Instituto Juan March
de Estudios e Investigaciones

29 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Biochemistry and Regulation of Programmed Cell Death

Organized by

J. A. Cidlowski, H. R. Horvitz, A. López-Rivas and C. Martínez-A.

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PROGRAMME

THE BIOCHEMISTRY AND REGULATION OF PROGRAMMED CELL DEATH

MONDAY, May 23rd

Registration

Genes and Gene Products Implicated in Programmed Cell Death
Chairperson: T. Honjo

S. Nagata - Fas Ligand and Fas; a Death Factor and its Receptor.

P.H. Krammer - APO-1 Mediated Apoptosis in Normal and Malignant Lymphocytes.

G. Nuñez - Regulation of Cell Death by Bcl-2 and Bcl-x.

D.R. Green - Fas, Ras, Apoptosis and Immune Homeostasis.

E.A. Harrington - Integration of Cell Proliferation and Programmed Cell Death (Apoptosis) by c-Myc.

Control of Apoptosis in Tumor Cells and AIDS
Chairperson: D.R. Green

T.G. Cotter - Oncogene Expression in the Control of Apoptosis in Tumour Cells.

R. Buttyan - Anti-Apoptosis Genes and Prostate Cancer.

J.T. Isaacs - Mechanism of Programmed Death of Non-Proliferating Androgen-Independent Prostatic Cancer Cells Induced by Thapsigarcin.

J.C. Ameisen - Programmed Cell Death (Apoptosis) and AIDS Pathogenesis.

OPEN SESSION

H.R. Horvitz - Cell Death in Development and Disease.

M.C. Raff - Social Controls on Cell Survival and Death.

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TUESDAY, May 24th

Programmed Cell Death in Development
Chairperson: S. Nagata

H.R. Horvitz - Genetic Control of Programmed Cell Death in the Nematode *Caenorhabditis Elegans*.

J. Yuan - The Genes that Control Programmed Cell Death: from Worm to Mammal.

L.M. Schwartz - Cloning and Characterizing Putative Cell Death Genes from Moths and Mice.

R.W. Oppenheim - Different Types of Programmed Cell Death in the Developing Spinal Cord.

M.D. Jacobson - Possible Mechanisms of Programmed Cell Death and Bcl-2 Protection.

The Role of Programmed Cell Death in the Immune System
Chairperson: M.C. Raff

J.J. Cohen - Regulation of Apoptosis in the Immune System.

J.J.T. Owen - Programmed Cell Death during Selection of Immature T Cells in the Thymus.

T. Honjo - Signals that Can Rescue B Cells from Antigen-Induced Apoptotic Death.

C.Martínez-A - Apoptosis during B Cell Development and Activation.

Short Oral Presentations:

M.J.Fernandez-Sarabia - R-ras and Other Proteins that Associate with Bcl-2.

M. Esteban - Role of the Interferon-Induced Human Protein Kinase (p68) in the Induction of Apoptosis.

Z. Molnár - Vital Assay for Neuronal Cell Death in Organotypic Cultures.

U. Hibner - Apoptosis Induction Results from Uncoordinated Cell Cycle Regulation.

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WEDNESDAY, May 25th

Signals Implicated in Apoptosis
Chairperson: R.W. Oppenheim

S. Orrenius - Recent Studies of Signal Transduction in Lymphocyte Apoptosis.

J.A.Cidlowski- DNA Degradation during Apoptosis: Enzymes and Regulation by Signals.

Chairperson: J.J.T. Owen

A. Eastman - Intracellular Signal Transduction in Apoptosis.

M.K.L.Collins- Regulation of the Apoptotic Response to DNA Damage by Oncogenes and Cytokines.

A.López-Rivas- Mechanism of Induction of Apoptosis by Cytotoxic Agents.

Concluding Remarks.

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INTRODUCTION

A. López-Rivas and C. Martínez-A.

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Understanding how organisms develop, differentiate and maintain homeostasis remains one of the foremost problems in the biological sciences. Recent appreciation by the scientific community that apoptosis or programmed cell death plays a major role in these fundamental processes has stimulated great interest and excitement in understanding the mechanism, biology and control of the apoptotic process.

Often referred to as programmed cell death, apoptosis has been extensively reported in the scientific literature during the last 5 years through morphologic studies in many tissues where death was taking place as a consequence of a physiological phenomenon. In these studies the presence of scattered single cells with highly condensed chromatin has been one of the parameters indicating the presence of apoptotic cell death. In a later phase the chromatin appears distributed into sub-cellular structures called apoptotic bodies.

However, apoptosis has not only been observed in the balanced situation of physiological tissue turnover but also in the elimination of specific cell subsets that occurs in embryogenesis. Apoptosis has also been implicated as the mechanism of cell elimination during tissue regression in metamorphosis and during tumour growth and regression.

Because apoptotic cells are rapidly, and specifically, engulfed *in vivo* by neighbouring cells in order to keep cell debris to a minimum and to allow shrinkage of a tissue without disruption of its basic architecture, these morphological tissue studies probably greatly underestimate the number of deaths occuring. Indeed, it has been proposed that many cells in the normal animal are undergoing apoptosis, and that those which fail to enter this cell death program are rescued by essential "survival factors".

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In contrast to our appreciation of cell proliferation, our understanding of the mechanism of apoptosis is still rudimentary. The aim of this meeting has been to bring together for the first time the leading scientists to present and debate recent progress in the study of programmed cell death. The workshop held in Madrid has highlighted studies on apoptosis in diverse model systems ranging from nematodes to man. This unique comparative approach has helped to define both common and cell type specific features of the apoptotic process and extended our understanding of its relevance in development, differentiation and disease. In the meeting the mechanisms of activation and suppression of apoptosis were presented in term of both signal transduction and molecular biology of efector molecules. Other speakers analysed the tissue specific control mechanisms and recent findings of genes critical to programmed cell death. Furthermore, the aspects of the role of apoptosis in cancer, autoimmunity and AIDS were covered and discussed.

Finally, in the name of all participants we would like to thank Andrés González and the Fundación Juan March for their invaluable support in the organization of this superb meeting.

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Genes and Gene Products Implicated in Programmed Cell Death

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Fas ligand and Fas; a Death Factor and its Receptor

Shigekazu Nagata, Osaka Bioscience Institute,
Suita, Osaka 565, Japan

Homeostasis in vertebrates is tightly regulated by cell death as well as by cell proliferation. The death of cells during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover are "programmed cell death", which are mediated by a process called "apoptosis".

The anti-human Fas antibody has a cytolytic activity against human cell line expressing the Fas antigen (Fas). Molecular cloning of human Fas cDNA indicated that Fas is a cell surface protein of 45 kDa belonging to the TNF/NGF receptor family, and it mediates apoptosis (1). A domain of about 80 amino acids homologous to the TNF type I receptor in the cytoplasmic region of Fas is responsible for the apoptotic signal transduction (2). We have recently identified the natural Fas ligand in a cytotoxic T cell line (3). The Fas ligand is a type II membrane protein and a member of TNF family. The Fas ligand is expressed some CTL cell lines and activated splenocytes suggesting an involvement of the Fas system in CTL-mediated cytotoxicity.

Chromosomal mapping and genetic analysis of mouse Fas and Fas ligand genes indicated that mouse lymphoproliferation mutation (*lpr*) and generalized lymphoproliferative disease (*gld*) are mutations in the Fas and Fas ligand genes, respectively (4-6). In one allele of *lpr*, an early transposable element (ETn) is inserted in intron 2 of Fas gene, which causes premature termination and aberrant splicing of Fas mRNA (5). In other allele of *lpr*, a point mutation causes a replacement of amino acid in the cytoplasmic region of Fas, and abolishes the activity to transduce the apoptotic signal (4). On the other hand, in *gld*, a point mutation in the receptor-binding domain of the Fas ligand inactivates its activity (6). Since mice homozygous at *lpr* or *gld* locus develop lymphadenopathy, and suffer from autoimmune disease, it is likely that the Fas system plays an important role in development of T cells.

In addition to thymocytes, the Fas mRNA is expressed in the liver, heart, lung and ovary (7). Administration of agonistic anti-mouse Fas antibody into mice induced apoptosis in the liver, and quickly killed the mice causing a liver damage (8). These findings suggest an important role of the Fas system in programmed cell death in the liver, and a possible involvement of Fas in pathological tissue damage such as fulminant hepatitis.

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APO-1 MEDIATED APOPTOSIS IN NORMAL AND MALIGNANT LYMPHOCYTES.

P.H.Krammer

In the context of negative growth regulation of normal and malignant lymphocytes, the monoclonal antibody anti-APO-1 was raised. Anti-APO-1 induced programmed cell death, apoptosis.
Anti-APO-1 defined the novel cell surface molecule APO-1, identical to the Fas antigen. The APO-1 antigen was purified to homogeneity and found to be a transmembrane glycoprotein of 48 kD relative molecular weight, identical to the Fas antigen. Peptides of the purified APO-1 molecule were sequenced and the APO-1 cDNA was cloned. The APO-1 molecule is a novel member of the TNF receptor superfamily. The APO-1 mediated apoptosis signal required crosslinking of the APO-1 antigen. Two requirements for induction of apoptosis in the APO-1 system were identified: expression of the APO-1 antigen and an intact apoptosis signalling pathway. In human peripheral blood T and B lymphocytes, expression of the APO-1 antigen and susceptibility to anti-APO-1 induced apoptosis were dependent on the stage of activation and differentiation. APO-1 mediated apoptosis was found to be an essential mechanism of peripheral T and B lymphocyte deletion and tolerance.

Heterogenous APO-1 expression was found on a variety of human tumor cell lines *in vitro* and on cells from various tumors taken directly from patients. These malignancies include pre-T-ALL, pre-B-ALL, CLL, various other tumors of the lymphoid series, B lymphoblastoid cell lines, glioblastomas, mammary carcinomas, colon carcinomas and soft tissue tumors. Susceptibility of corresponding *in vitro* cell lines to induction of anti-APO-1 mediated apoptosis was heterogenous. In contrast, cells obtained directly from ATL patients were almost completely sensitive. Resistance to APO-1 mediated apoptosis may be determined, at least in part, by a bcl-2 independent anti-apoptotic program.
These data may help to understand programmed cell death, apoptosis, on the molecular level, to assess the role of APO-1 expression in diagnosis and prognosis, and to test apoptosis as a concept of a rational intervention strategy in various diseases.

Tumorimmunology Program, Division of Immunogenetics, German Cancer Research Center, INF 280, 69120 Heidelberg, FRG.

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Regulation of Cell Death by Bcl-2 and Bcl-x.

Gabriel Nuñez.
Department of Pathology, The University of Michigan Medical School. Ann Arbor, Michigan.

Most examples of cell death in animals are controlled by a genetic program that is activated within the dying cell. The apoptotic process is further regulated by a set of genes that acts as repressors of cell death. Of these, *bcl-2* is expressed in a variety of embryonic and postnatal tissues which suggests a critical role for *bcl-2* in organogenesis and tissue homeostasis. Within lymphoid tissues, Bcl-2 is highly expressed in the earliest stages of lymphoid maturation (pro-B cells and $CD4^-CD8^-$ thymocytes), downregulated at the pre-B/IgM^+IgD^- and $CD4^+CD8^+$ stages and upregulated in mature B and T cells. Similarly, the expression of Bcl-2 is downregulated in B cells of the GC, a site associated with extensive apoptosis and selection of B-cells. Together these findings indicate that diminished expression of Bcl-2 is a common feature of those developmental stages characterized by extensive apoptosis and clonal selection. Thus, diminished Bcl-2 expression may facilitate the demise of lymphocytes after a death signal. Consistent with this is the finding that susceptibility to glucocorticoid-induced cell death in developing B cells correlates with the amount of endogenous Bcl-2 protein expresssed by the cells. Thus, developmental regulation of Bcl-2 may serve as a mechanism to modulate the susceptibility to cell death and ensure appropiate lymphoid selection during development.

Bcl-x is a new member of the *bcl-2* gene family which was recently isolated from gene libraries by hybridization with a *bcl-2* probe and by expression cloning using brain cDNA libraries transfected into death-susceptible B cells (González-García et al. unpublished). Two *bcl-x* cDNAs, *bcl-x*$_L$ and *bcl-x*$_S$, were isolated in the human. The predicted Bcl-x$_L$ protein displays high level amino acid and structural homology to Bcl-2. As with Bcl-2, transfection of *bcl-x*$_L$ into IL-3-dependent cells prevented their apoptotic cell death following growth factor deprivation. The predicted *bcl-x*$_S$ protein differs from Bcl-2 in that an internal region of 63 amino acids displaying the greatest homology to Bcl-2 has been deleted by alternatively splicing of the primary *bcl-x* mRNA transcript. Although transfection of *bcl-x*$_S$ failed to inhibit cell death, it in fact, facilitated PCD by inhibiting the death suppresor activity of Bcl-2. *Bcl-x* mRNA is expressed in a variety of tissues in the chicken. Importantly, the expression of the *bcl-x*$_L$ and *bcl-x*$_S$ forms appears differentially regulated in human tissues. In order to further assess the role of *bcl-x*, the murine *bcl-x* homolog has been isolated and characterized. Expression analysis have shown that *bcl-x*$_L$ is the major *bcl-x* mRNA form expressed during embryonic and postnatal

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development when compared to other *bcl-x* mRNA species. In addition to *bcl-x$_L$*, another form of *bcl-x* (termed *bcl-x$_\beta$*) is expressed in murine tissues. *Bcl-x$_\beta$* results from an unspliced *bcl-x* transcript and its predicted product lacks the hydrophobic C-terminal domain present in Bcl-x_L and Bcl-x_S. The function of Bcl-xβ is unknown. Both murine *bcl-x$_L$* and *bcl-x$_\beta$* are highly expressed in bone marrow and thymus but downregulated in spleen and lymph nodes. Electron microscopic studies indicate that the bulk of the Bcl-x_L protein, like Bcl-2 localizes to the periphery of mitochondria which suggest that both proteins function in a similar manner to prevent cell death. *Bcl-x$_L$* is expressed at higher levels than *bcl-2* during embryonic development which offers a plausible explanation to account for the normal development of mice with targeted disruption of the *bcl-2* gene.

The mechanism whereby Bcl-2 and Bcl-x_L inhibit cell death is poorly understood. One possibility is that Bcl-2 directly or indirectly interferes with the function or activation of proteins involved in the delivery of cell death signals. One of the putative effector proteins is the product of the tumor suppressor gene *p53*. Wild-type p53 is required for some forms of PCD in thymocytes, and its expression in transfected cells triggers PCD or cell-cycle arrest. Interestingly, we find that Bcl-2 can inhibit p53-mediated apoptosis. Cells co-expressing wild-type p53 and Bcl-2 remain viable but are arrested throught the cell cycle. Expression of c-myc rescued the cells from cell cycle arrest and allowed cell proliferation. Interestingly, Bcl-2 and c-myc expression interfered with the nuclear localization of p53 during a critical period of the G1 phase of the cell cycle when cells are susceptible to p53-induced cell death.

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Fas, Ras, Apoptosis, and Immune Homeostasis

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Apoptosis induced by ligation of the CD95 molecule has been implicated in function of some cytotoxic T cells, and is likely to play roles in the maintenance of immune homeostasis. The latter is based on the consequences of mutations affecting the function of this receptor or its ligand. Despite the probable importance of CD95, the signal transduction events mediating the induction of apoptosis have not been elucidated. Further, the effects of the lpr mutation in CD95 on immune regulation in vivo have not been examined in detail. Both of these problems will be addressed.

Based on considerations regarding the role of oncogenes in driving apoptosis versus proliferation of cells (e.g., Myc), we speculated that the Ras pathway might function in signal transduction events leading to apoptosis in some cases. Therefore we examined the effects of CD95 ligation on Ras activation. Ras and MAP kinase are activated within minutes of exposing Jurkat cells to anti-Fas antibody. The activation of Ras was found to be critical for subsequent Fas-induced apoptosis. This was demonstrated using several techniques including transient expression of a dominant negative Ras protein (Ras^{Asn17}), electroporation of a neutralizing anti-Ras antibody, and microinjection of either Ras^{Asn17} protein or the anti-Ras mAb. In all cases, inhibition of Ras function significantly inhibited apoptosis induced by ligation of CD95.

Activation of Ras by CD95 may occur via the activation of a guanine nucleotide exchange factor, Vav. Ligation of CD95 causes a rapid activation of Vav activity, in a manner that does not appear to involve a role for tyrosine kinases. One mechanism whereby Vav can be activated without tyrosine phosphorylation is via lipid mediators such as ceramide.

We therefore examined ceramide release following ligation of Fas, and found a simultaneous release of ceramide and depletion of sphingomyelin, as expected if sphingomyelinase is activated. Further we found that synthetic ceramides induce Vav, Ras, and apoptosis in Jurkat cells. Finally, we observed that apoptosis induced by exposure of Jurkat cells to ceramides or to sphingomyelinase is dependent upon Ras activation.

To address the possible roles for CD95 in immune homeostasis we examined central and peripheral deletion of T cells in response to in vivo administration of a superantigen, SEB. We employed young (4-6 week old) lpr/lpr mice that displayed no obvious immune abnormalities and in which mature T cells were fully responsive to in vitro challenge. While we could find no effect of the lpr mutation on SEB-induced negative selection in the thymus, we found a dramatic effect on SEB-induced deletion of mature T cells in the periphery. That is, responsive cells expanded as in +/+ controls, but the subsequent drop in cell number was profoundly delayed in the lpr mice. This decline in cell number was associated with apoptosis in the T cells from +/+ mice, which was absent in the cells from SEB-injected lpr animals. Thus, CD95 is likely to play an important role in the homeostatic process of peripheral deletion.

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INTEGRATION OF CELL PROLIFERATION AND PROGRAMMED CELL DEATH (APOPTOSIS) BY C-MYC

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Regulation of multicellular architecture involves a dynamic equilibrium between cell proliferation, differentiation with consequent growth arrest, and cell death. Apoptosis is a form of active cell death characterised by destruction of chromatin, cellular blebbing and condensation, and vesicularisation of internal components.

The c-*myc* proto-oncogene encodes an essential component of the cell's proliferative machinery and its deregulated expression occurs in most neoplasms. Intriguingly, c-*myc* is also a potent inducer of apoptosis in cells deprived of growth factors or forcibly arrested with cytostatic drugs. Myc-induced apoptosis is dependent upon the level at which it is expressed and deletion mapping shows that regions of c-Myc required for apoptosis overlap with regions necessary for co-transformation, autoregulation, inhibition of differentiation, transcriptional activation and sequence-specific DNA binding. Moreover, induction of apoptosis by c-Myc requires association with c-Myc's heterologous partner, Max. All of this strongly implies that c-Myc drives apoptosis through the transcriptional modulation of target genes.

Two simple models can be invoked to explain the induction of apoptosis by c-Myc. In one, death arises from a conflict in growth signals that is generated by the inappropriate or unscheduled expression of c-Myc under conditions that would normally promote growth arrest. In this "Conflict" model, induction of apoptosis is a not a normal function of c-Myc but a pathological manifestation of its deregulation. The other model holds that induction of apoptosis is a normal obligate function of c-Myc that is usually suppressed by the action of specific survival factors. We show that c-Myc-induced apoptosis in fibroblasts is suppressed by the insulin-like growth factors and PDGF independently of cell cycle or whether apoptosis is triggered by low serum or by DNA damage. In the case of serum deprivation, death proceeds by a p53-independent mechanism.

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Control of Apoptosis in Tumor Cells and AIDS

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ONCOGENE EXPRESSION IN THE CONTROL OF APOPTOSIS IN TUMOUR CELLS.

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Cancer has generally been thought of as a disease that results from a perturbation of cell proliferation rates. However, recent evidence has indicated that abnormalities in cell death, particularly via apoptosis, may also be a major contributory factor in the genesis of malignant disease. In this regard we have identified two oncogenes c-myc and c-abl, which have a regulatory role to play in apoptosis. Elevated expression of myc enhances apoptosis, whereas increased levels of abl inhibit apoptosis in tumour cells. In relation to abl an increased drug resistance and prolonged life-span was seen in the CML cell line K562 which over-expresses this gene. Both characteristics being reversed, when expression of the gene was down-regulated with antisense oligonucleotides. CML cells from newly diagnosed patients showed a similar drug resistance and prolonged life-span pattern. These data indicated that aberrant expression of genes which regulate apoptosis may lead to tumour development and affect the outcome of chemotherapy.

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Anti-Apoptosis Genes and Prostate Cancer

Ralph Buttyan, Ph.D.

Metastatic prostate cancer is treated by castration. This therapy is initially useful because most prostate cancer cells are dependent on male (androgenic) steroids and will die by means of apoptosis. Unfortunately, a fraction of the cancer cells inevitably survive this therapy and it is these hormone-refractory cancer cells that must be recognized and targeted for novel therapies to reactivate the apoptosis pathway. In the past, androgen-dependency was believed to be a simple matter of the androgen receptor content of the cancer cell. With the advent of useful antibody probes for detecting and measuring androgen receptors, there is no simple correlation can be established between receptor content and hormone-dependence of prostate cancer. Rather, in this overview, evidence will be presented that escape from hormone-dependence occurs through genetic mechanisms involving direct alterations in the expression or function of gene products that directly suppress apoptosis. This hypothesis derives from a number of studies of gene activity associated with prostate regression in the rat as well as studies of primary specimens of human prostate cancers. Moreover, the use of a prostate cancer cell line, LNCaP, has enabled us to establish an *in vitro* model to study prostate cell apoptosis and test the effects of individual gene products on the apoptotic response.

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MECHANISM OF PROGRAMMED DEATH OF NON-PROLIFERATING ANDROGEN-INDEPENDENT PROSTATIC CANCER CELLS INDUCED BY THAPSIGARGIN. Yuzo Furuya and John T. Isaacs, Baltimore, MD

Although lethal in metastatic patients, <5% of androgen independent prostatic cancer (PC) cells proliferate/day making anti-proliferating cytotoxic therapy of limited value. Methods for inducing the death of non-proliferating androgen independent PC cells are urgently needed. One approach is to activate the pathway for cellular suicide, termed programmed cell death (PCD) within these cells. For example, Thapsigargin (TG), a sesquiterpene γ-lactone, is a cell permeable, potent inhibitor (IC_{50} = 30nM) of endoplasmic reticulum (ER) Ca^{2+} pumps within androgen-independent PC cells. TG treatment thus depletes the ER pool of Ca^{2+} resulting within minutes in a 2-3 fold elevation in intracellular free Ca^{2+} (Ca_i) which generates a diffusible messenger increasing permeability of the plasma membrane allowing influx of extracellular Ca^{2+} sustaining elevation in Ca_i for hrs. This leads to morphological changes and new protein synthesis within 6-12 hr. Within 24 hr, cells arrest in G_0 and irreversibly lose proliferative ability. During the next 24-48 hr, the cells fragment their DNA and undergo fragmentation into apoptotic bodies. To determine whether induction of PCD by TG is due to depletion of ER pools of Ca^{2+} or elevation in Ca_i, calbindin-D gene, encoding a 28Kd calcium binding protein, was transfected into PC cells. Calbindin-D expressing clones, confirmed by immunohisto-chemical and Western analyses, were isolated. Exposure of parental and control transfectants to 500nM TG for 36 hr results in a 3-4 fold elevation in Ca_i and in ≥50% of cells undergoing PCD even when subsequently maintained in TG free media. In contrast, 36 hr TG treatment of calbindin-D expressing transfectants produces less than a 2 fold increase in Ca_i and ≥60% inhibition of the number of cells undergoing PCD. If calbindin-D transfectants are exposed to TG for ≥ 48 hr, however, this protection is lost, consistent with influx of extracellular Ca^{2+} eventually exceeding the binding capacity of intracellular calbindin-D thus sufficiently elevating Ca_i in these calbindin transfectants to activate PCD.In conclusion, TG can sustain an elevation in Ca_i derived from the extracellular Ca^{2+} activating PCD in non-proliferat-ing androgen independent PC cells.

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PROGRAMMED CELL DEATH (APOPTOSIS) AND AIDS PATHOGENESIS

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We have proposed[1,2] that the pathogenesis of AIDS may be related to the inappropriate induction of PCD in various cell populations, including CD4+ T cells and neurons, as a consequence of indirect interference of HIV with inter- and intracellular signaling; and that modulation of cell signaling may therefore have therapeutic implications in HIV-infected persons. Experimental support for this model has been provided by *in vitro* observations from several laboratories including ours, indicating that CD4+ and CD8+ T cells from HIV-infected persons are abnormally programmed for PCD[3-6]; that the cytopathic effect of HIV in CD4+ T cells is related to PCD[7,8]; that HIV envelope-mediated CD4 crosslinking can induce PCD in uninfected CD4+ T cells[6,9-11]; and that HIV-mediated PCD induction in immature T cells[12] and in hematopoietic progenitors[13] may prevent the renewal of mature peripheral T cells[10].
Using pathogenic and non pathogenic models of primate lentiviral infections, we have addressed the question of the significance of CD4+ and CD8+ T-cell PCD to AIDS pathogenesis. Primates explored included HIV-1 infected chimpanzees; SIVagm-infected african green monkeys; rhesus monkeys infected with the nonpathogenic molecular clone SIVmac251, that do not develop disease; and rhesus monkeys infected with the pathogenic wild type SIVmac251 strain that induces an AIDS-related disease. CD8+ T-cell PCD was observed both in pathogenic and non pathogenic models of chronic lentiviral infection. In contrast, CD4+ T-cell PCD was only detected in rhesus monkeys infected with the pathogenic strain of SIV, as in HIV-1 infected humans, suggesting that abnormal priming of CD4+ T cells for PCD during chronic lentiviral infection is closely related to AIDS pathogenesis[14].
We initially proposed[1,2] that abnormal priming of CD4+ T cells for PCD may be related either to envelope-mediated CD4 crosslinking, or to an inhibitory effect of HIV on the capacity of accessory cells, such as monocytes, to provide effective cosignals to activated T cells. Several laboratories including ours have reported that PCD induction in normal mature T cells can be regulated by accessory cells and various cytokines[15-18]. Since a switch from the type 1 to type 2 cytokine profile has been recently proposed to be involved in AIDS progression[19,20], we have addressed the question of the possible relationship between type1/type 2 cytokines and activation-induced T-cell PCD in HIV-infected persons.
Our results suggest that, in HIV-infected persons, the progressive loss of CD4+ TH1-cell function is not related to a TH1 to TH2 switch, but to the fact that activation of the CD4+ TH1 cells results in their rapid deletion by PCD. T-cell PCD induction involves an abnormal susceptibility of the TH1 cells to normal (very low) levels of the type 2 cytokines IL-10 and IL-4, that may be related to a defect of the type 1 cytokine IL-12 by accessory cells;

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accordingly, PCD of T cells from HIV-infected persons is prevented by the addition of either IL-12 or anti-IL-10 and anti-IL-4 antibodies[21].

Our findings have two implications. The first one is that cytokine-mediated PCD may play a general role in TH1/TH2 regulation, type 1 or type 2 cytokines contributing to TH1 or TH2 expansion by inducing PCD in the converse TH CD4+ cell population. The second implication directly relates to AIDS pathogenesis. If accessory cell dysfunction represents, as we initially proposed, a major pathological feature in HIV-infected persons, it is tempting to speculate that accessory cells may secrete the cytokines or express the cell surface molecules that normally play an essential role in TH1 PCD induction during an efficient process of TH1 to TH2 switch in the absence of cytokines that normally allow the concomitant expansion of the TH2 CD4+ cells. Accordingly, a progressive loss of TH1 CD4+ cells would occur in HIV-infected persons in the absence of a compensatory expansion of TH2 CD4+ cells. In such a conceptual framework, an abortive TH1/TH2 switch could at least partly account for both the progressive CD4+ T-cell dysfunction and depletion that lead to AIDS.

Analysis of the lentiviral, and cellular genes involved in the control of T-cell PCD may contribute to the understanding of AIDS pathogenesis, and to the delineation of therapeutic strategies aimed at the prevention of the disease.

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CELL DEATH IN DEVELOPMENT AND DISEASE

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Like the processes of cell division, cell differentiation and morphogenesis, the process of cell death constitutes a fundamental aspect of animal development. Naturally-occurring or programmed cell death is a normal and often major feature of the development of a wide diversity of cell and tissue types. In general, cell death appears to be an active process on the part of the cell that dies, involving continuing gene expression and alterations in the set of genes expressed. Specific genes have been identified that are causally involved in the process of cell death and that function within cells that die to cause cell death. The misregulation of programmed cell death seems likely to be involved in a variety of human diseases. For example, the cellular increase associated with the cancerous growth in follicular lymphoma can result from blocks in programmed cell death. By contrast, the cellular loss associated with neurogenerative disorders and with AIDS might result from too much programmed cell death. Thus, an understanding of programmed cell death should provide new routes toward the discovery of pharmaceutical agents that might be used to treat such diseases. The molecular mechanisms responsible for programmed cell death appear to be highly conserved, with comparable genes and proteins functioning in animals as diverse as nematodes and humans. Studies of the biology of cell death in different animals should be complementary and promise to reveal a pathway for programmed cell death that might well be universal among animals.

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SOCIAL CONTROLS ON CELL SURVIVAL AND DEATH

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We have been exploring the possibility that all vertebrate cells are programmed to kill themselves unless they are continuously signalled by other cells not to do so. Some cells clearly operate in this way, but it is uncertain how general this death-by-default mechanism is.

Oligodendrocytes and their precursors work in this way. They need signalling molecules secreted by other cells to avoid programmed cell death (PCD) *in vitro*. About 50% of newly formed oligodendrocytes normally die in the developing rat optic nerve, and this death can be inhibited by the experimental delivery of exogenous survival factors, suggesting that it may reflect a competition for limiting amounts of survival factors.

There is large-scale cell death in the normal developing rat kidney and much of it can be inhibited by treatment with either EGF or IGF-1, suggesting that it may reflect a lack of adequate survival factors.

Rat lens epithelial cells and chondrocytes can both survive for weeks when cultured at high density in the absence of exogenous signalling molecules, but they undergo PCD when cultured at low density. Conditioned medium from high-density cultures promotes the survival of the cells in low-density cultures, suggesting that these cells depend on signals from other cells of the same type to avoid PCD.

A variety of cell types, including anucleate cytoplasts, undergo PCD when treated with a high concentration of staurosporine, even in the presence of protein synthesis inhibitors, suggesting that in most cells all the protein components of the death program are in place. Blastomeres seem to be an important exception: they do not need signals from other cells to survive and they do not undergo PCD in response to staurosporine.

It seems then that cell survival, like cell proliferation, requires signals from other cells. Such "social controls" ensure that our cells only survive when and where they are needed and only divide when new cells are required.

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Programmed Cell Death in Development

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GENETIC CONTROL OF PROGRAMMED CELL DEATH IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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Of the 1090 nuclei generated during normal development of the *Caenorhabditis elegans* hermaphrodite, 131 undergo programmed cell death. We have characterized developmentally, genetically and to some extent molecularly the roles of 11 genes that function in all 131 of these programmed cell deaths. Three of these genes control the onset of the death process, seven act in the phagocytosis of dying cells by their neighbors, and one functions in the digestion of the DNA of cell corpses. Three additional genes specify which cells will or will not express this cell death program. Some of these genes show structural and functional similarities to genes that act in cell death in vertebrates. For example, the nematode gene *ced-9*, which protects against programmed cell death in *C. elegans*, is similar to the human oncogene *bcl-2*, which also protects against cell death. The nematode gene *ced-3*, which causes cell death in *C. elegans*, is similar to the human gene that encodes the enzyme interleukin 1-beta converting enzyme, which can cause the programmed death of mammalian cells. These findings suggest that molecular mechanisms responsible for programmed cell death are broadly conserved among organisms as diverse as nematodes and humans.

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THE GENES THAT CONTROL PROGRAMMED CELL DEATH: FROM WORM TO MAMMAL

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Cell death is a common aspect of normal animal development, tissue homeostasis and pathogenesis. More and more evidence indicates that cells possess a suicide mechanism. Our goal is to isolate vertebrate genes that might act in the process of programmed cell death. One approach is to isolate vertebrate homologs of *C. elegans* cell death genes. Two genes, *ced-3* and *ced-4*, have been isolated that function in controlling the onset of programmed cell death in *C. elegans*. The *C. elegans* gene *ced-3* is homologous to the mammalian interleukin-1β converting enzyme (ICE). We have shown that overexpression of ICE induced rat-1 fibroblasts to undergo programmed cell death. Expression of cowpox virus gene crmA, a specific inhibitor of ICE, protected the death of chicken dorsal root ganglia neurons induced by nerve growth factor deprivation. Thus, the genes in ICE/Ced-3 family may mediate cell death during vertebrate neuronal development. The other approach is to identify directly the vertebrate genes that might be involved in controlling programmed cell death. The model we are using is the interdigital cell death during chicken limb morphogenesis. Seventeen monoclonal antibodies have been isolated in this laboratory that recognize specifically those interdigital cells undergoing programmed cell death. These antibodies recognize not only the interdigital dying cells but also dying cells everywhere in the embryos. These antibodies are excellent markers for cells that are undergoing programmed cell death. Experiments are on going in this laboratory using these monoclonal antibodies to examine cell death under different conditions to address the question whether mechanism of programmed cell death is involved in these cell deaths. These experiments will lead to a better understanding of the mechanisms of programmed cell death in vertebrates.

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CLONING AND CHARACTERIZING PUTATIVE CELL DEATH GENES FROM MOTHS AND MICE

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In the 1960's, several laboratories demonstrated that the programmed death of cells in developing animals could be delayed or prevented, by judicious treatment with inhibitors of RNA or protein synthesis. These observations were interpreted as suggesting that programmed cell death (PCD) requires *de novo* gene expression. Subsequent experiments by Horvitz and colleagues [1] elegantly demonstrated the existence and importance of such genes. Many laboratories, including ours, are now actively trying to clone and characterize putative cell death genes from a wide variety of organisms. We have focused our studies predominantly on two PCD model systems: metamorphosis in moths and negative selection of T cells in mouse.

Working with the tobacco hawkmoth *Manduca sexta*, we have been examining the death of the intersegmental muscles (ISMs) and the motor neurons that innervate them. These cells die in response to a defined hormonal trigger following the emergence of the adult moth[2]. The ISMs are composed of sheets of giant cells (each cell approximately 5mm long and 1mm in diameter) that can be easily removed from the animal for study. *In vivo* labeling experiments with ^{35}S-methionine, followed by 2D PAGE analysis, suggests that 9 the expression of 9 genes is repressed and an additional 29 are up-regulated at the time of cell death. Using a variety of screening protocols, we have cloned or identified 3 of the repressed genes (actin, myosin heavy chain[3], and a 28kD subunit of the multicatalytic proteinase (MCP)) and ten of the up-regulated genes (polyubiquitin [4] and apolipoprotein III [5]). Levels of ubiquitin mRNA and protein increase greater than ten fold during the first few hours of death. As well, the enzymatic pathway that utilizes ubiquitin for tagging and degrading cellular proteins is likewise increased. We have examined ISM MCP and found that there is a dramatic increase in its proteolytic activity. Correlated with these changes is the appearance of four new subunits within the moiety. These data suggest that ubiquitin-dependent proteolysis plays a major role in the death of the ISMs.

Many of the genes isolated from the ISMs have predicted protein sequences that are not readily identifiable. We have generated polyclonal antibodies against these proteins and have found that they label dying neurons and muscles in *Manduca*. In order to extend these studies, we have begun to search for mammalian homologs of these genes. Using PCR and library screening, we have isolated a mouse homolog for the 18-56 gene. The mouse gene shares 91% identity with the moth sequence. Another clone, 18-44a, shares 55% identity with an expressed sequence tag (EST) from the human brain. We plan to use these genes to study PCD during mouse development.

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Recently, we have begun to examine the death of T cells in the mouse thymus during negative selection. Using a T cell Receptor (TCR) transgenic mouse, we have isolated several genes that are either activated or repressed during the apoptotic death of these cells. One of the up-regulated genes encodes *nur77*, an immediate early gene of the steroid/thyroid receptor super family[6]. Both cell death and *nur77* expression are initiated with treatments that mimic TCR engagement. When *nur77* activity is blocked by antisense, cell death in a substantial number of the cells is prevented.

A second gene whose expression is up-regulated with TCR-induced death is *apt-4*. Transcripts for this gene appear about 4 hours after triggering cell death. We do not currently know the identity of the protein product for *apt-4*. Nevertheless, we are very interested in this gene since its blockade with antisense prevents T cell hybrid death in response to not only TCR engagement, but also glucocorticoid and irradiation. These data suggest that *apt-4* acts downstream in the cell death pathway as an essential gene in the apoptosis program of these cells.

It is our hope that the identification and characterization of these various cell death associated genes will provide insight into the molecular mechanisms that mediate cell death.

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Different Types of Programmed Cell Death in the Developing Spinal Cord

Ronald W. Oppenheim, Ph.D.

Programmed cell death (PCD) of neurons in the developing nervous system has previously been thought to represent a common and uniform type of degeneration known as naturally occurring or target-dependent neuronal death. In this type of PCD post-mitotic neurons are thought to undergo considerable differentiation, including innervation of targets, prior to degeneration. And the neurons that die are thought to do so as a result of failing to obtain sufficient trophic support from their synaptic targets. Although it is true that this is a common and widespread form of PCD, it is becoming clear that there are also other different forms of PCD in the nervous system. For example, it has long been know that precursor cells in the early forming neural plate and neural folds undergo PCD during morphogenetic events such as invagination, evagination, neural tube closure, etc., and that this death occurs prior to differentiation and target innervation. We have recently examined two additional forms of cell death in the spinal cord that also appear to differ from the naturally occurring, target-dependent type of degeneration. One of these occurs shortly after neural tube closure and involves both proliferating precursors as well as early post-mitotic cells and occurs independent of interactions with targets. The other type involves a discrete population of motoneurons in the cervical (non-limb) segments of the spinal cord whose death differs in several respects from the later, target-dependent death of motoneurons in other spinal cord regions. In vitro and in vivo studies will be presented comparing the similarities and differences in three of the above categories of spinal cord PCD: (1) Target-dependent motoneuron death; (2) Target-independent motoneuron death; and (3) Early death following neural tube closure.

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POSSIBLE MECHANISMS OF PROGRAMMED CELL DEATH AND BCL-2 PROTECTION

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The mechanism of programmed cell death (PCD) is unknown. An important clue is provided by the Bcl-2 protein, which can protect many cell types from PCD, although it is not known where or how Bcl-2 acts. We have investigated the involvement of mitochondrial respiration , the nucleus, oxygen, and mitotic cyclin-dependent kinases in PCD, and find that none of these is required for the execution of the cell death programme, or for the ability of Bcl-2 to protect against PCD.

Mitochondrial respiration was excluded by inducing PCD in a cell line that lacks mitochondrial DNA (called rho-0 cells), and thus contains a defective respiratory chain. The nucleus was excluded by removing nuclei from a human fibroblast line, a rat oligodendroglial line, and subclones of these lines overexpressing *bcl-2* and then inducing PCD either by treatment with the protein kinase inhibitor staurosporine or by withdrawal of survival factors. Death was assayed by time-lapse video recording, electron microscopy, loss of mitochondrial function, and late loss of plasma membrane integrity. We find that, like intact cells, enucleated cells (cytoplasts) undergo PCD and are saved by Bcl-2. These findings suggest that PCD is orchestrated by a cytosolic regulator, which has multiple intracellular targets.

To sudy the role of oxidation in PCD, we treated cells grown in anaerobic culture conditions (<40ppm O_2), including rho-0 cells, with various PCD inducers. Under these conditions, cells were resistant to tumour necrosis factor-α but not to staurosporine-induced PCD. These findings suggest that reactive oxygen species may not play a central role in the effector mechanism of PCD. We also show that staurosporine induces an active form of cdc2 kinase, with a time-couse that parallels induction of PCD. Evidence will be presented which suggests that cdc2 kinase is not required for PCD, but may modulate sensitivity to inducers of PCD.

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The Role of Programmed Cell Death in the Immune System

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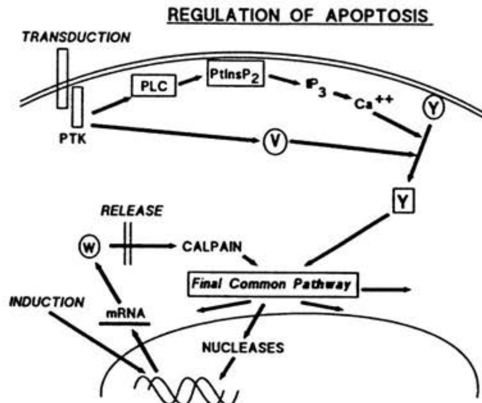
REGULATION OF APOPTOSIS IN THE IMMUNE SYSTEM. J. John Cohen, Department of Immunology, University of Colorado Medical School, Denver, Colorado 80262, USA.

Apoptosis is the physiological mechanism by which cells die when it is appropriate or desirable that they should do so [1]. It is particularly evident in the immune system, which regulates itself by clonal expansion, activation and elimination. There are a great many situations in the course of the development and function of the immune system in which cells may die [2]. For T cells, this probably begins in the thymus when the cell first rearranges its genes for the T cell receptor for antigen (TCR). Perhaps 2 times out of 3, the rearrangement will be out of the correct reading frame and the cell will be unable to transcribe that gene. If the other allele also suffers that fate, the cell cannot mature to become a T cell and dies by apoptosis. If the rearrangement is successful, the TCR is displayed on the surface. Cells that chance to have a TCR which reacts with self-MHC (either with or without a peptide in the antigen-binding cleft) at high avidity are eliminated; the process, negative selection, involves apoptosis [3]. Cells with no affinity for self-MHC are of no use to the immune system; they also die, by apoptosis that is probably induced by endogenous glucocorticoids [4,5]. In the periphery, self-reactive cells undergo apoptosis, in this case via a process that probably involves the surface receptor APO-1/Fas [6]. After antigenic stimulation, excess cells die when the level of growth factors falls below a certain limit [7]; others may die by an APO-1/Fas mechanism [8]. All lymphocytes are extremely sensitive to damage or incorrect signaling, and will invoke the apoptotic response instead of repair in most circumstances [9,10].

Studies of the biochemical mechanisms of apoptosis reveal a final common pathway involving cell shrinkage, DNA damage, and membrane changes that lead to recognition by nearby phagocytic cells. But there are cell-specific triggering pathways, and these are connected to different biochemical transducers. We are studying similarities and differences between apoptosis as induced by different agents in different cell types. One prevalent but not inevitable mechanism involves the activation of the calcium-dependent proteinase, calpain [11]. We are determining how this enzyme becomes activated and what its relevant substrates are. Calpain is involved in *induction* [12] mechanisms, which require new protein synthesis for apoptosis, as well as *release* [13] mechanisms in which apoptosis occurs when protein synthesis is blocked. It is not, however, involved in *transduction* mechanisms, which depend entirely of preformed molecules for apoptosis. Cytotoxic T cells and the Fas ligand are two examples of transducers. From these results we can tentatively construct a model for apoptosis signaling, but there are many holes left in this puzzle.



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PROGRAMMED CELL DEATH DURING SELECTION OF IMMATURE T CELLS IN THE THYMUS.

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The thymus is the site of generation of immature T cells expressing a wide range of T cell receptor (TCR) specificities. These immature T cells are characterised by the expression of both CD4 and CD8 antigens (i.e. they are 'double positive'). They are exposed to intense selection within the thymus so that potentially auto-reactive cells are induced to undergo programmed cell death (negative selection)[1] whereas cells with the potential to recognise foreign peptides in the context of self MHC molecules are induced to mature into single positive $CD4^+$ or $CD8^+$ T cells (positive selection)[2]. These observations raise interesting questions as to what determines these very different responses through the same receptor complex and what is the nature of the signalling pathways involved.

Before returning to these questions, it is important to recognise that $CD4^+8^+$ thymocytes normally have a 3-4 day

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lifespan within the thymus during which they are subject to selection. However if they are removed from the thymic environment thymocytes die rapidly, suggesting that all cells, irrespective of receptor specificity, receive signals from the thymic stroma which maintain their viability. We have evidence that this is the case[3] and that cells released from the thymic stroma behave differently in response to signals compared to cells within the thymus. Hence studies directed at determining the nature of the signals involved in either positive or negative selection should be carried out on thymocytes which retain their normal contacts with thymic stromal cells.

We have developed various <u>in vitro</u> protocols to maintain thymocyte - stromal cell interactions. These involve either organ cultures of mouse embryo thymus or tissue slices of thymus derived from newborn mice. In addition, in order to analyse specific interactions between particular stromal cell types and thymocytes, we have established systems for purifying stromal cell components and reaggregating them with thymocyte populations[4]. Using these approaches, we have shown that, in part, the different responses of programmed cell death or cell maturation depend upon interaction of thymocytes with specific stromal cell types, suggesting that co-stimulatory signals imparted by stromal cells modulate responses through the T cell receptor. In addition, we have initiated studies aimed at determining the nature of the signalling pathways involved using both assays of phosphoinositol hydrolysis and inhibitors of specific signalling molecules.

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SIGNALS THAT CAN RESCUE B CELLS FROM ANTIGEN-INDUCED APOPTOTIC DEATH

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Apoptosis (programmed cell death) has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. By crosslinking the antigen receptor (surface immunoglobulin; sIg) on WEHI231 B lymphoma cells, we found that B lymphoma cells underwent apoptosis. Such antigen-induced apoptosis of WEHI231 was completely blocked by simultaneous stimulation of CD40 on B cells[1]. Similarly, we found that strong crosslinking of sIg of the peritoneal and spleen B cells of normal mice induced apoptosis of mature B cells *in vitro* as well as *in vivo*, suggesting that interaction with membrane-bound self-antigens may eliminate self-reactive mature B cells by apoptosis. Antigen receptor-meidated B cell apoptosis is also blocked when a signal is transduced via the CD40 molecule on the B cell surface. Rescuing effects of CD40 stimulation is blocked by the inhibition of transcription. To understand the molecular mechanizm by which CD40 stimulation prevents cell death, genes induced by CD40 stimulation is being isolated. Since the ligand of CD40 (CD40L) is expressed on activated T helper cells, B cells may escape from apoptosis and are activated when the immune system interact with foreign antigens, which normally able to activate T helper cells. Moreover, sIg crosslinking fails to induce apoptosis of both bcl-2-transgenic mice and autoimmune disease-prone New Zealand mice[2,3]. In these mice, defect in sIg-mediated apoptosis of mature B cells may allow generation of self-reactive B cells, resulting in the pathogenic consequences.

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APOPTOSIS DURING B CELL DEVELOPMENT AND ACTIVATION

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Apoptosis or programmed cell death (PCD), is an important physiological mechanism for elimination of B and T cell populations at various stages during their development. The hallmarks morphological changes associated with apoptosis include membrane blebbing, cytoplasmic and nuclear condensation, disruption of the cytoskelton, and DNA fragmentation. For apoptosis to occur, it is accepted that an extracellular ligand is required to initiate a cascade of signaling in the cell that couples to gene regulatory changes that ultimately lead to cell death. The complex regulation of apoptosis and cell survival involves particular combination of signals and therefore, a number of extracellular stimuli, artificial inducers and inhibitors of signal transduction and metabolism, may enhance or diminish apoptosis in lymphocytes. An increasing number of gene products such as FAS/APO-1, TNF-R, and NGF-R, as well as transcription factors like steroid receptors, c-myc, and p53, are now known to be involved in cell death. Other proteins, including intracellular molecules i.e. Bcl-2, and Bcl-x as well as membrane receptors like CD40 and CD20 are involved in rescue from cell death.

Cell death plays a pivotal role in sorting of B lymphocytes during cell maturation. The developmental program is tightly regulated since it has to promote the generation of functional B lymphocytes but at the same time, the demise of non-functional as well as self-reactive B cells. By using either B cell lines at different stages of development, Pre-B cells and immature B cells, or mature peripheral B cells, we have characterized the role of Bcl-2 and Egr-1 in preventing the apoptosis induced under different experimental conditions. Furthermore, since B cell differentiation is also associated with differential expression of cell surface receptors, we have analyzed the role of some of those molecules in preventing apoptosis, with special emphasis in the role that CD2 might have, by signaling B lymphocytes.

Finally, the characterization of a number of compounds for their ability to block apoptosis in B cells, based on the fact that inhibition of PCD can occur at four different levels: (1) functional antagonism to an PCD inducer stimulus; (2) interception of a PCD-inducing stimulus; (3) interferences with a signal transduction cascade and (4) blockade of catalytic enzymes participating in cellular suicide will be discuss.

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R-*ras* and other proteins that associate with Bcl-2.

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We have isolated mammalian cDNAs encoding proteins that associate with Bcl-2. We have identified one of these proteins as the *ras*-related protein R-*ras* p23 (Fernandez-Sarabia and Bischoff, 1993). R-*ras* p23, a non-transforming member of the superfamily of *ras* proteins, interacts with Bcl-2 in the two-hybrid system, and co-precipitates with Bcl-2 from mammalian cell extracts (Fernandez Sarabia and Bischoff, 1993). The association is dependent on the nucleotide state of R-*ras* p23, only the nucleotide depleted form of R-*ras* p23 will associate with Bcl-2. We are currently analysing the biological significance of this asociation with respect to programmed cell death, as well as identifying effectors of R-*ras* p23. Towards this goal, we have observed that R-*ras* p23 is able to interact with the Raf-1 protein kinase, an effector of H-*ras* p21, in a GTP-dependent manner (Spaargaren et al., 1994), and studies to determine the functional outcome of this association are in progress.
Other proteins that also interact specifically with Bcl-2 and R-*ras* will be presented.

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ROLE OF THE INTERFERON-INDUCED HUMAN PROTEIN KINASE (p68) IN THE INDUCTION OF APOPTOSIS.

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Apoptosis (also called programmed cell death) occurs during embryonic development, synaptogenesis of neurons, elimination of self-reactive B and T lymphocytes, cytotoxic killing of target cells, and homeostasis in developing and adult organisms (1). Only few genes have been implicated in apoptosis, like the tumor suppressor p53 and the proto-oncogenes c-myc and c-rel. The observations that the tumor suppressor and the proto-oncogenes are involved in the control of apoptosis underscore the importance of apoptosis in carcinogenesis. In addition, apoptosis can also play an important role in the control of viral infections by eliminating the infected cells. To learn about the mechanism of induction of apoptosis, we have investigated whether an interferon-inducible gene, the ds-RNA dependent protein kinase (p68), implicated in antitumor and antiviral effects of interferon could have a role in apoptosis. The human p68 kinase is a serine/threonine kinase that is autophosphorylated upon binding to dsRNA and in the presence of ATP. The activated kinase then phosphorylates the alpha subunit of the initiation factor eIF-2, leading to inhibition of the translation of the initiation process.To stuy the role of p68 kinase *in vivo* , an inducible vaccinia virus expression system was developed. Upon expression of wild type p68 kinase, a severe inhibition of protein synthesis was observed (2). Inhibition of protein synthesis correlated temporally with autophosphorylation of p68 kinase. As a result of this translational block, vaccinia virus production was drastically reduced by about 100-fold (3). In addition, expression of p68 kinase resulted in a rapid cell death with characteristics of apoptosis (4). Expression of catalyticaly inactive p68 kinase did not result in apoptosis, demonstrating that a functional kinase is required for this process. Moreover, infection of HeLa cells with a mutant of vaccinia virus lacking the E3L gene, which encodes a dsRNA binding protein that acts as an inhibitor of p68 kinase, also resulted in apoptosis. Our findings demonstrate that p68 kinase functions as a key mediator of apoptosis and, this in turn, contributes to the antiviral and antitumor effects of interferon.

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VITAL ASSAY FOR NEURONAL CELL DEATH IN ORGANOTYPIC CULTURES Richard Adams[1*], Zoltán Molnár[2], Matthew Nesbit [2] and Colin Blakemore[1,2] MRC Research Centre in Brain and Behaviour[1] and University Laboratory of Physiology[2], Parks Road, Oxford OX1 3PT, UK.

Cell death is an important feature of the development of the nervous system. We have been investigating methods of assaying cell mortality. Our aim was to find a vital assay that can be used to map patterns of cell death and to use this to follow natural events as well as the effects of experimental manipulations. Acridine orange is a vital dye that crosses the plasma membrane and stains the nucleus with a green emission on excitation with light of 488nm. Propidium iodide also binds to chromatin but differs from acridine orange in two respects: it cannot cross the plasma membrane at normal resting membrane potentials and it has a longer Stoke's shift, giving a red emission of light when excited by the blue 488nm laser line. Thus, a short staining of tissue with a mixture of these dyes produces green fluorescence in all cell nuclei with an additional red fluorescence of the nuclei of those cells that are at a depolarized potential – largely indicative of dead or dying neurons in culture. We use confocal microscopy to image the 3-D distribution of stained nuclei within organotypic cortical and thalamic cultures and thalamo-cortical co-cultures stained for 30-60 minutes with 10µg/ml of each of these dyes. The red and green emissions were separated and collected simult-aneously by two photomultiplier channels and the two 3-D datasets were reconstructed into a two-colour projection.

In slices of P0 rat cortex kept in culture for several days we see occasional dead cells throughout the depth of the tissue but a predominance of such cells within the subplate and marginal zone - regions of transient cells known to be lost at this stage of development (Luskin and Shatz, 1985 J. Neurosci **5**:1062). Second, we have compared the survival of thalamic neurons in slices of E16 and P2 thalamus cultured with and without cortex in the same dish. Preliminary results are consistent with the idea that the cortex provides a soluble trophic component that prolongs the survival of neurons within the slice of thalamus (Cunningham *et al.* 1987 Dev. Brain Res. **37**:133).

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APOPTOSIS INDUCTION RESULTS FROM UNCOORDINATED CELL CYCLE REGULATION.

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The unfolding of the developmental program and the organization of multi cellular organisms require that cell numbers in differentiating and differentiated tissues are regulated. This is done by two distinct processes : control of cell proliferation and differentiation to a post-mitotic stage and control of cell survival or their elimination by apoptosis.

Transformed cells, in addition to defects in cell cycle regulation, appear highly susceptible to induction of programmed cell death. Moreover, several genes have been identified as implicated in the control of both proliferation and apoptosis.

We have studied the expression of the *c-myc* oncogene following apoptosis induction by surface IgM crosslinking of WEHI 231 B lymphoma cells. Contrary to some experimental systems, sustained *c-myc* induction correlates with survival rather than apoptosis of the WEHI 231 cells. Moreover, anti-IgM treatment of both WEHI 231 wild type cells and two mutant cell lines resistant to apotosis, induce expression of genes associated with growth arrest (the *gadd* gene family), suggesting that ligation of surface IgM is a growth arrest signal in these cells. Intersestingly, the patterns of expression of different *gadd* family members differ in apoptosis sensitive and resistant cells.

The analysis of our results as well as data from the literature lead us to propose a model for control of apoptosis induction. We propose that apoptosis can arise in cycling cells or in resting cells submitted to proliferative signalling through antonymy of signalling pathways, i.e. a situation where a cell simultaneously engages into incompatible pathways of proliferation and cell cycle arrest. Antonymy arises in cells incapable of integration of contradictory signals, i.e. cells irreversibly commited to either proliferation or arrest and responding to a contradictory signal. In turn, the irreversible commitment arises by uncoupling of signal transduction from coordinated pathways (as in transformed cells with constitutive expression of growth associated genes or in terminally differentiated post-mitotic cells).

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Signals Implicated in Apoptosis

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RECENT STUDIES OF SIGNAL TRANSDUCTION IN LYMPHOCYTE APOPTOSIS

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A variety of agents and treatments can trigger apoptosis in thymocytes and T lymphocytes by signal transduction mechanisms, which are either unknown or only partly identified. In some instances, these pathways appear to share common components. Thus, the p53 protein has been found to play an important role in thymocyte apoptosis triggered by DNA damage (1,2), and intracellular Ca^{2+} elevation appears to mediate apoptosis in several experimental models, including TCR/CD3-mediated apoptosis in thymocytes, activated T lymphocytes, and T cell hybridoma (3,4). In activated T cells and T cell hybridoma, but not in immature thymocytes, the Ca^{2+} requirement has been linked to the involvement of calcineurin in the signalling pathway leading to apoptosis. Further, the apoptotic killing of thymocytes by various immunotoxicants has also been found to involve a sustained elevation of intracellular Ca^{2+} (5,6).

Subpopulations of thymocytes and T lymphocytes exhibit different sensitivity to apoptosis-inducing agents. Although the immature $CD4^+CD8^+$ thymocytes are highly sensitive to apoptosis induced by glucocorticoids and radiation, they are less sensitive than peripheral T lymphocytes to apoptosis triggered by intracellular Zn^{2+} chelation (7). Moreover, the immature $CD4^-CD8^-$ thymocytes, which are more resistant than the $CD4^+CD8^+$ cells to glucocorticoid-, radiation-, and thapsigargin-induced apoptosis, are highly sensitive to apoptosis induced by topoisomerase II inhibitors. Thus, the relative sensitivity of various lymphocyte populations to apoptosis varies with the agent used to trigger apoptosis.

The possible relationship between oxidative stress and apoptosis has attracted increased interest recently, Thus, a large number of studies have indicated that

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prooxidants can trigger, and antioxidants can prevent, apoptosis in different experimental models (8) In recent studies we have found that induction of apoptosis in thymocytes by various agents is associated with decreased intracellular levels of antioxidants (GSH, vitamin E) and, conversely, that various oxygen radical spin traps (DMPO, TMPO, TEMPO) can inhibit apoptosis in thymocytes. This protective effect is observed with different inducers of apoptosis (methylprednisolone, etoposide, thapsigargin, TPEN), suggesting that redox modulation may play a central role in the regulation of apoptosis. The level at which redox modulation of apoptosis may occur is not known, although the recently discovered sensitivity of various transcription factors to redox regulation offers an attractive possibility which, however, has to be tested experimentally.

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DNA DEGRADATION DURING APOPTOSIS: ENZYMES AND REGULATION BY SIGNALS.

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Apoptosis is a form of programmed cell death that occurs under numerous developmental and physiological conditions which require the selective elimination of cells from tissues and organs without the production of an inflammatory response. The initiation of apoptosis is now well recognized to be regulated by a diverse group of compounds and signals including steroids, hormones, ions, growth factors, oncogenes, and drugs. The apoptotic phenotype in response to these agents includes reduction of volume, compaction of intracellular organelles, DNA degradation and generation of apoptotic bodies which leads to cell suicide without an inflammatory response. We have conducted studies to decipher the biochemical mechanisms that mediate glucocorticoid and immunosuppressant-induced cell death in mouse and rat lymphoid cells.

Our research has focused on the DNA degradation component of the apoptotic process in lymphocytes since this step appears to comprise the point of irreversible commitment to cell death in these cells. The goal of our work has been to identify, purify, and clone the gene(s) for the nuclease(s) responsible for DNA degradation during the process of apoptosis. To facilitate these efforts we have developed assays to quantitate total nuclease activity, high molecular weight DNA fragmentation, and internucleosomal DNA degradation activity. Using these assays and other genetic approaches we have now identified a novel low molecular weight nuclease (NUC 18) whose activation correlates temporally with DNA degradation in apoptotic lymphocytes in response to several kinds of apoptotic stimuli.

NUC 18 requires Ca^{++} and Mg^{++} for optimal activity in vitro and is inhibited by divalent cation sequestering agents. Moreover, known inhibitors of apoptosis such as Zn^{++} and aurintricarboxylic acid completely inhibit this nuclease activity. This enzyme has now been purified to apparent homogeneity and specific high affinity antibodies have been prepared against this protein. Partial amino acid sequence analysis of the purified protein reveals that this nuclease is a novel nuclear member of the cyclophilin family of proteins. Human recombinant cyclophilin A, prepared in bacteria, has biochemical and pharmacological properties identical to native NUC 18. Mechanistic studies on the regulation of this nuclease activity suggest that this enzyme is a constitutive nuclear protein that is "activated", but not induced, by apoptotic signals. Supported by DR 32078 from the National Institutes of Health.

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Intracellular Signal Transduction in Apoptosis

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Although there are many genes and drugs that can either cause or prevent apoptosis, there is little understanding of the signals required to induce the morphological and biochemical changes that characterize this mode of cell death. Experiments in this laboratory were designed to identify an endonuclease that causes the DNA digestion associated with apoptosis, determine how it is regulated, and thereby begin to characterize the signal transduction pathways involved.

For a number of years, it was generally accepted that the endonuclease involved in apoptosis was Ca^{2+}/Mg^{2+}-dependent, and was activated by increases in intracellular Ca^{2+}. However, there are many exceptions in which cells exhibit no increases in intracellular Ca^{2+} or do not contain this endonuclease. Many endonucleases exist which could potentially be involved in the process, and one identified by this laboratory is deoxyribonuclease II (DNase II) (1). DNase II is activated by decreasing intracellular pH rather than increases in intracellular Ca^{2+}. We have investigated intracellular pH changes in human HL-60 cells undergoing apoptosis induced by cytotoxic agents and have observed intracellular acidification of close to 1 pH unit below extracellular pH (2,3). Cells were sorted on the basis of their intracellular pH and only the acidic cells demonstrated the morphology and DNA digestion of apoptosis. The intracellular acidification is due to selective inhibition of pH regulation while the cells retain an electrochemical gradient across their membrane. A possible explanation for these results is dephosphorylation and thereby inactivation of the Na^+/H^+ antiporter and other pH regulators.

To investigate the significance of acidification further, we investigated the intracellular pH following removal of IL2 from an IL2-dependent cytotoxic T lymphocyte cell line CTLL-2. These cells also underwent acidification, and this correlated with the appearance of digested DNA (4). Growth factors presumably keep cells alive by activating an intracellular kinase cascade, but which protein phosphorylations are important for survival are unknown. One protein that is phosphorylated as a consequence of growth factor stimulation is the Na^+/H^+ antiporter. This is consistent with withdrawal of the growth factor leading to dephosphorylation of the antiporter and hence intracellular acidification.

Do these results confirm the involvement of DNase II in apoptosis? We have attempted to prevent activation of DNase II by incubating CTLL-2 cells at pH 8.0 so that acidification would only result in a neutral pH. Upon withdrawal of IL2, the cells underwent acidification to pH 7.2 as expected. This should be inadequate to activate DNase II, yet the cells still digested their DNA suggesting that DNase II may not be involved in this model. If the pH change is not inducing DNA digestion, what is? We have always observed pH shifts during apoptosis consistent with the possibility that phosphatases are activated during the process. We have also been able to prevent DNA digestion by incubation of cells with phosphatase inhibitors. Hence, our current

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hypothesis is that activation of phosphatases may be important for apoptosis, but that antiports may be only one target for the dephosphorylation; other targets may be more critical for survival. Proof of which endonucleases are involved in apoptosis will have to wait until genetically manipulated cells are produced that express only specified endonucleases.

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Regulation of the apoptotic response to DNA damage by oncogenes and cytokines.

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Apoptosis induced by a variety of DNA damaging treatments in the murine bone marrow-derived cell line, BAF3, can be inhibited by interleukin 3, interleukin 4, or insulin-like growth factor 1. The extent of DNA damage and its rate of repair is not affected by cytokines; rather they allow cells to pass a checkpoint where apoptosis occurs. Using cell lines expressing mutant interleukin-2 receptor β chains, we can demonstrate that tyrosine kinase stimulation and optimal MAP kinase activation are not required for cytokine protection against DNA damage. Over-expression of the Bcl-2 gene product also protects cells against apoptosis induced by DNA damage. In this case, cells do not proliferate but arrest and become refractory to cytokine stimulation, due to specific inhibition of the MAP kinase signalling pathway. Constitutive c-Myc over-expression in these cells renders them unable to survive in sub-optimal cytokine and more sensitive to DNA damage, without affecting their rate of cell cycle progression. When apoptosis is triggered in BAF3 cells by a variety of conditions, the first irreversible event occurs close to the time at which DNA cleavage is detected. We have therefore purified and characterised a nuclease capable of fragmenting chromatin in response to physiological signals.

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MECHANISM OF INDUCTION OF APOPTOSIS BY CYTOTOXIC AGENTS.

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A wide variety of cells have been shown to undergo apoptosis in vitro and in vivo when deprived of essential growth/survival factors. In cultures of bone marrow-derived haemopoietic cells removal of interleukin-3 leads after several hours to the initiation of a process of apoptosis with condensation of chromatin, degradation of DNA into oligonucleosome-length fragments, decrease in nuclear and cellular volume and cell death. Unlike other cells of haemopoietic origin such as T and B cells in which changes in calcium metabolism appears to play an important role in apoptosis, in these haemopoietic precursor cells programmed cell death takes place even in the absence of extracellular calcium and alterations in the intracellular calcium content elicited by ionophores do not induce apoptosis. Furthermore, elevation of cellular calcium inhibits apoptosis and maintain the cells in a viable noncycling state.

A number of antineoplastic drugs and treatments exert their cytotoxic effect by inducing apoptosis and the drug and radiation resistence of many tumors could be ascribed to the failure of certain cancer cells to commit apoptosis due to deregulation of bcl-2 expression or mutations in p53 anti-oncogene. Inhibitors of DNA precursor synthesis are very effective as cytotoxic agents against tumour cells and an imbalance in deoxynucleotides pools have been suggested to be a crucial event leading to cell death after certain treatments. Dihydrofolate reductase (DHFR) and ribonucleotide reductase (RR) catalyse key steps in the novo production of deoxynucleosides triphosphate and their activities may limit the ability of the cell to synthesise or repair DNA. Therefore inhibitors of these enzymes, such as methotrexate and hydroxyurea, have been used in the therapy of neoplasias. We have used these inhibitors to perturb deoxynucleotides metabolism in the haemopoietic cell line BAF3 and study the effect of deoxynucleotides imbalance in apoptosis in these cells. Treatment of BAF3 cells with either of these inhibitors in the continuous presence of IL3, causes cell death after 15 hours. Analysis of DNA revealed that after 8 hours there is activation of endogenous endonuclease that produces the ladder pattern characteristic of apoptosis. The study of deoxynucleosides triphosphate pools indicates that the inhibitors produce an imbalance situation and in the case of methotrexate, there is a rapid decrease in the dTTP pool whereas dATP remains normal. Since the previous experiments were performed in the presence of IL3, these results indicate that apoptosis can be directly induced in BAF3 by blocking dNTP de novo synthesis and perturbing dNTP balance, even in the presence of other signals from the IL3 receptor.

In the present study we describe changes in dNTP pools that precede chromatin fragmentation in a model of apoptosis driven by deprivation of the cytokine IL3. In BAF3 cells, IL3 withdrawal leads to a rapid decrease in the size of dATP, dTTP and dGTP pools without affecting the dCTP levels. This imbalance in dNTP pool precedes DNA fragmentation and is mainly caused by a loss of ribonucleotide reductase (RNR) activity and a decrease in precursor uptake through the salvage pathway. Readdition of IL3 leads in the short term to a rapid restoration of normal dNTP pools through a protein synthesis-independent mechanism by increasing precursor incorporation through the salvage pathway, without an increase in RNR activity. This study suggests that dNTP pool imbalance after IL3 deprivation could be responsible for nucleotides misincorporation during DNA synthesis and repair and may therefore serve as a signal to the cell to initiate a death program to prevent the accumulation of mutations. Removal of IL3 from BAF3 cells overexpressing bcl-2 or treatment of these cells with dNTP synthesis inhibitors also results in the loss of dNTP without DNA fragmentation, indicating that bcl-2 can inhibit apoptosis despite dNTP imbalance.

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POSTERS

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Bcl-2 MEDIATES THE NEUROTROPHIN SURVIVAL RESPONSE

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Developing neurons undergo apoptosis if they fail to obtain an adequate supply of neurotrophins from their targets. How neurotrophins suppress apoptosis is unknown. As experimental over-expression of exogenous bcl-2 prevents apoptosis in cultured neurons deprived of NGF-related neurotrophins (NGF, BDNF and NT-3) we investigated a role for endogenous bcl-2 in mediating the action of neurotrophins. Expression of antisense but not sense *bcl-2* RNA in BDNF-dependent neurons killed these neurons in the presence of BDNF. Ciliary neurons, which are not rescued by bcl-2 overexpression following withdrawal of ciliary neurotrophic factor (CNTF) were unaffected by antisense *bcl-2* RNA. BDNF caused a transient elevation of *bcl-2* mRNA expression in BDNF-dependent neurons, but neither CNTF nor the related factor GPA affected *bcl-2* mRNA expression in ciliary neurons. Our findings indicate that neurotrophins can regulate neuronal viability via the expression of the well-characterised cell death-suppressor *bcl-2* and substantiate the hypothesis that different molecular mechanisms control apoptosis in different neuronal types.

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IL-7 INDUCES CD25 EXPRESSION AND RESCUES HUMAN ACTIVATED T CELLS FROM APOPTOSIS INDUCED BY GLUCOCORTICOIDS.

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Glucocorticoids are among the most potent and widely used anti-inflammatory and immunosuppresive drugs which induce apoptosis in activated mature T cells, cortical thymocytes and a number of tumor T cells. Glucocorticoid-induced T cell death can be abrogated by IL-2. Although IL-7 is also a potent growth factor for T cells, its role in rescuing T cells from glucocorticoid-induced apoptosis has not yet been tested. To reach this aim, we analyzed the viability and expression of CD25 antigen on human PHA-activated T cell or different isolated human T cell clones in the presence or absence of IL-2, IL-4 or IL-7 and dexamethasone. Our in vitro results indicated that treatment with all three lymphokines increased the number of living PHA-activated T cells in the presence of dexamethasone. When αβ or γδ T cell clones were tested, only IL-2 and IL-7 were able to augment viability of the cultures and to prevent glucocorticoid-induced apoptosis. CD25 expression was increased in all cell types when treated with IL-2 or IL-7 in the presence of dexamethasone, while IL-4 had little or no effect. The highest levels of CD25+ cells were found in IL-7 + dexamethasone treated cultures. Exogenously added IL-2 had no synergy with IL-7 on inducing CD25 expression. Anti-IL-2 mAb did not inhibit IL-7-induced CD25 indicating that IL-7 effect is not dependent on endogenously produced IL-2. Our results indicate that IL-7 may be an important lymphokine involved in the rescue of activated T cells from apoptosis induced by glucocorticoids.

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c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines

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We have investigated the mechanism by which deregulated expression of c-Myc induces death by apoptosis in serum-deprived fibroblasts. We demonstrate that Myc-induced apoptosis in low serum is inhibited by a restricted group of cytokines, principally the insulin-like growth factors and PDGF. Cytokine-mediated protection from apoptosis is not linked to the cytokines' abilities to promote growth, and is evident both in the G_1 pre-commitment (mitogen-dependent) and the post-commitment (mitogen-independent) $S/G_2/M$ phases of the cell cycle and also when apoptosis is induced by cytotoxic drugs. We conclude that c-Myc-induced apoptosis does not result from a simple conflict of growth signals but appears to be a normal physiological aspect of c-Myc function that is regulated by the environmental context in which the cell exists.

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THY-1 TRIGGERS MOUSE THYMOCYTE APOPTOSIS THROUGH A *bcl-2*-RESISTANT MECHANISM.

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Programmed cell death (PCD) plays an important role during thymocyte development, since a vast majority (97%) of mouse cortical thymocytes die in thymus, while only 3% of these cells are rescued from cell death and positively selected. Although it seems well established that thymocyte fate depends upon appropriate surface-expressed TCR, little is known about the molecular mechanim(s) responsible for the massive thymocyte elimination occuring in thymus. We report here that Thy-1 is capable of triggering mouse thymocyte death *in vitro* through a *bcl-2*-resistant mechanism. We have previously shown that Thy-1 is involved in mouse thymocyte adhesion to thymic stroma through interaction with an epithelial cell ligand. To examine the Thy-1 signalling functions in thymocytes, we have mimicked its interaction with stromal cells by culturing mouse thymocytes onto tissue culture plates coated with mAb directed at distinct Thy-1 epitope regions. MAb recognizing determinants in a defined Thy-1 structural domain, but not others, were found to induce marked thymocyte apoptosis as evidenced by morphological and biochemical data. Use of a quantitative DNA blot assay indicated that Thy-1-mediated thymocyte apoptosis was not blocked by RNA or protein synthesis inhibitors, EGTA nor by cyclosporin A, and differed, therefore, from "activation-driven cell death" (ADCD). Moreover, Thy-1$^+$-transfected, but not wild-type AKR1 (*Thy-1$^-$d*) thymoma cells underwent apoptosis following ligation with apoptosis-inducing, Thy-1-specific mAb. In contrast to thymocytes, the latter event was inhibitable by RNA and protein synthesis inhibitors, an indication that thymocytes, but not thymoma cells, contain the molecular components necessary for Thy-1-driven apoptosis. We further showed that Thy-1-triggered thymocyte death is a developmentally regulated process operative in fetal thymocytes from day 17 of gestation, but not in peripheral T cells. Finally, it is of major interest that Thy-1-mediated apoptosis, which was found to be readily detectable in thymocytes from *bcl-2*-transgenic mice, represents a so far unique experimental system for studying *bcl-2*-resistant thymocyte death mechanism(s).

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Fas and Diethylmaleate Induce Apoptosis by Different Pathways

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Apoptosis can be induced by various substances. Fas and TNF receptor (TNF-R) are cell surface proteins which trigger apoptosis either by stimulation with the corresponding ligand or specific monoclonal antibodies. Diethylmaleate (DEM) reduces cellular stores of glutathione by forming a thioether conjugate in a reaction catalyzed by glutathione-S-transferase, and induces apoptosis. Here, we examined whether glutathione plays a role in Fas or TNF-R-mediated apoptosis.

Mouse L929 fibroblasts overexpressing human Fas were incubated with several substances known for their anti-oxidant activity, followed by treatment with apoptosis-inducing agents: anti-human Fas antibody murine TNF or DEM. The following antioxidants were tested for their ability to block apoptosis: γ-glutamyl-cysteinylglycylethyl ester that can be transported into cells and is split intracellularly to glutathione; reduced γ-L-Glu-L-Cys which has been shown to be a substrate for microsomal glutathione transferase; deferoxamine which is an inhibitor of ferrochelatase; α-Tocopherol that breaks lipid peroxidation chain reactions.

All antioxidants mentioned above inhibited the DEM-induced apoptosis in the Fas-overexpressing L929 cells. On the other hand, these antioxidants could not inhibit the apoptosis induced by anti-Fas antibody or TNF. These results indicate that reactive oxygen intermediates do not play a major role in Fas- or TNF-R-mediated apoptosis, and the apoptotic signal transduction mechanism by Fas or TNF-R is different from that involved in DEM-mediated apoptosis.

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Regulation and Function of bcl-2 During B Cell Development.

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Homeostasis in the central and peripheral B cell pools is controlled by a continous production of B cell precursors in the bone marrow, as well as by the rate of survival and death of B cells. The high rate of cell death during B cell development is due, in part, to a selection process by which B cells that fail to display surface IgM or those that express antigen receptors for self are clonally eliminated by apoptosis. The intracellular signals that control the survival of developing B cells are largely unknown. A clear candidate for such a role is the bcl-2 proto-oncogene, whose product promotes cell survival by inhibiting apoptotic cell death. The physiological role of the endogenous Bcl-2 protein is unclear because its expression in developing B cells and its ability to modulate apoptosis are undetermined. We have studied in detail the expression of Bcl-2 during B cell development by three-color flow cytometric analysis. Our results shown a striking developmental regulation of the Bcl-2 protein in B lymphocytes. Bcl-2 is highly expressed in pro-B and mature B cells but downregulated at the pre-B and immature B cell stages of development. Importantly, expression of Bcl-2 perfectly correlates with susceptibility to apoptosis mediated by dexamethasone in vivo. Targeting of Bcl-2 to pre-B cells and immature B cells rescued the cells from dexamethasone-induced apoptosis. In order to assess Bcl-2 in the process of clonal deletion of self-reactive immature B cell precursors, neonatal C57BL/6 mice were injected intraperitoneally with $F(ab')_2$ goat anti-mouse IgM polyclonal antibody which mimmics the delection process. Two days following injection, there was a selective elimination of IgM^+IgD^- immature B cells expressing low levels of Bcl-2. In contrast, Bcl-2^{high} IgM^+IgD^- immature B cells and IgM^+IgD^+ mature B cells largely survived anti-IgM treatment. Importantly, overexpression of a bcl-2 transgene in Bcl-2^{dull} IgM^+IgD^- immature B cells failed to prevent their anti-IgM-induced cell death. These results strongly suggest that downregulation of Bcl-2 at the pre-B and immature B cells stages facilitates appropriate selection of developing B cells following physiological cell death signals. Yet, clonal deletion at the immature B cell stage but not glucocorticoid-induced apoptosis is independent of Bcl-2. After or during clonal selection, Bcl-2 is upregulated in mature B cells and maintains peripheral B cell survival.

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A CALCIUM-DEPENDENT NUCLEASE FROM APOPTOTIC RAT THYMOCYTES IS HOMOLOGOUS WITH CYCLOPHILIN: RECOMBINANT CYCLOPHILINS A,B AND C HAVE NUCLEASE ACTIVITY

Jenifer Walter Montague

Apoptosis is an important physiological process that involves the deletion of specific cells in a controlled and timely manner. A biochemical hallmark typifying apoptosis is cleavage of DNA by a calcium-dependent nuclease. We have identified and purified an 18 kD nuclease (NUC18) whose activity is associated with this apoptotic DNA fragmentation in glucocorticoid-treated rat thymocytes. Two peptides resulting from trypsin digestion of NUC18 were sequenced and found to have remarkable similarity to rat cyclophilin A (CypA) and other members of the Cyp family. Cyps are peptidyl-prolyl cis-trans isomerases first identified by their high-affinity binding to the immunosuppressant drug Cyclosporin A (CsA). Cyclophilins have been identified in many diverse species, including mammals, yeast, bacteria, Drosophila, and Neurospora. Peptide #1 of NUC18 (TVVFGK) corresponds to residues 125-130 of rat CypA (HVVFGK), with the exception of the His/Thr mismatch, and peptide #2 corresponds exactly to the first 16 residues of rat CypA (VNPTVFFDITADGEPL). Western blot analyses were performed with antibodies (Abs) directed against CypA and NUC18. Both sets of Abs recognize CypA and an 18 kD nuclear protein produced in thymocytes of glucocorticoid-treated rats. The cross-reactivity of the Abs indicates the two proteins are immunologically similar, as well as sequentially similar.

Because the isolation scheme of NUC18 was based on its ability to degrade DNA, we decided to next examine the immunologically related Cyp for possible nuclease activity. CypA cDNA was introduced into bacterial cells so that production of the resulting 18 kD protein could be induced by adding IPTG. This 18 kD protein was tested for nuclease activity by electrophoresis through an SDS-polyacrylamide gel impregnated with ^{32}P-labeled DNA. The SDS is removed and the nuclease is activated by Ca^{2+} and Mg^{2+} at 37 ^{0}C. The gel is dried down and exposed to film. Nuclease activity is detected by the appearance of "holes" (sites of labeled DNA loss) in an otherwise black autoradiograph. This assay demonstrates that the induced Cyp protein is capable of degrading DNA. Additional assays reveal that Cyp nuclease activity requires Ca^{2+} for activation and is inhibited by Zn^{2+} and aurintricarboxylic acid, two known inhibitors of nucleases. Cyp shows the same response to activators and inhibitors as NUC18.

We obtained purified Cyps A, B and C (18 kD, 21 kD, and 22 kD, respectively) to examine the possibility that the nuclease activity observed with our genetic approach resulted from a bacterial contaminant and not the induced CypA. The nuclease activity in the radioactive gel nuclease assay corresponds exactly to the size of the Cyp protein in that lane. Additionally, resolubilized CypA crystals were tested in this same manner to ensure that a contaminant was not responsible for the apparent nuclease assay. The samples of resolubilized CypA crystals also display a Ca^{2+}-dependent nuclease activity.

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A classical means of testing Cyps for nuclease activity is a method in which Cyp is directly incubated with excess substrate DNA (linearized pUC18) in the presence or absence of ions. With this assay all three forms of Cyp again demonstrate the ability to degrade DNA, as detected by an increase in low molecular weight DNA with a concomitant decrease in the amount of substrate DNA in an EtBr-stained agarose gel. Cyps B and C could produce low molecular weight DNA within one hour of incubation, a time frame that corresponds with the apppearance of DNA degradation during apoptosis. To further demonstrate that the DNA degradation results from the Cyp itself, we added to these assays the high-affinity ligand for Cyp, CsA. This drug is known to inhibit the peptidyl-prolyl isomerase activity of Cyp, but appears to actually enhance the nuclease activity of Cyp.

In summary, we have shown that an apoptotic nuclease, NUC18, has sequence homology with CypA and other members of the Cyp family, and that NUC18 and CypA are immunologically related. Further, we have shown that Cyps demonstrate the ability to degrade DNA in a time frame that corresponds to apoptotic DNA degradation and that this nuclease activity of Cyps can be enhanced by the high-affinity ligand CsA. We are continuing with genetic and biochemical studies to determine the involvement of Cyp proteins in apoptosis.

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A DECREASE IN THE DEOXYRIBONUCLEOTIDE POOLS IS AN EARLY EVENT IN APOPTOSIS INDUCED BY INTERLEUKIN-3 REMOVAL

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Apoptosis or programmed cell death is a physiological proccess of cell homeostasis by which unwanted cells are triggered to die. This homeostatic function is exerted in relation to tissue dinamics specially in continuosly renewing tissues, as the hematopoietic system, where the steady state is achieved by a balance between cell death and cell division. Activation of an endonuclease activity has been proposed to be a necessary event in the onset of apoptosis, although a model to explain the cascade of events leading to such activation remains elusive. Differents antineoplastic agents, acting on their target cells by inhibiting *de novo* synthesis of dNTPs, have been reported to kill cells by a mechanism involving apoptosis.

Methods:BAF3 cells were cultured in RPMI with 10% fetal bovine serum and 10% conditioned medium from the IL-3-producing cell line Wehi-3B; dNTP were determined enzymatically by DNA polymerase; ribonucleotide reductase activity was measured by the Dowex-1-borate column method.

Results and discussion: Previous results from our laboratory have shown that inhibition of the enzyme thymidilate synthase with 5'-fluor-2'-deoxyuridine, to perturb deoxyribonucleotides metabolism, drives the hematopoietic precursor cell line BAF3 to an apoptotic programm, even in the presence of interleukin-3 (IL-3). In the present study we show that IL-3 withdrawal leads to a rapid decrease in the size of dATP and dTTP pools, that precedes DNA fragmentation. This is accompained by a dramatic loss of ribonucleotide reductase activity. Removal of IL-3 from BAF3 cells overexpressing the protooncogene BCL-2 also result in the loss of dNTPs, although with a slower kinetic. Readdition of IL3 restores normal dNTP pools even in the absence of protein synthesis, without a parallel increase in ribonucleotide reductase activity. We are now studying the putative short term modifications affecting at different steps of dNTP metabolism. In conclussion, this provides a novel mechanism to understand the initiation of apoptosis following growth factor removal and suggest that an unbalance of dNTP pool is an essential switch for commiting the cells to apoptosis in this model of cell death.

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CELLULAR SUSCEPTIBILITY TO APOPTOSIS BY THE E1A ONCOGENE REQUIRES BINDING TO p105-rb/p107/p60 and p300 CELLULAR PROTEINS.

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The mechanisms of cellular resistance to anticancer drugs and radiation are multiple and may depend on specific oncogenic alterations, induction of apoptosis or other factors. The oncogene E1a confers susceptibility to apoptosis induced by DNA damaging agents and p53 protein mediation is required for efficient execution of the death program.

In order to test if the apoptosis of E1a transformed cells requires binding to the retinoblastoma gene product (p105) and other cellular proteins, p60, p107 and p300, we studied the effect of cisplatin (CP) and ionizing radiation on murine keratinocytes (PAM 212) transfected by several E1a mutants that bind to different subset of proteins. Cell viability and dose response assays were evaluated by the crystal violet method, thymidine uptake and flow cytometry.

Keratinocytes transfected by the wild type E1a oncogene or the mutant d1787N (which binds to p60, p105, p107, and p300) showed a lethality to CP (10 ug/ml) 4 times higher than controls and 3 times to radiation (5 grays) while the keratinocytes carrying the mutants NTd1598 (binding to p105, p107 and p60) and d1922 (binding only to p300) showed similar sensitivity to DNA damage agents of the control cells carrying only the G418 resistant gene. Apoptosis (after 24 hours) studied by DNA fragmentation was only observed in the cells carrying the wild type E1a or the mutant d1787N and not with the keratinocytes transfected with the other mutants. P53 protein expression, revealed by western-blotting, showed strong positivity in the wild type E1a expressing cells and mutants that bind to all four proteins together. Intermediate levels, between the cells carrying an intact E1a gene and the neo-control keratinocytes, were observed with the other mutants From these results, we conclude that cell sensitivity to anticancer drugs and radiation induced by the E1a oncogene requires binding to a specific set of proteins, and that mutants which do not bind to p105, p107 or p300 do not undergo apoptosis after treatment with DNA damaging agents.

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CELL KILLING MEDIATED BY THE TNF RECEPTOR AND APO-1/Fas DOES NOT FOLLOW THE SAME PATHWAY

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Tumor necrosis factor (TNF) receptors and the APO-1/Fas cell surface molecule are members of the tumor necrosis factor/nerve growth factor (NGF) receptor superfamily which have been implicated in induction of cell death in various cells. In this study we compared the killing pathways mediated by both receptor molecules in TNF-sensitive L929 cells stably transfected with APO-1/Fas cDNA. Morphologically, TNF-induced cell death resembled necrosis. In contrast, APO-1/Fas-mediated cell killing followed an apoptotic pattern as shown by the appearance of membrane blebbing, nuclear condensation and nonrandom DNA degradation. Experiments with several inhibitors revealed that the mechanism of TNF- and APO-1/Fas-mediated cell killing was substantially different. TNF cytotoxicity was mediated by reactive oxygen intermediates (ROI) generated during mitochondrial respiration. However, APO-1/Fas-induced cell killing did not reveal any involvement of mitochondria or ROI because neither mitochondrial inhibitors nor antioxidants exerted a protecting effect. Moreover, several inhibitors of calcium metabolism, ADP ribosylation and phospholipase action inhibited TNF cytotoxicity, but failed to protect against APO-1/Fas-mediated apoptosis. These data suggest that the mechanism of TNF receptor and APO-1/Fas-mediated cell death may be different in a single cell type. Hence, cells may undergo different cell death programs depending on the activating stimulus.

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CELL NUCLEUS AND DNA FRAGMENTATION NOT REQUIRED FOR APOPTOSIS

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Apoptotic cell death is characterized by a sequence of cytological alterations including membrane blebbing and nuclear and cytoplasmic condensation. Activation of an endonuclease which cleaves genomic DNA into internucleosomal DNA fragments is considered to be the hallmark of apoptosis. However, no clear evidence exists that DNA degradation plays a primary and causative role in apoptotic cell death. Here we show that L929 cells enucleated with cytochalasin B still undergo apoptosis either induced by treatment with menadione, an oxidant quinone compound, or by triggering APO-1/Fas, a cell surface molecule involved in physiological cell death. Incubation of enucleated cells with the agonistic monoclonal anti-APO-1 antibody revealed the key morphological features of apoptosis. Moreover, in non-enucleated cells inhibitors of endonuclease blocked DNA fragmentation, but not cell death induced by anti-APO-1. These data suggest that DNA degradation and nuclear signaling are not required for apoptotic cell death.

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List of Invited Speakers

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Workshop on

THE BIOCHEMISTRY AND REGULATION OF PROGRAMMED CELL DEATH

List of Invited Speakers

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Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

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10 **Workshop on Engineering Plants Against Pests and Pathogens.**
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Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

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19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanes-sian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic. P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

24 Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.
Organized by P. D. Coleman, F. Mora and M. Nieto-Sampedro. Lectures by J. Avila, Y.-A. Barde, P. D. Coleman, C. W. Cotman, Y. Lamour, M. P. Mattson, A. Matus, E. G. McGeer, B. S. Meldrum, J. Miquel, F. Mora, M. Nieto-Sampedro, V. H. Perry, M. P. Rathbone, G. S. Roth, R. R. Sturrock, H. B. M. Uylings and M. J. West.

25 Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.
Organized by J. J. Bujarski, S. Schlesinger and J. Romero. Lectures by V. Agol, P. Ahlquist, J. J. Bujarski, J. Burgyán, E. Domingo, L. Enjuanes, S. P. Goff, T. C. Hall, A. S. Huang, K. Kirkegaard, M. M. C. Lai, T. J. Morris, R. F. Ramig, D. J. Robinson, J. Romero, L. Roux, S. Schlesinger, A. E. Simon, W. J. M. Spaan, E. G. Strauss and S. Wain-Hobson.

26 Workshop on Cellular Interactions in the Early Development of the Nervous System of Drosophila.
Organized by J. Modolell and P. Simpson. Lectures by S. Artavanis-Tsakonas, J. A. Campos-Ortega, S. B. Carroll, C. Dambly-Chaudière, C. Q. Doe, R. G. Fehon, M. Freeman, A. Ghysen, V. Hartenstein, D. Hartley, Y. N. Jan, C. Klämbt, E. Knust, A. Martínez-Arias, M. Mlodzik, J. Modolell, M. A. T. Muskavitch, B.- Z. Shilo, P. Simpson, G. M. Technau, D. van Vactor and C. Zuker.

27 Workshop on Ras, Differentiation and Development
Organized by J. Downward, E. Santos and D. Martín-Zanca. Lectures by A. Balmain, G. Bollag, J. Downward, J. Erickson, L. A. Feig, F. Giráldez, F. Karim, K. Kornfeld, J. C. Lacal, A. Levitzki, D. R. Lowy, C. J. Marshall, J. Martín-Pérez, D. Martín-Zanca, J. Moscat, A. Pellicer, M. Perucho, E. Santos, J. Settleman, E. J. Taparowsky and M. Yamamoto.

28 Human and Experimental Skin Carcinogenesis
Organized by A. J. P. Klein-Szanto and M. Quintanilla. Lectures by A. Balmain, F. F.

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Becker, J. L. Bos, G. T. Bowden, D. E. Brash, A. Cano, C. J. Conti, G. P. Dotto, N. E. Fusenig, J. L. Jorcano, A. J. P. Klein-Szanto, E. B. Lane, H. Nakazawa, G. Orth, M. Quintanilla, D. R. Roop, J. Schweizer, T. J. Slaga and S. H. Yuspa.

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The Centre for International Meetings on Biology
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