Recombinational activity for exogenous plasmid substrates is conferred, but whether this SCID/SCID RAG-1 element confers normal or abnormal recombinase activity has not yet been determined (*Weaver*, 1989). Thus, inability of these mice to join

coding regions of V, D, and J heavy segments provides a sufficient explanation for the absence of T and B cells in the mutant (*Weaver*, 1989). *Schuler* et al. (1990) reported a high frequency of abnormal Igh and TcR β gene rearrangements in transformed immature SCID lymphoid cells, which typically involved large J segment-associated deletions resulting from attempted D-J recombination.

Normal murine T cells have a variety of functions connected with interactions between cells involved in the immune response. T-cell function involves production of the T-cell receptor (TCR), a set of transmembrane glycoproteins, that provide a direct counterpart to the antibodies produced by B cells. The TCR must recognise a foreign antigen of unpredictable structure and recognise histocompatibility (*Lewin*, 1985; *Carbonari* et al., 1990).

The TCR is actually a complex (TCR/CD3), and is made of either alpha-beta (α-β) chain TCR or gamma-delta (γ-δ) chain TCR, both associated with a constant CD3 element. Alpha-beta chain TCR is found predominantly on peripheral blood T cells and central lymphoid organ T cells. Gamma-delta TCR is present on an immature minor population of cells, predominantly in bone marrow. During intrathymic differentiation, genes are first expressed for the CD3 proteins and then the TCR, however, the TCR/CD3 complex will not appear on the cell surface if either TCR alpha or beta chain is absent. Transfection of TCR alpha or beta genes into mutant cells deficient in synthesis will restore surface expression, therefore, it is thought that one of the TCR genes is a limiting determinant (*Carsten* et al., 1989).

In adult thymocytes from SCID/SCID mice, TCR alpha, beta and gamma genes are in the germline configuration with the presence of beta and gamma transcripts. Examination of the delta locus showed a restricted number of sub-germline bands consistent with attempted diversity-delta-2 to joining-delta-1 rearrangement. This confirms that there may be an ordering of TCR recombinational events during T-lymphocyte differentiation, with delta rearrangement occurring first and representing a selective advantage for this recombination (*Caroll* and *Bosma*, 1989).

Rescue of the SCID/SCID mouse immune system by transgenic introduction of productively rearranged Ig genes has resulted, to a limited extent, in B-cell maturation to IgM synthesis (*Fried* et al., 1989). Alpha and beta TCR chain transgenic introduction has rescued SCID/SCID thymocytes to $CD4^+/CD8^+$ maturation, but further proliferation and maturation occurred only in transgenic mice expressing MHC. This shows the importance of appropriate thymic MHC-TCR interaction for T-cell development (*von Boehmer* and *Blüthmann*, 1989).

Croitoru et al. (1990) identified intraepithelial leukocytes (IEL) in SCID/SCID mice lacked CD3 expression and mRNA for the V.7 V region gene of the T cell receptor. They concluded that these IEL differ from classical T cells in their ability to differentiate and express CD8 and do not require T cell receptor expression for their localisation to the intestine.

'Leaky' SCID/SCID mice

"Leakiness" in the SCID/SCID mouse refers to the somatic expression of immunoglobulin (Ig) by a SCID/SCID mouse population as it ages under non-specific pathogen-free conditions. This may indicate that the penetrance of this mutation is not complete (*Caroll* et al., 1989). Forty-one percent of SCID/SCID mice older than 9 months and 15% of younger

SCID/SCID mice produce a limited clonal diversity of serum Ig as shown by isoelectric focusing (*Gibson* et al., 1989). An oligoclonal pattern usually of between 1 and 12 clonotypes is seen with little sequential variation in these patterns, thereby, suggesting that the leaky phenotype occur at the level of B-cell precursor. Since this is prior to VDJ rearrangement, B cells have little subsequent potential for expansion and differentiation (*Gibson* et al., 1989).

Leakiness is strongly determined by reactivity to autoantigens as shown by B-cell hybridomas generated from spleens of leaky SCID/SCID mice, which are specific for host cell nuclei, erythrocytes and platelets as well as to the enteric pathogens of *Enterobacter* or *Serratia* origin (*Kearney* et al., 1989).

At the molecular level, limited differentiation of T lymphocytes can be shown in leaky SCID/SCID mice. When spleen cells from leaky SCID/SCID mice are cultured *in vitro* and probed for TCR expression they show the expansion of only 1-5 clones per spleen, but the majority of cells from these clones have apparently normal TCR gene rearrangements (*Carroll* et al., 1989).

Injection of purified B-cell hybridoma antibody into neonatal SCID/SCID mice induces both T- and B-cell development. The T-cell population has been said to expand with Ig production as a stimulus; $CD3^+$ cells are present ($<10^5$) and skin grafts can be rejected, but the CD4/CD8 ratio remains skewed (*Kearney* et al., 1989). This illustrates the co-ordinate development of both cell lineages in the leaky phenomenon, and when taken together the data for T- and B-cell differentiation in leaky SCID/SCID mice favours an epigenetically driven reversion of mutational events rather than a stochastic process of chance productive gene rearrangements (*Ansell* and *Bancroft*, 1989). The question is what role the microflora and/or its products play in this reversion.

MATERIAL AND METHODS

Animals

Inbred SCID/SCID mice were obtained from the Johns Hopkins University Oncology Center Animal Resources Division, Baltimore, MD, USA; the original SCID/SCID mice came from M.J. Bosma, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, USA. Currently they are housed in micro-isolators, but are being caesarean-derived and placed into the germfree environment.

Inbred germfree DBA/2Wg mice, originally obtained from Walberg, Oak Ridge National Laboratory in l969; inbred germfree C3H/HeSchGN mice, originally obtained from the A.R. Schmidt Co. (now Harlan Sprague Dawley, Inc.); inbred BALB/c nu/nu mice, originally obtained from the Department of Radiation Medicine, Massachusetts General Hospital, are maintained by brother-sister matings in flexible plastic film isolators and treated according to the standard procedures in use at the LOBUND Laboratory for germfree mice (*Trexler*, 1959; *Wagner*, 1959).

All mice are free of *Pneumocystis carinii* and of all murine viruses, with the exception of latent leukaemia virus, which is carried by all strains of germfree mice. Sacrifice was by cervical

Table 2: Fasting glucose levels of SCID/SCID C.B-17 mice

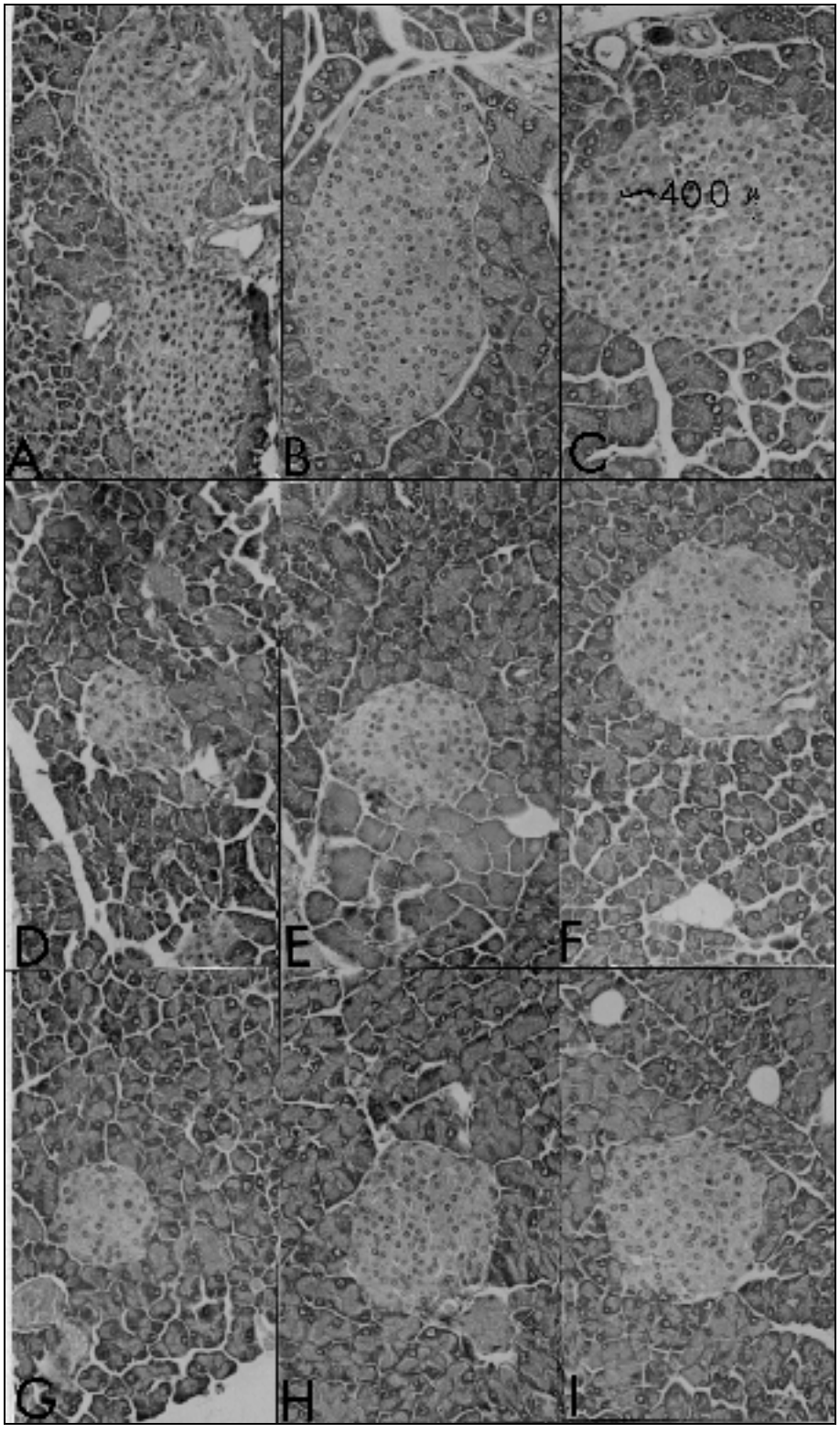
Strain	Birth date	Age	Blood glucose (mg/100 ml)
SCID/SCID	09/04/89	26 wk	52 <40
C.B-17	10/30/89	17 wk	152 147
CFW/Bel	12/17/89	$10^{1}/_{2}$ wk	112 130
C3H/HeNSch	01/25/90	5 wk	92 112
DBA/2Wg	08/27/89	26 wk	92 93
C57Bl/6J	01/25/90	5 wk	120 130
BALB/c nu/nu	08/21/89	27 wk	80 100

dislocation; tissue specimens were removed, placed into Bouin's fixative for 1 hr, then placed into 70% EtOH until paraffin embedding and sectioning at 5 mm. Slides were either stained with routine haematoxylin and eosin or with Gomori stain, which permitted the separation and identification of alpha and beta islet cells in the pancreas.

Blood glucose monitoring

Blood glucose levels were sampled by retro-orbital bleeding (Riley, 1960) via a haematocrit capillary tube, with a minimum of two. Mice were under plane II ether anaesthesia for this procedure. Blood samples were tested by a Tracer II blood glucose monitor and test strips (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250, USA), and are reported in mg/dl by digital display. Preliminary studies included two mice of each strain as described in the results (Table 2); the confirmative study included 8 male SCID/SCID adult mice and 5 female SCID/SCID adult mice; controls were 7 male and 5 female C.B-17 adult mice. All mice were sampled in the morning between 7 and 10 a.m. after an overnight 12 hr fast; water was supplied *ad libitum*.

Figure 1: SCID/SCID male pancreas (A); SCID/SCID female pancreas (B); pregnant SCID/SCID female pancreas (C). Normal exocrine structure; hyperplastic (est.~400 mm) islet of Langerhans; elliptically-shaped islet of Langerhans in (B); endocrine cellular morphology normal. Balb/c nu/+ male pancreas (D); Balb/c nu/+ female pancreas (E); Balb/c nu/+ pregnant female pancreas (F). Normal exocrine structure; islet of Langerhans slightly enlarged in pregnant female (F), ~225 mm; DBA/2Wg male pancreas (G); DBA/2Wg female pancreas (H); DBA/2Wg pregnant female pancreas (I). Fasting state serous cells; small sized islet of Langerhans (est. 175 mm).
All tissues are stained with H & E. (215x)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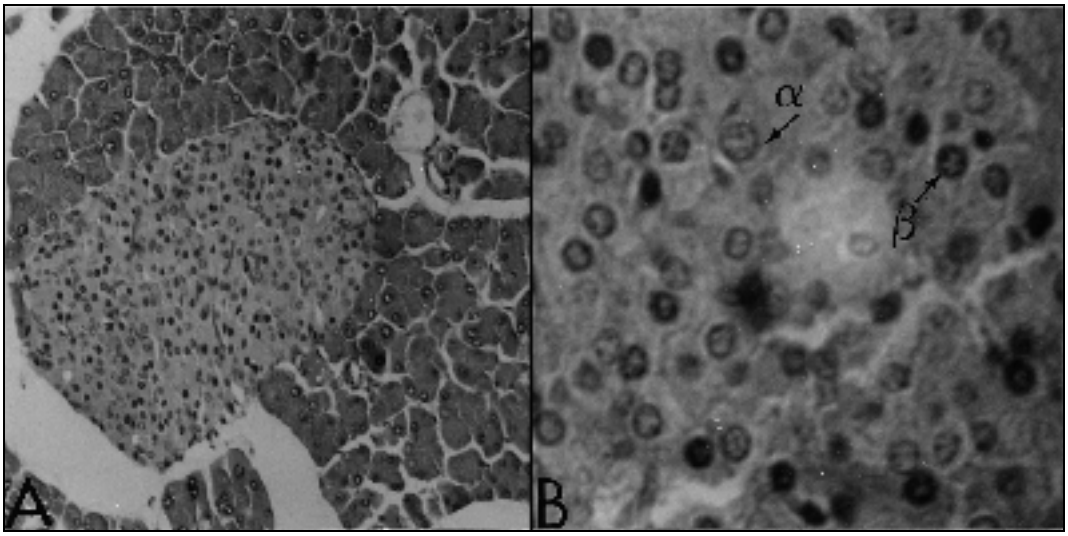

Figure 2: Photomicrograph of SCID/SCID mouse pancreas. Photomicrograph of adult female pancreas stained with trichrome stain. Alpha cells are hyperplastic.
A. 240x; B. 480x.

RESULTS

SCID/SCID mouse lymphoid follicles in the spleen, mesenteric and popliteal lymph nodes and the 'medullary' thymus consisted of stromal cells, histiocytes, and granulocytes and were devoid of lymphocytes (*Bealmear* et al., 1990). Erythroid hyperplasia and some megakaryocytosis were observed in the red pulp of the spleen. Haematocrits were normal and peripheral blood and bone marrow were characterised by 'leukocytosis', lymphopenia. Peyer's patches were sparse and devoid of lymphoid cells. Hepatic, lung, and renal parenchyma were normal; cardiovascular architecture had no congenital defects or myopathy. Adrenal cortex and medulla appeared hypertrophic and warrant further investigation of the juxta-medullary x-zone layer (*Arey*, 1963). The ovaries had follicles and eggs in all stages of development, however the testicles appeared to have few sperm. Skeletal muscle and central nervous system were normal.

The pancreas was grossly of normal size and consistency for all strains taken in the study; no masses, nodularity, or sites of ectopic (i.e., metastatic) tissue were found. No gross systemic disease was noted; all mice were considered to be in a general state of good health.

Exocrine and endocrine components of the pancreas were examined microscopically. The exocrine component of all strains was examined for congenital duct anomaly, signs of regressive changes (i.e., fatty infiltration, atrophy, etc.), inflammatory processes (i.e., acute or chronic pancreatitis), and tumours (both cystic and carcinomatous). None of the abnormalities listed were observed. A non-uniform staining of serous cytoplasm was noted in C3H/HeNSch female, and DBA/2Wg pregnant female sections (*Borrillo* and *Bealmear*, 1990). This was attributed to normal physiological changes in the digestive phase of these mice.

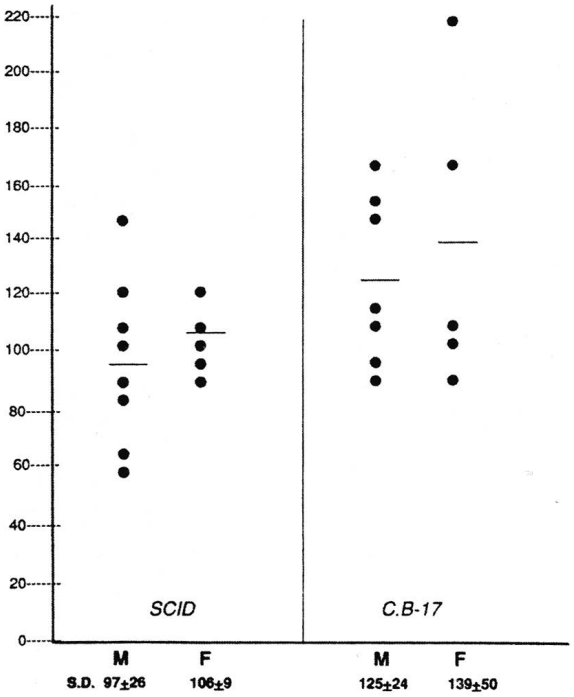

Figure 3: Fasting blood glucose levels (mg/dl) of SCID/SCID vs. C.B-17 Mice. All mice were older than 12 wk of age. Female mice were nongravid.

All microscopic examination of at least 50 slides from pancreas sections from SCID/SCID mice, stained with haematoxylin and eosin, revealed islets which were either round or oval in shape, and averaged >400 mm (Figure 1). No inflammation or fibrosis of the islets or surrounding exocrine pancreas was noted. There was no evidence of hyalinisation or dysplasia and excess mitotic activity was not evident.

Examination of liver sections did not show evidence of ectopic islet cells. Gomori and Masson staining of SCID/SCID mouse pancreas (Figure 2) demonstrated an abnormal central abundance of pink staining alpha (glucagon-producing) cells mixed within the normally predominating beta (insulin-producing) cells, which stain blue.

Small and medium-sized islets from all the other strains examined (seven strains representing male and female (both pregnant and nonpregnant) were identical in appearance to those of the SCID/SCID mouse, except for their diameter, which averaged <300 mm. Pancreatic sections from all controls showed a predominance of small islets. However, since large and medium-sized islets comprise most of the total volume, subsequent estimation of islet size included only those islets with diameters of at least 100 mm.

Preliminary male SCID/SCID fasting blood glucose levels (Table 2) were decreased in comparison to all strains and ages sampled (*Borrillo* and *Bealmear*, 1990), a hypoglycaemic state not seen during nonfasting. Female fasting blood glucose levels showed no trend. This original study was confirmed in a larger sampling of 13 SCID/SCID mice and their C.B-17 controls (Figure 3).

DISCUSSION

The islet hyperplasia may be age dependent, because as mice age, the growth rate of islets tends to accelerate. In the Wellesley rat strain, which tends to be obese, 50% of the males and 5% of the females become glycosuric between the ages of 16 and 55 weeks with nearly all exhibiting extreme islet cell hyperplasia between 12-30 months (*Jones*, 1964). Obese hyperglycaemic V strain mice, as a result of their Mendelian recessive transmission, allow the study of lean and obese litters. Using this strain, *Bleisch* et al., (1952) showed the islets of Langerhans to be hyperplastic predominantly in obese mice (i.e., 50-60 g at 12 months) (*Black* et al., 1988). Although not obese, the SCID/SCID mouse does maintain its hyperplasia with age, but does not have an associated hyperglycaemia or the characteristic diabetic lesions, i.e., beta cell degranulation with vacuolisation, islet hyalinisation, or leukocyte infiltration. Rather, an association between immune function and islet size should be considered because of the nature of the SCID/SCID defect. We postulate that the immune system may play a role in suppression of normal long-term islet cell growth, a role that should not always be viewed as an 'autoimmune pathology'. An overexpression of this suppressive role may lead to diabetes, just as an undersuppression may lead to hyperplasia or malignancy. In this model, the short term regulation of islet cells would still be under blood glucose control, and in the SCID/SCID mouse could account for the alpha cell hyperplasia; an imbalance among the cells of the immune system may be responsible for the suppression of islet-cell glucose receptor antibody and upset the delicate balance between glucose and insulin. Other endocrine glands, their secretions, and their synergistic effect(s) on the immune system should be studied before the severe combined immune deficiency defect can be clearly defined.

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