Instituto Juan March
de Estudios e Investigaciones

38 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Immunodeficiencies of Genetic Origin

Organized by

A. Fischer and A. Arnaiz-Villena

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The lectures summarized in this publication were presented by their authors at a workshop held on the 6th and 7th of March, 1995, at the Instituto Juan March.

Depósito legal: M. 16.682/1995
I. S. B. N.: 84-7919-538-X
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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INTRODUCTION

A. Fischer and A. Arnaiz-Villena

This booklet is a collection of summaries of presentations at the "*Immunodeficiencies of Genetic Origin*" workshop held in Madrid on 6-7 March 1995.

The idea to celebrate such a meeting was put forward because of the overwhelming information published in the last years on the precise molecular and genetic anomalies which underlay many inmunodeficiencies. Abnormal surface molecules, defective tyrosin-kinases and mutated genes among others have been spotted in children with immune system dysfunctions.

Andrés González rapidly materialized the proposal and Alain Fischer and Antonio Arnaiz-Villena called most of the scientists who have reported the molecular basis of inherited deficiencies, and others who are in the front line of research on gene therapy, thymic ontogeny and immune genes "*knock out*" mice. The scientists response was good and most of them accepted the invitation. The magnificent and internationally reputed organization of these series of meetings was maintained at its highest level by Andrés González, as usual.

Only inherited immunodeficiencies affecting lymphocytes were studied in the workshop. Theoretically it is possible to find out the genetic defects at any level of immunological ontogeny and functionality; thus, examples of deficiencies were presented at the following immune response steps:

1.- **Antibody presenting cells or B-T cell contacts:** mutations at CD18 (LFA-1) molecule or CD40 ligand; "*bare lymphocyte syndrome*" with absence of HLA class II presenting molecules and TAP-2 deficiency (peptide transporter molecule for HLA class I antigens) may also be classified within this item.

2.- **B-T cell specific genes rearrangement defects:** progress in recombinase defects was shown and multiple enzymes with genes in different chromosomes were involved.

3.- **B cell antibody production:** Bruton agammablobulinemia due to a defect of *btk* (Bruton tyrosin-kinase).

4.- **T cell recognition:** defect at the CD3 complex has been described; ϵ and γ chain mutant families there exist.

5.- **T cell response:** a defect in ZAP-70 tyrosin-kinase coupled to CD3 is responsible for a defective transmission signal and a severe immunodeficiency is associated to a defective γ subunit which is common to the receptors for IL-2, IL-4, IL-7, IL-9 an IL-15.

6.- **T cell cooperation:** CD40 ligand deficiency could also be included here. In addition, abnormal IL-2 production and reduction/lack of NF-AT have been detected without knowing the precise gene involved.

7.- **Toxic metabolites:** accumulated in ADA and PNP deficiencies result in a serious immunodeficiency, which may be treated by adequate gene therapy.

8.- There are **other inmunodeficiencies,** whose genetic defect is pin pointed but the mechanisms by which immunodeficiency is established are still obscure; these are the cases of the Wiskott-Aldrich syndrome and the Fas deficiency.

In addition, artificially impaired genes in mice ("*knock out*" mice) showed how a lack of certain surface proteins and interleukins affected the immune function; the last advances in T-cell ontogeny were also detailed (i.e.: The precise nature of pre-T cell α chain).

The problems of gene therapy were also stressed by the example of the adenosin-deaminase deficiency treatment; the infusion of culture - expanded peripheral blood T cells modified by retroviral mediated ADA gene-transfer can be an effective therapy for this severe immunodeficiency.

In summary, the precise physiology of the immune system is being clarified by these inherited natural (human) or "*knock out*" (mouse) occurring genetic errors.

It is likely that the study of immunodeficiencies of genetic origin will be one of the most productive field in biology. This study has been possible due to the widespread availability of new technology, including the existence of specific monoclonal antibodies against surface molecules, molecular genetics, easy gene amplification, Ca^{++} and tyrosin-kinases study methodology.

Immunology and physiology in general will benefit of the discoveries and preventive and even curative medicine will be used to fight anomalies.

FIRST SESSION: T-CELL DIFFERENTIATION AND DEFICIENCIES

Chairman: Max Cooper

T LYMPHOCYTE FUNCTION AND ONTOGENY IN GENE-TARGETED MUTANT MICE

Tak W. Mak

Ontario Cancer Institute,Toronto, Ontario/Canada

T lymphocytes recognize their antigen peptides and Major Histocompatibility Complex products with the use of their T cell antigen receptors (TcR). In addition to the α and β chains of TcR, the interaction between T cells and their target cells or antigen presenting cells is also assisted by a series of other cell surface polypeptides. Most notable of these are CD4 and CD8, which are selectively expressed on mature helper/induces and killer/suppressor T cells, respectively. Upon engagement of their ligands, a series signals are being transduced intracytoplasmically via some of these molecules and their associated proteins. Perhaps the most important enzyme in this signal transduction process is the lymphocytes specific tyrosine kinase lck. Another important component is the cell surface tyrosine phosphatase CD45. This molecule is alternatively spliced and the different isoforms are expressed on the various hemopoietic and lymphopoictic cells. Signaling through the TcR-CD4/CD8-lck-CD45 complex is thought to be insufficient to activate T lymphocytes. A co-stimulatory signal is believed to be essential. Many investigators have suggested that CD28, a ligand for B7/BB1 is an essential co-stimulatory signal. In an attempt to gain better understanding on the roles of these molecules in T lymphocyte functions and ontogeny, we generated a series of mutant mice with disruptions in these genes. These mutant mice are being analyzed in order that we can evaluate the importance of these genes in T cell development.

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Identification of 3 Different Genes that Encode Products Involved in V(D)J Recombination and DNA Repair. Guillermo E. Taccioli, Zhiying Li, #Tanya M. Gottlieb, *Tracy Blunt, #Nicholas J. Finnie, Jocelyne Demengeot, *Anne Priestley, Ryushin Mizuta, Yijie Gao, +Tomas Otevrel, *Alan Lehman, +Thomas Stamato, *Penny Jeggo, #Stephen P. Jackson, and Frederick W. Alt. The Howard Hughes Medical Institute, Children's Hospital, Center for Blood Research and Department of Genetics, Harvard University Medical School, Boston. #Wellcome/CRC Institute and Department of Zoology, Cambridge University, UK. *Medical Research Council, Cell Mutation Unit, University of Sussex, Brighton, UK.+Lankenau Medical Research Center, Wynnewood, Pa.

The V(D)J recombination reaction involves recognition of conserved recombination sequences (RS) that flank each germline V, D, or J segment, introduction of double strand breaks at the RS/coding sequence junctions, loss and/or addition of nucleotides at the coding junctions, and additional activities (e.g. polymerization and ligation) to complete the joining process. V(D)J recombination appears to be carried out by both pre-lymphocyte specific factors such as RAG-1, RAG-2 and TdT as well as generally expressed factors including gene products involved in double strand DNA break repair (DSBR) pathways.

We have previously shown that three independent Chinese hamster ovary (CHO) mutant cell lines that have defects in double strand DNA break repair (DSBR) also have defects in V(D)J recombination as assayed with transient V(D)J recombination substrates following introduction of the RAG-1 and 2 genes into these cell lines. These findings suggest that the DSBR and V(D)J recombination processes share common factors. In combination with its V(D)J defect, the mutant V-3 CHO line has a defect that affects V(D)J recombination in a manner essentially identical to that found in the context of the homozygous murine *scid* mutation : V(D)J recombination is properly initiated, RS joining occurs normally, but coding join formation is impaired. We have shown by cell complementation studies that the murine *scid* and V-3 mutations probably affect the same gene. Two additional, independent CHO cell DSBR mutants (xrs-6 and XR-1) can initiate V(D)J recombination properly, but are blocked in ability to form both RS and coding joins.

We have obtained both genetic and biochemical evidence which demonstrates that the human gene (XRCC-5; chromosome 2) which complements the xrs-6 mutant encodes the 80 kDa subunit of the Ku complex. The complex of the 80 and 70kDa Ku subunits binds to free double stranded DNA ends and forms the DNA binding component of the DNA-dependent protein kinase (DNA-PK) . The large catalytic subunit of DNA-PK (DNA-PKcs; approximately 450kDa), once activated through its interaction with DNA bound Ku can phosphorylate a number of proteins *in vitro* including transcription factors and p53. A variety of biochemical data indicate that DNA-PK activity is defective in the V-3/Scid cell lines due to a defect in the DNA-PKcs molecule. Furthermore, YACs containing the human DNA-PKcs gene can complement the defective DNA-PK activity, DSBR defect, and V(D)J recombination defect in the V-3 line. This and other data suggest that the DNA-PKcs is the XRCC-7 gene (human chromosome 8) which complements the V-3 (murine Scid) defect.

Finally, we have recently cloned a human cDNA sequence, which encodes a unique protein, based on its ability to restore normal V(D)J recombination activity to the XR-1 cell line. Expression of this cDNA sequence in XR-1 cells also substantially complements their DSBR defect. We have mapped the gene (XRCC-4 gene) encoding this cDNA to human chromosome 5. Current efforts are aimed at elucidating potential functions of the XRCC-4 gene product in DSBR and V(D)J recombination.

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Papillon-Lefevre Immunodeficiency

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The Papillon-Lefevre (P.F.) syndrome is caused by an unknown autosomic recessive genetic anomaly and is characterized by a severe periodontal inflammation and palmoplantar hyperkeratosis. 30 % patients show increased susceptibility to bacterial infections.

We have shown that patients with P.F. syndrome show usually normal percentages of peripheral blood lymphocytes analyzed by many CDs including integrins, CD2 and CD3. However, slightly low CD45 R0 and CD29 number of lymphocytes were recorded during a 7 year follow up, and all times a reduced number of CD18, CD11a and CD2 molecules were obtained in the corresponding positive cells: the high density LFA-1 and CD2 cells were strongly diminished. LFA-1 and CD2 molecules were normal as assessed by immunoprecipitation. It is postulated the existence of a common mechanism that regulate CD2 and LFA-1 expression, which is impaired in P.F. patients.

Apo-1/Fas/CD95 MUTATIONS IN HUMAN LYMPHOPROLIFERATIVE SYNDROME ASSOCIATED WITH AUTOIMMUNITY AND DEFECTIVE APOPTOSIS

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The Apo-1/Fas/CD95 antigen has been identified as a key cell surface receptor involved in apoptotic cell death. It was found defective in the lpr mouse. Fas expression and function were analysed in three children (including two siblings) with a lymphoproliferative syndrome and autoimmune evidence (thrombocytopenia, hemolytic anemia and neutropenia) in two of them. A restricted ß T-cell repertoire was detected in one of the 3 children both in double negative and single positive T-cells. A large genomic deletion encompassing the terminal part of the intracellular domain-encoding exon was responsible for the lack of detectable Fas cell surface expression in the most affected patient. Less severe clinical manifestation was found in the two related patients. Fas expression was normal in these patients but Fas-mediated T and B cell apoptosis was impaired because of a truncated protein resulting from a 2bp deletion within the intracytoplasmic domain.
This Fas gene-defect in humans is to our knowledge the first description of a complete Fas deficiency (the lpr mice have residual Fas expression) and confirms the crucial regulatory role of this molecule in lymphocyte cell death and may provide a molecular basis for susceptibility to autoimmune diseases in humans.
F.RIEUX-LAUCAT et al submitted.

MHC CLASS II DEFICIENCY, A DISEASE OF GENE REGULATION

Bernard Mach, M.D., Ph.D., University of Geneva Medical School

When it was shown that a form of primary immunodeficiency, MHC class II deficiency (also referred to as the Bare Lymphocyte Syndrome, BLS), was in fact a disease of gene regulation, involving transacting regulatory factor(s) (1), there was hope that solving the genetic basis of that rare disease might lead to the identification of essential regulators of MHC class II gene expression. MHC class II genes are indeed very tightly regulated and the control of their expression in certain selected cell types is an essential component of the control of the immune response.

MHC class II deficiency is an autosomal recessive disease characterized by severe immunodeficiency with multiple infections (2). The clinical picture results from an absence of HLA-DR, -DQ and -DP molecules on all cells of these patients. This unusual phenotype was in turn shown to result from a lack of transcription from the promoters of the different MHC class II genes. Although clinically homogeneous, the disease is genetically heterogeneous, with three complementation groups described, A,B and C (3,4).

A first clue to the regulatory defect responsible for this disease came from the observation that patients from BLS group B and C exhibit a characteristic defect in the binding of a specific protein complex, called RFX, to the X box motif of MHC class II promoters (5), while patients from complementation group A bind RFX normally. This defines two distinct types of molecular defects. Genetic complementation of cell lines corresponding to each of these two types of defects by cDNA libraries in expression vectors has now allowed the cloning and identification of the regulatory genes involved in each of these two types of defects. Patients from complementation group A (as well as some in vitro generated regulatory mutant lines that belong to the same group) have a normal promoter binding pattern but are **mutated in a novel MHC class II transactivator called CIITA** (6) (see Figure, bottom left). Patients from group C, characterized by the lack of binding of RFX in vitro and an unoccupied promoter in vivo, are indeed **mutated in the gene that encodes the large subunit of the RFX complex** (Steimle V, Durand B, Barras E, Zufferey M, Mach B, Reith W, in preparation). In both cases, introduction of the normal, non-mutated, form of the regulatory gene (as cDNA) into MHC class II negative B lymphocytes from BLS patients restores full expression of all MHC class II genes and cell surface molecules in several patients from each of these two groups. Although the gene mutated in complementation group B has not been cloned yet, it is tempting to suggest that it encodes the other subunit of RFX, since purified RFX heterodimers can correct MHC class II transcription in vitro in extracts from group B as well as group C patients (7). The two types of molecular defects identified in promoters of BLS patients, and the two transacting regulatory factors involved are schematically represented in the Figure (bottom left and right), with obvious consequences in terms of cell surface expression of class II molecules.

Somewhat unexpectedly, it was discovered that CIITA, the MHC class II transactivator mutated in BLS, is the mediator of this crucial induction process (8). Expression of the CIITA gene is, itself, very tightly controlled, induced for instance by interferon gamma, and it is both necessary and sufficient to activate MHC class II gene expression (8). In fact, CIITA behaves as the main regulator of MHC class II expression in a variety of biological situations.

Because of the importance of MHC class II expression in numerous pathological situations, including in disease that are not T lymphocyte-mediated, the novel MHC class II transactivators discovered in studying BLS patients should probably be considered for novel immunomodulation strategies. Contrary to many transcription factors, RFX and CIITA are both absolutely essential and remarkably specific for MHC class II gene expression. These properties make them excellent targets for immunomodulators that would act at the transcriptional level.

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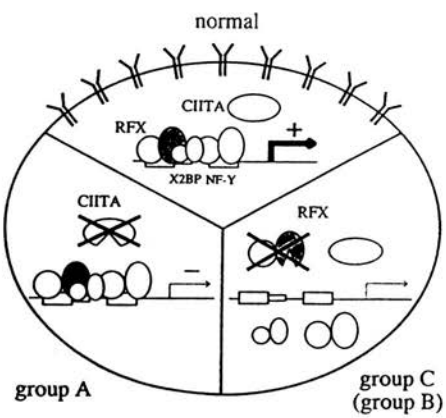

The two types of molecular defects in MHC class II deficiency

CD3 DEFICIENCIES IN MAN AND MOUSE

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The CD3 chains of the T lymphocyte antigen receptor (TCR) are multiple monomorphic components of uncertain stoichiometry most likely involved in signal transduction for T cell selection or activation through a range of protein kinases. The recognition of natural human defects (CD3γ, CD3ϵ) and the generation of mouse KOs (CD3δ, CD3ζ, CD3η) are helping to understand the redundant and the specialized roles of the invariant chains of the TCR/CD3 complex. The main effects of the CD3 deficiencies on T-cell development, selection and on TCR/CD3 assembly, structure, expression and signaling will be reviewed.

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X-SCID caused by IL2Rγ mutations

G. de Saint Basile, J. DiSanto, A. Dautry-Varsat, F. Le Deist, M. Cavazzana-Calvo, S. Hacein-Bey, S. Markiewicks, F. Rieux-Laucat, E. Morellon, A. Fischer

X chromosome-linked severe combined immunodeficiency disease (SCID X1) is a recessive hereditary disorder characterized by a complete absence of immature and mature cells and natural killer (NK) cells, whereas B cells are present in normal or elevated numbers. SCID X1 infants present with severe and persistent infections resulting in failure to thrive and early death in the presence of curative bone marrow transplantation (BMT). Mutation of the γ chain common (γc) to interleukin (IL) 2, 4, 7, 9 and 15 receptors has been shown to induce the SCID X1. However, although some mutations lead to the typical form of SCID X1, others mutations are responsible to mild forms of SCID where patient present with some T cell differenciation and function.

SCID X1 patients presenting with various phenotypes were therefore analyzed for γc chain mutation and some functions. In nine patients with typical form of SCID X1, multiple genetic defects in the extracellular region of the γc chain were detected, which affect in most cases γc membrane expression and in all cases, high affinity IL2 binding.

In an other patient, an atypical phenotype of SCID X1 was observed consisting in an absence of T and NK cells during the first year of life and in further developpement of an autologous mature T cell population with mild mitogen and antigen response. Raise in T cell counts as well as in T cell function occurs following an allogenic bone marrow transfert which fails to engraft. Identification of a γc nonsens mutation predicts a truncation of the major part of the intracytoplasmic γc region which precludes any γc-Jak3 association in this case. This mutation however, does not affect high affinity IL2 binding but partly reduce IL2 endocytosis. These data indicate that in certain circumstances, some accessory signaling pathways can partly overcome γc chain defect to allow T cell differenciation and function although a severe immune deficiency persists.

A familial atypical form of SCID X1 characterized by the presence of a low to normal

number of poorly functional and oligoclonal T cells of NK cells and by normal serum concentration of the Ig isotypes resulted in an other SCID-X1 patient from a 5 fold reduction γc expression . High affinity IL2 binding was normal. γc chain mutation in this patient consists in a substitution of the penultimate base of the first exon causing a splice defect. However alternative splice preserved a functional mRNA in 20% of splice products.

Since expression of a reduced number of γc chain causes a severe immunodeficiency, effective gene therapy of SCID X1 will probably require high level of γc expression. First data on γc retrovirus-infected EBV-B cell line of a SCID X1 patient indicate that selection of transduced B cell clones expressing normal γc chain occur during in vitro culture and that no evidence for a dominant negative effect of the mutated transcript on the normal γc expression was found.

Together, these data raise new questions on the role of the γc chain in T cell developement and differenciation and bring preliminary informations on the normal γc chain expression in transduced SCID X1 patient cells.

The role of the common cytokine receptor γ chain in X-linked severe combined immunodeficiency and in cytokine signaling. Warren J. Leonard, Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20817

X-linked severe combined immunodeficiency (XSCID) is the most common form of SCID, accounting for approximately half of all cases. Noguchi et al. (1993) demonstrated that mutations in the IL-2 receptor γ chain results in XSCID in humans. This finding was unexpected since mice and humans deficient in IL-2 have normal numbers of T cells, whereas patients with XSCID have absent or profoundly diminished numbers of T cells. The dilemma was resolved by the striking observations that IL-2Rγ is not only a component of the IL-2 receptor, but also is a critical component of the receptors for IL-4, IL-7, IL-9, and IL-15, and hence is also known as the common cytokine receptor γ chain, γ_c. Thus, inactivation of γ_c simultaneously inactivates at least five cytokine systems, explaining the severe immunodeficiency present in XSCID patients. The phenomenon of sharing of cytokine receptor subunits exists for two other sets of cytokines. IL-6, IL-11, oncostatin M, leukemia inhibitory factor, CNTF, and cardiotrophin 1 all share gp130 as a signaling molecule, whereas IL-3, IL-5, and GM-CSF all share the common β chain. These observations collectively have implications related to the understanding of cytokine pleiotropy and redundancy.

In the case of IL-2, it has been established that dimerization of the cytoplasmic domains of IL-2Rβ and γ_c is required for signaling, whereas the role of IL-2Rα is primarily to augment binding affinity and sensitivity of responsiveness to IL-2. A number of laboratories have sought to identify the signaling molecules which can associate with each of these chains. Considerable energy has focused on Janus family tyrosine kinases. IL-2 activates both Jak1 and Jak3, but not Jak2 or Tyk2. Corresponding to this observation, it has been demonstrated that Jak1 associates with IL-2Rβ and Jak3 associates primarily with γ_c, but Jak3 can also associate directly with IL-2Rβ. Interestingly, IL-4, IL-7, and IL-9 also activate Jak1 and Jak3, indicating that the Jak family kinases are not responsible for the differences in signals transduced by these different cytokines. Distinctive proteins, such as IRS-1, which is activated by IL-4, may help to determine specificity. Moreover, the Stat proteins activated by some of these cytokines are different. For example, IL-2 and IL-4 activate different Stat proteins. However, IL-2 and IL-7, which share a number of actions, activate the same Stat proteins in normal peripheral blood lymphocytes. We have clarified the molecular basis for this phenomenon by identifying the tyrosines which when phosphorylated serve as docking sites for the relevant Stat proteins. This serves to provide a molecular basis for some of the signals induced by IL-2 and other cytokines.

Finally, as part of an effort to understand γ_c in an in vivo model, we and others have inactivated the γ_c gene in mice by homologous recombination. The interesting phenotype of these mice contributes further to our understanding of cytokine pleiotropy and redundancy based on both similarities and differences in mice and humans lacking expression of this protein.

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FIRST SESSION: T-CELL DIFFERENTIATION AND DEFICIENCIES (Continuation)

Chairman: Alain Fischer

CD40/CD40L interaction and imunodeficiency

Raif S. Geha

Interaction between CD40 and CD40L play an important role in T-B cell collaboration. Stimulation of T cells via the TCR/CD3 that recognizes MHC class II peptide complex induces CD40L expression. Engagement of CD40 by CD40L induces expression of B7 molecules on B cells. Interaction of B7 with CD28/CTLA4 costimulates T cells to proliferate and secrete cytokines. Engagement of CD40 on B cells induces isotype switching to the immunoglobulin locus targeted by T cell cytokine e.g. IL-4 primes B cells to switch to IgE.

We have shown that T cell but not anti-CD40 directed isotype switching is deficient in X linked hyperIgM syndrome and that patients with this disease have mutations in the CD40L. We will present limited data on some unusual mutations.

We have created CD40 deficient mice by disruption of the CD40 gene. These mice fail to undergo immunoglobulin isotype switching in response to T cell dependent antigens and fail to develop germinal centers. B cells from these mice fail to elicit proliferation in allogeneic cells. This is reversed by anti-CD28. More importantly, *in vivo* injection of CD40-/- B cells specifically tolerizes the recipient T cells to alloantigens.

Because of its importance in B cell function, we studied the expression of CD40 in B cell ontogeny. CD40 was not detectable on pro-B cells. CD40 was expressed on pre-B cells; however CD40 ligation on pre-B cells did not transduce a signal as determined by CD23 expression and by proliferation in the presence of IL-4. Expression of the bcl-2 transgene permitted pre-B cells to respond to CD40 ligation.

The CD40 signalling complex was investigated. CD40 was associated on the cell surface with a 26kD protein with unique amino acid sequences. p26 association with CD40 was contingent on the presence of the extracellular domain of CD40 and was esssential for CD40 mediated aggregation but not for CD40 mediated lymphotoxin α gene expression.

The transcriptional regulation of CD40L expression was investigated. There are four consensus NFAT sites in the CD40 regulatory region. These sites bind NFATc and NFATp, but are not transcriptionally active until the NFAT proteins associate with an AP-1 complex containing Fos and Jun proteins as determined by reporter gene luciferase assays in T cells transfected with multimers of the NFAT sites.

Wiskott-Aldrich Syndrome

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The Wiskott-Aldrich syndrome (WAS) is a monogenic defect, which maps to Xp11.23 between OATL1 and GATA 1. The gene that is defective in WAS has been cloned. It encodes a protein of 501 amino acid residues, which has been called the WAS protein. Several mutations have been identified in this protein in WAS patients. It contains several tyrosine residues in its amino-terminal portion that may be phosphorylated and bind to SH2 domains. It also has several stretches of polyproline in its carboxy-terminal portion that bind SH3 domains. It is not related to any protein in the data bank. Its function is at present unknown.

The genetic defect appears to affect blood cell lineages. Obligate female heterozygous carriers exhibit non-random X-inactivation of all leukocyte and megakaryocyte lineages and as well as in these lineages early in commitment. Phenotypically the thrombocytes and T lymphocytes are most profoundly affected and exhibit a pattern of disorganization of their cytoskeleton. As a consequence of this the T lymphocytes are bald and lack microvilli and consistently fail to respond mitogenically to insolubilized anti-CD3. The cell surface sialoglycoproteins are unstable in the T cell membrane.

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Mutations of the WASP Gene Cause Classic Wiskott-Aldrich Gene and X-linked Thrombocytopenia

Hans D. Ochs, Qili Zhu, Min Zhang, Jonathan M. J. Derry, R. Michael Blaese, Anne Junker, Shi-Han Chen, Uta Francke.

The Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia, small platelet volume, eczema, recurrent bacterial and viral infections, autoimmune disease, increased risk of malignancies and abnormal B and T cell function (1). In addition to the classic WAS phenotype, patients with congenital thrombocytopenia and small platelets, but without eczema or significant infections, and with normal or only mildly defective immune system, have been described (2). This milder form of WAS has been mapped to the same region of the human X chromosome (3). The gene responsible for the Wiskott-Aldrich syndrome (*WASP*) has been isolated, cloned and sequenced (4). The gene, composed of 12 exons containing 1823 basepairs, encodes a 502 amino acid proline rich protein that appears to be of central importance for the development of hematopoietic cell lineages. Sequence analysis of genomic DNA, originally carried out in three families with WAS, revealed three independent mutations within exon 2, strongly suggesting that the newly identified gene was associated with WAS (4). To confirm that the *WASP* gene is responsible for the majority of WAS patients irrespective of clinical phenotype, we selected for mutation analysis the affected members of twelve unrelated WAS families with clinical symptoms and immunologic abnormalities that ranged from mild to severe. To identify the approximate location of mutations within WAS cDNA, we first screened the entire cDNA with the dideoxynucleotide fingerprinting method. This procedure revealed abnormal sequences in all twelve patients studied and allowed us to predict the approximate location of each defect. Based on the screening results, we then amplified the implicated regions of the WAS cDNA for precise analysis of the mutation. Finally, based on the mutations identified by direct sequence analysis of cDNA, we selected and designed primer pairs that allowed amplification of the corresponding regions of genomic DNA.

Point mutations were the most frequent mutations observed, being present in eight of the eleven unique mutations identified. Two unrelated patients with a mild form of WAS were found to have a C $\rightarrow$T mutation at position 290, within exon 2, resulting in the substitution of arginine with cysteine at codon 86. Another missense mutation, affecting codon 125 of exon 4, was found in a patient with a severe phenotype. Two patients, both with severe symptoms of WAS, were found to have nonsense mutations resulting in direct stop codons within exon 1 and exon 10, respectively. In all instances, the same point mutations involving the same nucleotides were found in genomic DNA.

Point mutations resulting in deletions or insertions were observed in four familes. These included the skipping of exon 3 in a patient with severe WAS, deletion of exon 11 due to a splice site mutation in a patient with mild WAS, the deletion of 13 nucleotides within exon 9 due to a new splice site in a patient with mild symptoms, and the insertion of 114 nucleotides 5' of intron 9 due to alternative splicing.

Insertions and deletions of genomic DNA, and secondarily of cDNA, were observed in three unrelated WAS patients. These included the insertion of a single nucleotide in exon 1, a deletion and insertion of small DNA fragments, resulting in a new splice site and the addition of 13 nucleotides at the beginning of exon 11, and the deletion of a nucleotide in exon 10, all resulting in premature stop and moderate to severe clinical phenotypes.

Since the clinical phenotype of WAS often varies from family to family, but also within kinships (1), it is almost impossible to correlate the clinical phenotypes with location and type of mutations. However, in two of our patients with mutations affecting nucleotide 290 (C → T transition) the clinical symptoms were very mild, consisting only of thrombocytopenia, small platelets and, in one of the two cases, mild eczema. Both of these patients fulfill the criteria of X-linked congenital thrombocytopenia, suggesting that this variant is part of the spectrum of WAS. We have previously reported two unrelated Spanish families ("classic WAS") with mutations affecting the arginine at codon 86, in one case due to a G → T transversion of nucleotide 291 (Arg86Leu), and in the other case due to a G → A transition of the same nucleotide (Arg86His). These findings suggest that codon 86 is a hot spot and may be mutated in up to 25% of WAS patients.

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A NEW EXPERIMENTAL SYSTEM FOR STUDYING THE PATHOGENESIS OF THE WISKOTT-ALDRICH SYNDROME AND THE FUNCTION OF THE WASP GENE.

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The Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency whose responsible gene (WASP) has very recently been identified (Derry et al, Cell, 78:635). Nevertheless, the function of WASP remains unclear.

We have been working towards the development of an adequate experimental system to better understand the pathogenesis of WAS. A first step in this direction was the establishment of a panel of allospecific T cell lines from WAS patients (Molina et al, J. Exp. Med. 176:867). These cells have discriminated previously described abnormalities, showing that the CD43 alteration is a secondary defect origininated in the circulation, whereas surface morphology abnormalities are primary defects directly linked to the WAS gene. Furthermore, we showed a specific defect in the CD3 signal transduction pathway of WAS T cell lines that is by all indications directly linked to the WAS gene (Molina et al, J. Immunol. 151: 4383). The CD3-mediated signal is initially transduced but interrupted at an unidentified step. Interestingly, the allospecific stimulation mediated by the $\alpha/ß$ TCR chains is normally completed, resulting in vigorous proliferation.

Attempts to improve this experimental system have been successful by infecting the panel of allospecific T cell lines with the Herpesvirus Saimiri 488. This virus is able to immortalize T lymphocytes after interacting with a surface receptor not known yet. The resulting cells are functionally and phenotypically stable, and remain dependent of IL-2 for growth.

We report here the initial characterization of two WAS allospecific T cell lines and one normal counterpart after immortalization following HVS infection. These cell lines have been kept in culture for over 6 months, and during this time have maintained their phenotype and dependence of exogenous IL-2, although they do not need allospecific stimulation. The doubling time varies with every cell line, but generally is 2-4 days.

Upon stimulation with the anti-CD3 mAb OKT3, the immortalized WAS cell lines GOR1-HVS and GOR7-HVS failed to proliferate (left panel), whereas the allospecific RAJI cells trigger a vigorous proliferative response comparable to the immortalized normal counterpart DEE8-HVS, measured in a 72 h ^{3}H-thymidine uptake assay (right panel). This mirrors the findings of the WAS parental cell lines, and likewise, the early CD3 signal transduction events are intact, including phosphorylation of the CD3-ζ chain after OKT3 stimulation.

The surface phenotype of WAS-HVS cells does not show any difference respect either the HVS-immortalized normal cell line or the WAS parental cells. Under the scanning electronmicroscope, the examined GOR1-HVS line shows a cell surface completely devoided

of microvilli. This is a fundamental primary alteration in WAS found in both fresh PBLs and T cell lines. In this case, we have found a very homogeneous and consistent defect.

Finally, the recent cloning of the WASP gene has allowed us to address the expression of the WASP gene. By using a combination of a 5' sense primer with an internal antisense primer, we have detected by Reverse transcriptase-PCR WASP message in normal cells. However, we have not found WASP mRNA levels in GOR1-HVS cells. The DNA mutations contained in the WASP gene of our established cell lines remains to be determined.

Attempts to determine whether a normal WASP gene can rescue our WAS cell lines are currently in progress.

In summary, by immortalizing the allospecific WAS T cell lines, we have improved our experimental system aimed to the understanding of the pathogenic mechanisms of WAS.

The transcription factor PU.1 is necessary for macrophage proliferation

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PU.1 is an *ets* related transcription factor that is specific for macrophages and B lymphocytes. In serum free media, bone marrow derived macrophages are able to produce colonies. In the presence of M-CSF, proliferation of PU.1 transfected macrophages was enhanced in relation to the controls. A similar effect was observed in the presence of GM-CSF, but not in the presence of IL1, IL2, IL3, IL4, IL6 or G-CSF.

Macrophages transfected with antisense PU.1 showed a decreased response in proliferation to M-CSF in relation to macrophages transfected with the control vector. A decreased proliferation was also observed when antisense oligonucleotides were added to the culture media containing M-CSF.

Mutations by alanines of the serines 41 and 45, that are sites of casein-kinase depending phosphorylation, abrogate the proliferative effect of PU.1.

These experiments demonstrated that the transcription factor PU.1 is necessary for macrophage proliferation and also that phosphorylation of PU.1 is required for the proliferative effect.

A TYROSINE-CONTAINING MOTIF MEDIATES ER RETENTION OF CD3-ε AND ADOPTS A HELIX / TURN STRUCTURE

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The CD3-ε endoplasmic reticulum (ER) retention motif has been characterized by mutagenesis and nuclear magnetic resonance spectroscopy. Tyrosine 177, leucine 180 and arginine 183 are involved in ER retention. The motif forms an elongated α-helix in which the tyrosine and leucine residues are closely apposed, followed by a βI' turn that places arginine 183 in the vicinity of leucine 180. The structure formed by tyrosine 177 and the leucine in position +3 is reminiscent of the β-turn structure adopted by tyrosine-containing endocytosis signals. Moreover, substitution of the transferrin receptor (TfR) internalization sequence by the CD3-ε motif still allowed the rapid internalization of the TfR and, conversely, the chimeric protein resulting from the substitution of the CD3-ε motif by the endocytosis signal of the low density lipoprotein receptor was ER located. These data support the idea of a functional homology between the two types of signal.

SECOND SESSION: B CELL DIFFERENTIATION DEFICIENCIES

Chairman: Fred S. Rosen

B CELL DIFFERENTIATION DEFECTS IN X-LINKED AGAMMAGLOBULINEMIA, IgA DEFICIENCY AND COMMON VARIABLE IMMUNODEFICIENCY

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Mutations in the Btk gene have been shown to be responsible for the arrest in B cell differentiation that occurs in boys with X-linked agammaglobulinemia (XLA) and the B lineage cells in female XLA carriers that use the X chromosome containing the defective Btk gene. The cellular abnormality is evident by the late pro-B to pre-B cell stage in differentiation. Our studies suggest that signals generated through the CD19 molecule and the IL-7 receptor are important in normal pro-B cell differentiation and that these signal pathways are impaired by function-loss mutations in the Btk gene. Selective IgA deficiency (IgA-D) and common variable immunodeficiency (CVID), the two most common primary immunodeficiencies, represent polar ends of a disease spectrum that reflect a common genetic predisposition. One clue to the identity of a susceptibility gene is the frequent occurrence of an extended complex of ancestral MHC genes featuring deletion of the C4A complement gene in family members having either IgA-D or CVID. C4A can bind to antigen-antibody complexes and can guide these to receptors on B cells to facilitate activation by antigen. An alternative hypothesis, that a class II gene in the ancestral MHC haplotype predisposes to the immunodeficiency, is rendered less likely by the absence of IgA deficiency in a group of Sardinian individuals who are homozygous for an MHC haplotype featuring an ancestral cross-over between the disease-associated class II genes and the region of the MHC gene complex containing the C4A gene.

X-linked agammaglobulinemia: A tyrosine kinase disease

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X-linked agammaglobulinemia (XLA) was the first immunodeficiency identified (Bruton 1952) and is characterized by reduced levels of immunoglobulin and B cells, resulting in a propensity for recurrent bacterial infections (Lederman & Winkelstein 1985, Sideras & Smith 1995). In a collaborative positional cloning project, a cDNA enrichment technique was employed using yeast artificial chromosomes from the implicated region of the X-chromosome (Vetrie et al. 1993, Vorechovsky et al. 1994). The same gene was also identified by searching for novel protein-tyrosine kinases (PTKs) (Tsukada et al. 1993).

The gene involved in XLA encoded a novel cytoplasmic PTK named Bruton's agammaglobulinemia tyrosine kinase (Btk). In another collaborative effort we could demonstrate that the mouse immunodeficiency Xid (X-linked immunodeficiency) is caused by a mutation in the mouse Btk gene (Thomas et al. 1993). Btk belongs to a new family of cytoplasmic PTK's formed by Tec, Itk (also named Tsk and Emt), reviewed in Smith et al. 1994, and Bmx (Tamagnone et al. 1994).

Btk is expressed in most hematopoietic cell lines with the absence of T lymphocytes and plasma cells (Smith et al. 1994). Btk represents the first cytoplasmic PTK in which mutations have been found to cause a hereditary disease. This gives us the unique possibility to make genotype-phenotype correlations in patients to directly delineate functionally important regions in the Btk molecule. In total more than a hundred mutations have been found. An international study group has recently been formed that will provide a mutation database. Each patient will be assigned a unique patient identity number and details on the mutation will be given.

We have recently used molecular modeling techniques to investigate the effect of mutations causing amino acid substitutions. A model of the kinase domain indicated structural clustering of functionally important residues (Vihinen et al. 1994).

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UNEQUAL CROSSING OVER AS SOURCE OF IGHC LOCUS VARIABILITY

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The Immunoglobulin Heavy chain Constant region (IGHC) genes are a multigene family located on chromosome 14 and organised in a 350-kb region as follows: M-D, G3-G1-EP1-A1, GP, G2-G4-E-A2. Their high reciprocal homology leads through meiotic mispairings to deleted and duplicated haplotypes.

The frequencies of these haplotypes in 668 healthy blood donors from 5 different Italian regions were determined by Pulsed Field Gel Electrophoresis and the genes involved in the rearrangements were characterised by Southern blotting with several IGHC probes and by densitometric evaluation of the films. 20 deleted (1.50%) and 60 duplicated (4.50%) haplotypes were identified, all in heterozygous individuals except for two homozygous deletions. The deletions/duplications involved one or more genes. GP-A2, A1-E and G4 duplications, and A1-E and GP-A2 deletions were the most common. In the same survey 5 triplicated haplotypes (two of them spanning from the A1 to E genes) were also found. All the rearranged haplotypes seem to be the result of unequal crossing-over. The difference between the number of duplications and deletions was significant in Sardinia, Lombardy, Puglia and in the total of 668 subjects ($p<0.001$). The crossing over events should produce an equal number of deleted and duplicated haplotypes. Several mechanisms can be postulated for this imbalance: i) negative selection against deleted haplotypes; ii) positive selection for duplications; iii) genetic drift.

Ig A deficiency. Clinical and immunological heterogeneity

Ig A deficiency (Ig A-D) is the most common primary immunodeficiency (PID). These patients form a very heterogeneous group ranging from asymptomatic cases to those presenting allergic manifestations, repeated infections, or autoimmune or malignant diseases. In some cases Ig A-D is associated with Ig G2 deficiency and may benefit from G. Globulin therapy Follow-up is lacking in most published series and very little is known of Ig A -D patients who develop more profound immunodeficiency during their life time.

Common variable immunodeficiency (CVID) is a PID diagnosed in most cases in young adults. Delayed diagnosis is very common and it usually takes some years for correct therapy to be established. The evolution of Ig levels over the years before definitive diagnosis is made is usually not reported and we do not know whether an Ig A-D was previously present.

Autoimmune diseases was diagnosed in 8.3 % of our series of 144 symptomatic patients.

The mothers of two girls have Ig A-D, and SLE was diagnosed in one of them.

Three cases of Ig A-D were diagnosed at 7, 8 and 14 years of age,two of them presented with autoimmune phenomena in two cases (chronic active hepatitis and SLE-like disease) and the third with repeated infections in the third All three were diagnosed of CVID 1, 6 and 12 years later, respectively. G.Globulin has been established with correction of the autoimmune and infectious manifestations (including the negativization of autoantibodies).

The main immunological abnormalities in these three cases are : Ig G2 deficiency , low CD4 number in the second case (with high ANA and anti-DNA antibodies), normal lymphocyte response to mitogens and anti-CD3.

CD40L expression in stimulated T-cells was also studied in those patients and other CVID and was normal in the majority.

Ig A-D and CVID may represent polar ends of the spectrum reflecting one underlying genetic defect. Genetic and immunological studies of symptomatic Ig A-D cases and closer follow-up will be needed to understand the causes and immunological evolution of these two common diseases.

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THIRD SESSION: GENE THERAPY

Chairman: Maxime Seligmann

T Lymphocyte Gene Therapy for ADA Deficiency (SCID): Results of the initial trial with 4 years observation.

R. Michael Blaese, Kenneth Culver, Charles Carter, Harvey Klein, Melvin Berger, Craig Mullen, Jay Ramsey, Linda Muul, Steven Rosenberg, Thomas Fleisher, Gene Shearer, Mario Clerici, Richard Morgan, A Dusty Miller, and W. French Anderson.

Two girls with severe combined immunodeficiency caused by an autosomal recessive defect in the gene for adenosine deaminase have been treated by retroviral-mediated gene therapy using periodic infusions of gene-corrected autologous T lymphocytes. Neither child had a HLA-matched sibling donor for bone marrow transplantation and both had objective evidence of incomplete immune reconstitution with PEG-ADA enzyme replacement therapy. Autologous peripheral blood T cells were obtained by apheresis, the T cells were induced to proliferate by stimulation with the anti-T cell receptor monoclonal antibody OKT3 and interleukin 2, and the induced proliferating T cells were then transduced by repeated exposures to supernatants containing the LASN retroviral vector. The T cells were expanded 20-100 fold in culture over 9-12 days and were then reinfused into the patients without selection for transgene expressing cells. The period of culture expansion was brief to maintain polyclonality of the T cell repertoire in each cell population. Each child received 11-12 infusions of these cultured, gene-modified cells during the first 23 months of the protocol and have then been under close observation for an additional 2 years to evaluate the overall effects of the treatment. Patient 1 has experienced restoration of total T cell numbers to the normal range. The concentration of ADA enzyme activity in her circulating lymphocytes has risen to roughly half heterozygote levels with an LASN vector DNA signal on Southern analysis equivalent to 0.5 to 1 copy of vector per circulating mononuclear cell. Anergic prior to beginning this treatment protocol, she acquired normal delayed hypersensitivity skin test reactivity, experienced reconstitution of cytokine production and cytolytic T cell activity, and enhanced antibody (isohemagglutinin) production during the first months of this treatment. In addition, she is reported to have experienced fewer episodes of infectious diseases requiring antibiotic treatment and has been able to regularly attend public school. The elevated peripheral blood T cell count and increased level

of ADA in the cells from patient 1 have persisted for the duration of this observation period demonstrating an unexpectedly long life span for peripheral T cells and showing that silencing of the integrated LASN vector has not been sufficient to eliminate therapeutic effectiveness.

Patient 2 had a more limited and variable immune deficiency before beginning the protocol and evaluation of her response is less definitive. She also experienced normalized total T cell counts and restoration of normal delayed hypersensitivity skin test reactivity. Isohemagglutinins rose from barely detectable to the normal range within 3 months of initiation of therapy. Cytolytic T cell function to influenza improved following the start of gene therapy while responses to allogeneic cells spontaneously improved before this treatment. The efficiency of gene transfer into the T cells from patient 2 was only about 10% of that achieved in patient 1 and her T cells underwent senescence in culture at day 13-16 compared to day 30+ for patient 1. These differences in intrinsic T cell behavior and proliferative potential may explain the presence of only 0.1 to 1% LASN vector containing cells in the circulating blood of this patient with lymphocyte levels of ADA consistent with this limited number of gene-corrected cells. The elevated peripheral blood T cells in patient 2 returned to pretreatment levels after about one year. There have been no significant side effects associated with the treatments and no evidence of the appearance of recombinant replication competent retrovirus in either child. We conclude that although much remains to be learned, infusion of culture-expanded autologous peripheral blood T cells modified by retroviral-mediated ADA gene-transfer can be a safe and effective addition to the therapy for this severe primary immunodeficiency disease.

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Thymic selection

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T lymphopoiesis is tightly controlled by α and β T cell receptor (TCR) genes that rearrange in temporal order and form first the pre-TCR and then the αβ TCR. Both receptors associate with signal transducing CD3 molecules. Signaling by both, the pre-TCR as well as the αβ TCR is essential for T cell development as evidenced by the fact that defective genes which prevent formation of these receptors or interfere with the signaling cascade lead to an arrest in T cell development. The pre-TCR consists of the TCR β chain in disulfide linkage to the pre-TCR α (pTα) chain. Its function is to induce extensive cell proliferation of early thymocytes that have succeeded in the formation of a productive TCR β gene and is accompanied by allelic exclusion of the TCR β locus, CD4/8 coreceptor expression and TCR α rearrangement as well as other subtile changes that generate shortlived $CD4^+8^+$ thymocytes. Signaling by the pre-TCR requires CDε but not CD3 δ signal transducing proteins and the $p56^{lck}$ kinase plays a major role in signal transduction. The pTα gene is expressed in immature but not mature T cells including pro-T cells in fetal blood as well as immature T cells in sites of extrathymic T cell development.

The αβ TCR must bind to class I or II MHC molecules to rescue immature T cells from programmed cell death. This binding also decides whether the rescued cells become $CD4^+$ helper or $CD8^+$ killer cells. The correlation of class MHC restriction and functional phenotype does not always hold and possible reasons for this will be discussed.

FOURTH SESSION: OTHER IMMUNODEFICIENCIES

Chairman: Maxime Seligmann

Virus infection models affecting CD3 activation

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Secondary immunodeficiencies are a heterogeneous group of disorders in which the immune alteration is not the primary defect but the result of several pathologic conditions such as viral infections, atopy, cancer, etc... Although the molecular mechanisms leading to immunodeficiency are mostly unknown, some of them have been associated with T-cell activation defects though several membrane molecules. Moreover, the complete knowledge of the molecular mechanisms generating the immune defect could led us to a better understanding of the immune normal physiology.

Infections by some viruses have been associated with selective TCR/CD3 activation defects: human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). The immunological characteristics of individuals infected by EBV and CMV will be presented.

EBV infection causes T-lymphocyte anergy probably due to a selective and proximal TCR/CD3 signal transduction defect resulting in a low proliferation, IL-2 production and IL-2 receptor expression. It is believed that a viral homologue of IL-10 may be involved in EBV-induced immunosuppression.

CMV infection causes also a selective T-lymphocyte anergy, at least "in vitro". Peripheral mononuclear cells from CMV infected patients showed a low proliferation to IL-2 synthesis in response to bacterial antigens, anti-CD3 monoclonal antibodies and some lectins whereas stimulation through TCR/CD3 independent pathways was normal.

The putative molecular mechanisms involved in these two secondary immunodeficiencies will be discussed.

LEUKOCYTE ADHESION DEFICIENCY (LAD)

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Cellular adhesion plays an essential role in the onset and regulation of immune and inflammatory processes. Most leukocyte adhesive functions are absolutely dependent on the expression and function of the leukocyte-specific heterodimers LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18), which comprise the ß2 integrin subfamily (1). Their essential role in leukocyte binding to and migration through the endothelial layer in inflamed tissues is illustrated by the existence of an immunodeficiency disorder termed Leukocyte Adhesion Deficiency (LAD). LAD is an autosomal recessive disease caused by the deficient expression of the three ß2 integrins on the cell surface of leukocytes (1,2). LAD patients exhibit delayed umbilical cord separation, impaired pus formation, and recurrent bacterial and fungal infections of soft tissues, as a consequence of the lack of neutrophil migration into sites of inflammation (1-4). Two clinical phenotypes of LAD (Severe and Moderate or Partial), which normally correlate with the severity of the disease, have been defined according to the level of ß2 integrin expression on patient leukocytes (3). LAD is originated by heterogeneous mutations within the common ß2 (CD18) subunit gene (5,6), which is located on chromosome 21q22 (7). Several types of LAD have been defined (I-V) based on the size and levels of the CD18 subunit precursor, the CD18 messenger RNA, and the resulting phenotype (5). Analysis of Severe and Moderate LAD CD18 alleles has identified aberrant splicing events (8-11), missense mutations (9-15), and a 1 bp deletion (14) within the structural region of the CD18 gene.

We have investigated the molecular basis of the disease in two unrelated Severe patients (HS and ZJO)(16). Both patients share a complete absence of CD18 protein precursor and cell surface expression, but they differ in the level of CD18 mRNA, which is normal in HS and undetectable by Northern blot in ZJO. Determination of the primary structure of the patient HS CD18 mRNA revealed a 10 bp deletion between nucleotides 190-200 (CD18 exon 3), which eliminates residues 41-43 and causes a frameshift into a premature termination codon 17 bp downstream from the deleted region. The 10 bp frameshift deletion maps to a region of the CD18 gene where aberrant mRNA processing has been detected in HS and two other unrelated LAD patients. In the ZJO patient, amplification of lymphoblast CD18 mRNA demonstrated the presence of a non-sense mutation in the third nucleotide of the triplet encoding ^{534}Cys (TGC→TGA), within exon 12. Both genetic abnormalities were also detected at the genomic level, and affect the restriction pattern of their corresponding genes, thus enabling the detection of the mutant alleles among healthy heterozygous in family studies. The identification of two new LAD CD18 alleles, either carrying a non-sense mutation (ZJO) or a partial gene deletion (HS), expand the repertoire of genetic abnormalities causing LAD and further indicate that the heterogeneity of the molecular basis of LAD is comparable to that of other genetic diseases.

Integrin functional activity is absolutely dependent on the state of cellular activation and differentiation (17). To be completely effective, the ligand-binding activity of integrins must be turned "on" and "off" in a very precise manner and during a brief period of time (e.g. during bleeding)(17). Such a rapid regulation of the integrin functional activity takes place on the plasma membrane, and cellular agonists rapidly modulate the integrin ligand binding properties, in a process that has been termed "inside-out" signalling (17). The regulation of affinity for ligands has now been demonstrated for numerous integrins and can be accomplished *in vitro* by cell stimulation with phorbol esters, by cross-linking certain cell surface receptors, or by treatment with activating monoclonal antibodies (17). To study the influence of the cellular environment on the leukocyte integrins functional activity and analyze their involvement in hematopoitic cell differentiation, we have developped stable stable transfectants of the three ß2 integrins in cultured cell lines whose differentiation can be induced *in vitro*. Like on circulating leukocytes, the integrins expressed on U937 or K562 cells were expressed in a constitutively inactive state, as demonstrated by the lack of adhesion to their cellular counterreceptors or soluble ligands, the absence of CD18-dependent

intercellular aggregation and their inability to mediate adhesion to protein-coated plates. However, while ß2 integrin adhesive functions in U937 cells could be induced upon cellular activation, their function in K562 cells was unaltered by cellular agonists and could only be upregulated with activating monoclonal antibodies. Unlike the ß2 subfamily, ß1 integrins expressed on K562 cells acquired the high affinity ligand-binding state during cellular activation, threfore suggesting that K562 cells are defective in a putative ß2-specific component of the inside-out signalling parthway. This suggestion raises also the possibility of the existence of alternative LAD patients whose molecular basis would map to the ß2-specific component of the cellular "inside-out" signalling pathway. The acquisition of CD11b/CD18-dependent adhesive functions in *c-fes*-transfected K562 cells (18) would indicate the involvement of a tyrosine kinase in this putative ß2-specific inside-out signalling pathway.

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ATAXIA-TELANGIECTASIA: PATHOGENESIS AND POSITIONAL CLONING.

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Several lines of evidence are converging to produce new insights into the cancer susceptibility of ataxia-telangiectasia (A-T) patients and heterozygotes. Defective or damaged DNA is tumorigenic and can result from inherited or somatic changes. Inherited defects in mismatch repair genes that maintain the integrity of DNA have recently been identified in colon cancer families (1-3). Cancer-associated mutations of p53, Rb and p16, all part of a pathway leading to G1 cell cycle arrest and the time needed to repair DNA damage from metabolic processes, have also been identified. Ataxia-telangiectasia (A-T) cells do not respond normally to ionizing radiation, showing a poor p53 response, a foreshortened G1/S delay, and an accumulation of cells in G2/M (4-6). These cell cycle transition defects are believed to result in the replication of poorly edited DNA. This new understanding may also explain the 38% incidence of cancer in A-T homozygotes and the 5-fold increased risk of breast cancer in female A-T heterozygotes (7).

In addition to our studies characterizing the cell cycle traverse in A-T cells (8-11), we are attempting to isolate the major A-T gene by positional cloning (12-15). Linkage analysis and genotyping data on 176 families in the A-T International Consortium database localize the major A-T gene to a ~500 kb region, between the markers A4(S1819) and A2(S1818). They localize the Group A A-T gene (ATA) to a region that contains the ~500 kb region. The region of the Group C gene also contains the ~500 kb region (6). The data are compatible with linking Groups D and E as well as ATFresno to 11q22-23, but not NBS-V1 or NBS-V2 (6,16), suggesting the presence of other loci, not at chromosome 11q22-23, for the latter two rare clinical variants. Four non-linking but classical A-T families contain only single affecteds, leaving open the possibility that these may be spontaneous mutations rather than evidence for another classical A-T gene outside the 11q22-23 region. On the other hand, the 11q22-23 region may contain more than a single A-T gene within it.

Concannon et al. have recently identified a 42 kb cosmid from the candidate region which corrects the radioresistant DNA synthesis of A-T Group A fibroblasts, but not fibroblasts from patients in Groups C or D (17). In collaboration with this laboratory, we are isolating cDNAs from the region so as to sequence them and screen patient mRNAs and DNAs for mutations, insertions or deletions. We have already characterized one gene of approximately 18 kb; we have sequenced the entire cDNA (4.3 kb) but find no mutations in over 80 patients. We have also translated its protein, using cDNA templates from 13 patients, and find no new or truncated proteins. We have defined the genomic boundary of that gene and are subcloning the remaining 24 kb of the cosmid to establish a better genomic map. Meanwhile, others in the lab are isolating cDNAs and GC-rich genomic clones from the 24 kb region, which we are screening for mutations. If these efforts fail to identify an A-T gene, we will return to our analyses of the larger ~500 kb candidate region.

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TWO EARLY PHASES OF LYMPHOHEMATOPOIESIS IN THE MOUSE EMBRYO. M.A.R. Marcos and S.G. Copin, Centro de Biologia Molecular S.O., CSIC-UAM, Madrid, Spain; I. Godin and F. Dieterlen-L., Institut d'Embryologie, Nogent-sur-Marne, France; S. Morales and M.L. Gaspar, Instituto Carlos III, Madrid, Spain.

Various inducing microenvironments support hematopoiesis throughout mouse lifespan, from the earliest embryo sites to the adult BM.

Multipotent hematopoietic stem cells (HSCs) are detected in paraaortic splanchnopleura SPL/AGM, yolk sac (YS), blood and liver in early (8,5-12 days) mouse embryos. The most immature progenitors intrinsically differ from adult BM HSCs in that they are bigger in cell size, Sca-1 $(Ly6A)^{-}$, and $AA4.1^{+}c\text{-}Kit^{+}$. On the basis of timing of appearance, kinetics of Ag expression and decrease in cell size, the following ontogenical sequence of cell populations has been characterized: A) $c\text{-}Kit^{+}AA4.1^{-}Mac\text{-}1^{-}$ --▶ B) $c\text{-}Kit^{+}AA4.1^{low}Mac\text{-}1^{-}$ --▶ C) $c\text{-}Kit^{+}AA4.1^{low}Mac\text{-}1^{low}$ --▶ D) $c\text{-}Kit^{+}AA4.1^{high}Mac\text{-}1^{high}$. In organs of the same developmental age, the most mature phenotype is present in SPL/AGM, followed by blood/YS, and being the liver the most delayed site in terms of hematopoietic cell differentiation. Studies of quantitative RT-PCR reveal a rather ubiquituous expression of c-Kit and CD34 genes in the 9-12 days embryo. Recombinase-encoding RAG-1 gene is firstly expressed in SPL/AGM between days 9,5-11, being delayed 0,5 days its expression in the YS. In the liver, RAG-1 gene expression is detected for the first time at day 12 of gestation, as well as in simultaneous blood and omentum. Pre-B cell specific λ5 gene transcription only shows up in day 13-liver (scnsitivity levels of the assay: 1-10 cells). Genomic IgH DJ rearrangements are, however, detected from day 9,5 on, in the mouse embryo. Two clear-cut patterns of DJH usage are observed: 1) Predominant usage of J4 in SPL/AGM, YS, blood, omentum and pre-day 12,5 liver, and 2) Randomized utilization of the four Js in post-day 13 liver.

We conclude that emergent embryonic hematopoiesis proceeds through site-specific, ontogenical pathways and speeds of maturation. Early B-lymphoid generation do not pass through a λ5-encoded pre-B cell receptor-expressing stage.

POSTERS

Structure/function analysis of the tyrosine kinase pp56^{lck} involved in the establishment of immunocompetence.

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pp56^{lck} is a tyrosine kinase mainly expressed in T lymphocytes. pp56^{lck} has been shown to be required for several specific T cell processes. Interestingly, mice overexpressing pp56^{lck} display a similar immunodeficient phenotype than mice lacking pp56^{lck} expression. We have analyzed which subdomains of pp56^{lck} are important to yield its enzymatic activity and its biological function. To investigate the functions of these subdomains we constructed and analyzed a collection of chimeras and deletion mutants of pp56^{lck}. The conclusions from our studies and their interest for gene therapy will be discussed.

CD40L EXPRESSION IN HYPER IgM SYNDROME AND CVID

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Absence of the ligand of CD40 molecules (CD40L) in activated T cells has been demonstrated to be the cause of abnormal T-B cell collaboration resulting in Hyper Ig M syndrome (HIGM). The incidence of this genetic abnormality in other humoral Immunodeficiencies is not well known.

We are interested in the role of this molecule in different forms of Hypogammaglobulinemias and non X-linked HIGM. We have therefore studied 4 boys and 2 girls with HIGM syndrome, 12 cases of Common Variable Immunodeficiency (CVID) and normal controls. Three of the boys with HIGM have a classical family history of an X-linked disease and one of the girls, 7 years old, is a congenital rubella syndrome. CVID patients, 10 males and 2 females, were diagnosed at young adult age except 4 boys diagnosed between 2 and 8 years of age. The two female cases were diagnosed of Ig A deficiency some years earlier and controlled because of autoimmune phenomena or repeated infections.

PBL cells were stimulated with PMA and ionomycin, 6-8 h. and labeled with a MoAb anti-CD40L (TRAP-1, provided by Dr. L. Notarangelo) and FITC-goat anti-mouse (indirect IF) The results were measured by a FACScan cytometer (B-D). CD69 was used as an activation marker control

Absence of CD40L molecules in activated T-cells was detected in the three cases with X-linked forms, but the expression was normal in the fourth boy and the girls. In CVID a few cases had very low expression but the majority had normal or near normal values. Correlation with the number of B cells in peripheral blood, Ig levels and severity of the clinical manifestations will be presented.

Knowledge of the genetic defects will aid understanding these heterogeneous ID and may have prognostic and therapeutic implications.

BamHI AND PstI POLYMORPHISM OF THE HUMAN CD50 (ICAM-3) GENE.

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The differentiation antigen, CD50 (or ICAM-3), is an adhesion molecule defined as a 120 kD highly-expressed and hematopoetically-restricted leucocyte surface glycoprotein. CD50 seems to be preferentially used by lymphocytes in the LFA-1-mediated binding during the initial interactions of immune recognition and lymphocyte activation, when the other two LFA-1 counter-receptors, CD54 (ICAM-1) and CD102 (ICAM-2) molecules, are not yet up-regulated.

Using a Restriction Fragment Lenght Polymorphism (RFLP) assay, we demostrated two polimorphism in the CD50 genomic map. We have observed a PstI polymorphism characterized by two bands (4.2 and 4.4 kb). In addition a BamHI polymorphism, defined by two other bands (4.2 and 4.5 kb), was also observed. Both polymorphism are quite informative in the population analysis: 4.2 kb PstI allele is present in 19% of the population and 4.4 kb PstI allele is present in 81% of the population, while 4.5 kb BamHI allele is present in 78% of the population and 4.9 kb BamHI allele is present in 22% of the population.

These data open a easy way to analyze both CD50 alleles in heterozygous people (more than 50%, if we use any of both polymorphisms), being possible by this method to study some genetic disorders, as chromosome deletions, where CD50 gen could be involved.

THE HUMAN AUTOSOMAL SCID AND OMENN'S SYNDROME: AN INCREASED RADIOSENSITIVITY WITHOUT DNA REPAIR DEFECT.

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About 20% of patients with Severe Combined Immunodeficiency (SCID) present a phenotype characterised by the absence of T and B lymphocytes, together with the presence of NK cells. This SCID phenotype is inherited as autosomal recessive and resembles the scid mutation in mice by several criteria. We analysed the radiosensitivity of marrow GM-CFU and fibroblasts from several patients with T(-) B(-) phenotype, patients with the Omenn immunodeficiency syndrome, patients with X-linked T(-) B(+) SCID, and healthy controls. GM-CFU and fibroblasts from T(-) B(-) SCID and Omenn's showed increased radiosensitivity as measured by survival curves following γ-ray irradiation. This increased radiosensitivity was comparable to that observed for the murine scid.

In order to better understand the qualitative and quantitative aspects of the human SCID defect we went on to analyse the kinetics of DNA repair following double-strand breaks. This was performed using Pulse-Field Gel Electrophoresis (PFGE) on C14-Thymidine labelled fibroblast-DNA and quantification with the PhosphorImager. The CHO mutant xrs6 cell line together with the K1 parental line were used as controls. Only a 40% repair was achieved following 50Gray irradiation of the xrs6 cell line. The defect persisted after 24h and was also demonstrable using 30Gray and 40Gray doses. In contrast, the K1 parental cell line achieved a 90% DNA-repair after 2h. Murine scid skin fibroblasts repaired at a much lower rate (50% after 5h) after a 50Gray irradiation; The reparation was however completed after 24h. Finally, neither cells from the T(-) B(-) SCID patients nor from Omenn's showed any defect in the capacity to repair DNA-double strand breaks (90% repair after 3h).

In summary, although the human autosomal SCID patients have an increased radiosensitivity, this defect is clearly different from the DNA-repair abnormality seen in the xrs6 mutant and may also differ from the murine scid mutation.

CLINICAL AND IMMUNOLOGICAL STUDY FROM A HYPER-IgM IMMUNODEFICIENCY SYNDROME.

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The present study emphasizes the clinical and immunological heterogeneity in Hyper-IgM immunodeficiency syndrome, something referred to some years ago. Our patient was first seen at the Hospital General de Asturias in 1978 and a diagnosis of Dysgammmaglobulinemia with severe deficits of IgG and IgA and marked elevation of IgM was made on the basis of serum immunoglobulin levels, and recurrent infections of bacterial origin. Histological examination of the lymph nodes was performed but unfortunately no germinal centers could be evaluated.

Clinical Features: The patient, a 31 year old man, with Hyper-IgM serum (1250 mg/dl), low IgA (< 7 mg/dl) and low IgG (< 33 mg/dl), who represents a sporadic case with no similarly affected relatives, either male or female. The clinical history showed the following infections: pneumonia, otitis, sinusitis occurring at an early age, meningitis at age 15 but with no etiological agent being demonstrated, and meningitis at age 30 caused by Neisseria meningitidis. No hematological disturbances (anemia, thrombocitopenia and neutropenia) were present. The patient received no replacement Ig-globulin at the time of immunological study.

Immunological study: Phenotypic analysis of peripheral blood mononuclear cells was performed using monoclonal antibodies from B-D and FITC goat anti human IgM, IgD,$(F(ab)'_2$ fragment) purchased from Caltag, and Tago respectively. Positive cells: CD3 = 86%, CD4 – 31%, CD8 = 52%, CD19 = 8%, CD20 = 9%. CD20 IgM-bearing cells = 4% and CD20 IgD bearing cells = 7%. Peripheral blood mononuclear cells (PBMC) proliferation assay was induced by mitogens, anti-CD3 monoclonal antibody and alloantigens. PBMC when stimulated with pokeweed mitogen *in vitro* secreted IgM exclusively.

Analysis of CD40 ligand expression: CD40 ligand surface expression by activated T cells was made following the activation and staining protocol described in Cell 72, 291-300 (1993). CD40lg and an unrelated protein Leu8lg were donated by D. Hollenbaugh and A. Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, USA). Activated T cells from this HIM patient show surface expression of CD40 ligand (30%, MFI = 66) The histogram image is quite different from the normal control

probably because the percentage of lymphocyte T CD4+ is smaller for the patient.

	Normal Control		HIM patient	
	%	MFI	%	MFI
CD3	87	138	76	106
CD4	54	54	40	46
CD40Ig goat anti-human IgG 1/400	50	46	30	66
Leu8Ig goat anti-human IgG 1/400	100	6	100	8
CD69	100	118	100	108
Isotype control	100	4	100	7

Preliminary molecular study on CD40 ligand: Total RNA from stimulated lymphocytes was extracted by the guanidium thiocyanate phenol-chloroform method according to Chomczynski et al and subjected to reverse transcriptase PCR (BRL RT) as described in Cell 72, 291-300 (1993). (see poster number 128) Direct sequence of PCR-amplified CD40 ligand cDNA is now being performed. No mutations were found from residue 169 to 247, a sequence where previous studies (Immunological Reviews, 1994, N°138) have placed 30% of the CD40-ligand gene mutants.

Conclusions:

We have identified a sporadic presentation of a Hyper-IgM immunodeficiency syndrome in a male patient whose activated T cells show CD40 ligand expression and it does not look like he is not an X-linked form of Hyper-IgM. Sequence of his CD40-ligand is being performed. Other functional studies are being considered.

The Authors thank Dr. D. Hollenbaugh and Dr. A. Aruffo for the kindly donating the CD40Ig and Leu8Ig reagents.

A RAPID PCR-SSCP MUTATION ANALYSIS OF THE BRUTON'S TYROSINE KINASE GENE IN X-LINKED AGAMMAGLOBULINAEMIA

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The identification of the *BTK* (*B*ruton's *t*yrosine *k*inase) gene defective in human immunoglobulin deficiency X-linked agammaglobulinaemia (XLA) and characterisation of *BTK* exon-intron boundaries has now allowed the analysis of mutations and polymorphisms at the level of genomic DNA. Using Southern blot analysis and the polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) assay, amplifying all 19 exons and the putative promoter region with a single annealling temperature, mutations have been identified in 19 out of 24 unrelated patients diagnosed as having XLA. Apart from a large deletion involving exon 19, nine missense (F25S, R288W, I370M, M509V, R525P, N526K, R562W, A582V and G594R), two nonsense (E277X and R525X), five frameshift and two splice site mutations have been found affecting most coding exons and all major enzyme domains. No mutations or polymorphisms were detected in the putative promoter region. A single nucleotide deletion located in the last exon, resulting in a truncation of the eight carboxy-terminal residues of Btk and a typical XLA phenotype, indicates structural and/or functional importance of Btk helix I in the catalytic domain. The structural consequences of Btk defects due to deduced amino acid substitutions were analysed with computer-aided molecular modelling. A rapid and sensitive individual mutation analysis of the *BTK* gene can now be achieved in a one-day procedure.

PILOT LINKAGE MAPPING OF SELECTIVE IMMUNOGLOBULIN A DEFICIENCY AND COMMON VARIABLE IMMUNE DEFICIENCY: LACK OF EVIDENCE FOR AN INVOLVEMENT OF THE MHC REGION IN FAMILIES WITH AUTOSOMAL DOMINANT SEGREGATION

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Screening of close relatives of Swedish patients with selective immunoglobulin A deficiency (IgAD) and common variable immune deficiency (CVID) for serum immunoglobulin levels has identified 32 multiplex families, in 25 of which the disorders segregated as an autosomal dominant trait. A high relative risk for sibs, low phenocopy rate and a permanent phenotype make the two disorders, which have been proposed to represent an allelic condition, amenable to genetic linkage analysis. In a pilot multicentre linkage study, involving the 16 most informative multiplex families with dominant inheritance of IgAD/CVID and comprising a total of 49 affected and 43 unaffected individuals, we have attempted to confirm previously reported genetic linkage of the disease to the major histocompatibility complex (MHC) region. Surprisingly, using both parametric and nonparametric linkage analysis with a series of microsatellite markers at and flanking the MHC region, no evidence for genetic linkage was found ($Z = -5.48$, -6.39, -3.48, -3.19, -10.57 and -9.50 at zero recombination fractions at D6S291, D6S273, D6S306, D6S464, D6S299 and D6S285, respectively; $0.30 < p < 0.85$ at all loci in an extended sib-pair analysis, ESPA). Negative lod scores were found for 15 out of 16 families using 5 tightly linked polymorphic loci mapped to the MHC region, including PCR-typed polymorphism at codon 57 of *DQB1* chain ($Z = -3.64$ at zero recombination fraction; $p = 0.63$, $\chi^2 = 0.11$ in ESPA). In accordance with these results, no evidence for linkage to the MHC region was obtained by computing lod scores in retrospect, based on the previously published segregation data at the MHC region in multiplex families with IgAD/CVID in more than one generation. Assuming a major locus under the dominant model, we conclude that, in contrast to type I diabetes mellitus, the previously reported allelic associations of largely sporadic IgAD/CVID to this region should be explained by reasons other than a presence of closely linked, deficient gene(s) at the MHC region co-segregating with the disease in our families.

APO-1/Fas (CD95) mediated T cell receptor induced suicide

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The APO-1/Fas (CD95) cell surface receptor is a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily and mediates apoptosis. Peripheral activated T cells (ATC) from lymphoproliferation (lpr/lpr) mutant mice that express a reduced number of APO-1 have a defect in T cell receptor (TCR)-induced apoptosis. This suggests that TCR-induced apoptosis involves APO-1. We tested this hypothesis in various human T cells: (i) malignant Jurkat cells, (ii) an alloreactive T cell clone (S13), and (iii) peripheral ATC. TCR triggering via immobilized anti-CD3 antibodies or Staphylococcus Enterotoxin B (SEB) superantigen induced expression of the APO-1 ligand and apoptosis in these cells. Anti-CD3-induced apoptosis of Jurkat cells was demonstrated even in single cell cultures. In all cases apoptosis was substantially inhibited by blocking anti-APO-1 antibody fragments [F(ab')2 anti-APO-1] and soluble APO-1 receptor decoys [immunoglobulin fusion proteins (APO-1-Fc)]. The APO-1 ligand was found in the supernatant of activated Jurkat cells as a soluble cytokine. Based on these findings we propose that TCR-induced apoptosis in ATC can occur via an APO-1 ligand mediated autocrine suicide. These results provide a mechanism for suppression of the immune response and for peripheral tolerance by T cell deletion.

* Both authors contributed equally to this work.

PERSPECTIVES

A. Fischer and A. Arnaiz-Villena

The general feeling is that the meeting was successful in discussing and gathering the most interesting question on inherited immunodeficiencies.

There were established the genetic defects underlying many immunodeficiencies; the pre-α TCR chain in pre-T cells was identified in humans; several "*knock out*" mice and their immune function were described and gene therapy for ADA deficiency was show to be effective.

It is inferred that the following questions should be addressed in the near future:

1.- To test which deficiencies are susceptible for gene therapy.

2.- To do population studies both to calculate the precise carrier numbers and to establish if certain genetic anomalies accompanying deficiencies are not polymorphisms.

3.- Population studies are also necessary to explain why some deficiencies (i.e.: IgA) are very common and the possible advantage of the defective genes could also be studied.

4.- Many other deficiencies should be studied with the available molecular and genetic methodology.

5.- The common but still unexplained relationship between immunodeficiency and autoimmunity should be investigated.

6.- A more comprehensive effort to classify inherited (and other) immunodeficiencies should be tried; severe combined immunodeficiency should only be regarded as severe symptomatology.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 6th and 7th of March, 1995, at the Instituto Juan March.

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