ADVANCES IN

Immunology

EDITED BY

FRANK J. DIXON

Research Institute of Scripps Clinic
La Jolla, California

ASSOCIATE EDITORS

Frederick Alt
K. Frank Austen
Tadamitsu Kisimoto
Fritz Melchers
Jonathan W. Uhr

VOLUME 61

ACADEMIC PRESS
San Diego New York Boston
London Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

International Standard Serial Number: 0065-2776

International Standard Book Number: 0-12-022461-5

PRINTED IN THE UNITED STATES OF AMERICA
96 97 98 99 00 01 BB 9 8 7 6 5 4 3 2 1

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Robert T. Abraham (147), Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905

Iwan Aebischer (341), Institute of Immunology and Allergology, University of Bern, Inselspital, CH-3010 Bern, Switzerland

John P. Atkinson (201), Department of Medicine, Division of Rheumatology, Washington University School of Medicine, St. Louis, Missouri 63110

Jacques Banchereau (1), Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France

Claus R. Bartram[1] (285), Section of Molecular Biology, Pediatrics II, University of Ulm, D-89070 Ulm, Germany

Timothy C. Farries (201), Molecular Immunopathology Unit, MRC Centre, Cambridge CB2 2QH, United Kingdom

Larry M. Karnitz (147), Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905

M. Kathryn Liszewski (201), Department of Internal Medicine, Division of Rheumatology, Washington University School of Medicine, St. Louis, Missouri 63110

Douglas M. Lublin (201), Department of Pathology, Division of Laboratory Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Bernard Mach (327), Department of Genetics and Microbiology, University of Geneva Medical School, CH-1211 Geneva, Switzerland

Walter Reith (327), Department of Genetics and Microbiology, University of Geneva Medical School, CH-1211 Geneva, Switzerland

Isabelle A. Rooney (201), Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

[1] Present address: Institute of Human Genetics and Anthropology, University of Heidelberg, 69120 Heidelberg, Germany.

Klaus Schwarz (285), Section of Molecular Biology, Pediatrics II, University of Ulm, D-89070 Ulm, Germany

Beda M. Stadler (341), Institute of Immunology and Allergology, University of Bern, Inselspital, CH-3010 Bern, Switzerland

Janet Stavnezer (79), Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122

Viktor Steimle (327), Department of Genetics and Microbiology, University of Geneva Medical School, CH-1211 Geneva, Switzerland

Cees van Kooten[2] (1), Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France

[2] Present address: Department of Nephrology, AZL, Leiden, The Netherlands.

ADVANCES IN IMMUNOLOGY, VOL. 61

CD40–CD40 Ligand: A Multifunctional Receptor–Ligand Pair

CEES VAN KOOTEN[1] AND JACQUES BANCHEREAU[2]

Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France

I. Introduction

A decade has passed since monoclonal antibodies allowed the identification of the CD40 antigen. Meanwhile it has become clear that, in addition to soluble factors (cytokines), direct cellular interactions play a pivotal role in the regulation of immune responses. The CD40 antigen was independently identified in 1985 and 1986 by monoclonal antibodies reacting with carcinomas and B cells (antibody S2C6, antigen p50 (Paulie *et al.*, 1985)) and showing costimulatory effects for B lymphocyte proliferation (antibody G28-5, antigen Bp50 (Clark and Ledbetter, 1986)). This antigen was designated as CDw40 at the Third International Workshop on Leukocyte Antigens in Oxford in 1986, and CD40 at the Fourth Workshop in Vienna in 1989. A cDNA encoding CD40 was isolated in 1989 (Stamenkovic *et al.*, 1989) whose sequence demonstrated a relationship with the human low-affinity nerve growth factor receptor (LNGFR). These molecules are now considered as part of the tumor necrosis factor receptor superfamily. Cross-linking of CD40, in conjunction with IL-4, was then found to induce B cells to undergo long-term B cell growth, as well as isotype switching (Banchereau *et al.*, 1991; Banchereau and Rousset, 1991; Jabara *et al.*, 1990). In 1992, expression cloning using CD40–Fc fusion protein allowed the isolation of a CD40 ligand (CD40-L) expressed on activated T cells (Armitage *et al.*, 1992a), an observation which led to the demonstration of the key role of CD40–CD40-L interactions in T-cell-dependent B cell activation by many groups (Noelle *et al.*, 1992a). The CD40-L is now one of the members of the recently identified tumor necrosis factor superfamily. In 1993, it was demonstrated that a genetic alteration of the CD40-L was responsible for the X-linked hyper-IgM syndrome which is characterized by the lack of circulating IgG and IgA and the absence of germinal centers (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993; Fuleihan *et al.*, 1993; Korthäuer *et al.*, 1993). These phenotypes have been confirmed in murine "knockout" experiments where either the CD40 or the CD40-

[1] Present address: Department of Nephrology, AZL Building 1 C3P, Rijnsburgerweg 10, 2333 AA Leiden, the Netherlands.

[2] To whom correspondence should be addressed at Schering-Plough, Laboratory for Immunological Research, 27 chemin des Peupliers, BP 11, 69571 Dardilly, France. Fax: (33) 78.35.47.50.

L was inactivated by homologous recombination (Kawabe *et al.*, 1994; Xu *et al.*, 1994).

While the function of CD40 has principally been studied on mature B lymphocytes, more recent studies have shown the presence of biologically functional CD40 on other cell types, such as monocytes/macrophages (Alderson *et al.*, 1993), dendritic cells (Caux *et al.*, 1994), hematopoietic progenitors (Saeland *et al.*, 1992), epithelial cells (Galy and Spits, 1992), and endothelial cells (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995). Many aspects of CD40–CD40-L biochemistry and biology have been reviewed extensively in recent papers (Armitage and Alderson, 1995; Banchereau *et al.*, 1994; Bonnefoy and Noelle, 1994; Callard *et al.*, 1993; Clark and Ledbetter, 1994; Hollenbaugh *et al.*, 1994) and the reader is referred to these articles for additional details. In the present review, we will concentrate on the most recent developments, including (i) those concerning CD40 signal transduction, (ii) a comparison with other members of the superfamily, (iii) a functional expression of CD40 on cell types other than B cells, (iv) the *in vivo* relevance of CD40–CD40-L interactions.

II. CD40 as a Member of the TNF-Receptor Superfamily

A. Cloning and Structure of CD40

The CD40 antigen is a phosphorylated glycoprotein which migrates in SDS polyacrylamide gel electrophoresis as a 48-kDa polypeptide under both reducing and nonreducing conditions. It is a hydrophobic molecule with an acidic P_i of 3.2. A significant proportion of CD40 from the Burkitt lymphoma Raji cells and normal B cells is in a dimeric form whereas such dimers are virtually absent from carcinoma lines or EBV-transformed cells (Braesch-Andersen *et al.*, 1989; Paulie *et al.*, 1989).

A cDNA encoding CD40 was isolated by expression cloning from a library of the Burkitt lymphoma Raji (Stamenkovic *et al.*, 1989). The mature molecule is composed of 277 amino acids (AA) with a 193-AA extracellular domain including a 21-AA leader sequence, a 22-AA transmembrane domain, and a 62-AA intracellular tail. Therefore, CD40 has the structure of a typical type I transmembrane protein. The human CD40 gene is expressed as a single 1.5-kb mRNA species. The extracellular segment of CD40 displayed significant similarity (34.5% in the extracellular region) to the analogous protein domain of the p75 LNGFR (Johnson *et al.*, 1986). In particular, the 22 cysteine residues in CD40, which form four predicted protein domains of about 40 AA with 6 cysteine residues each (Fig. 1), are homologous to LNGFR and the other members of what is now called the TNF-R family (see Section II,B). This is in contrast to the intracellular

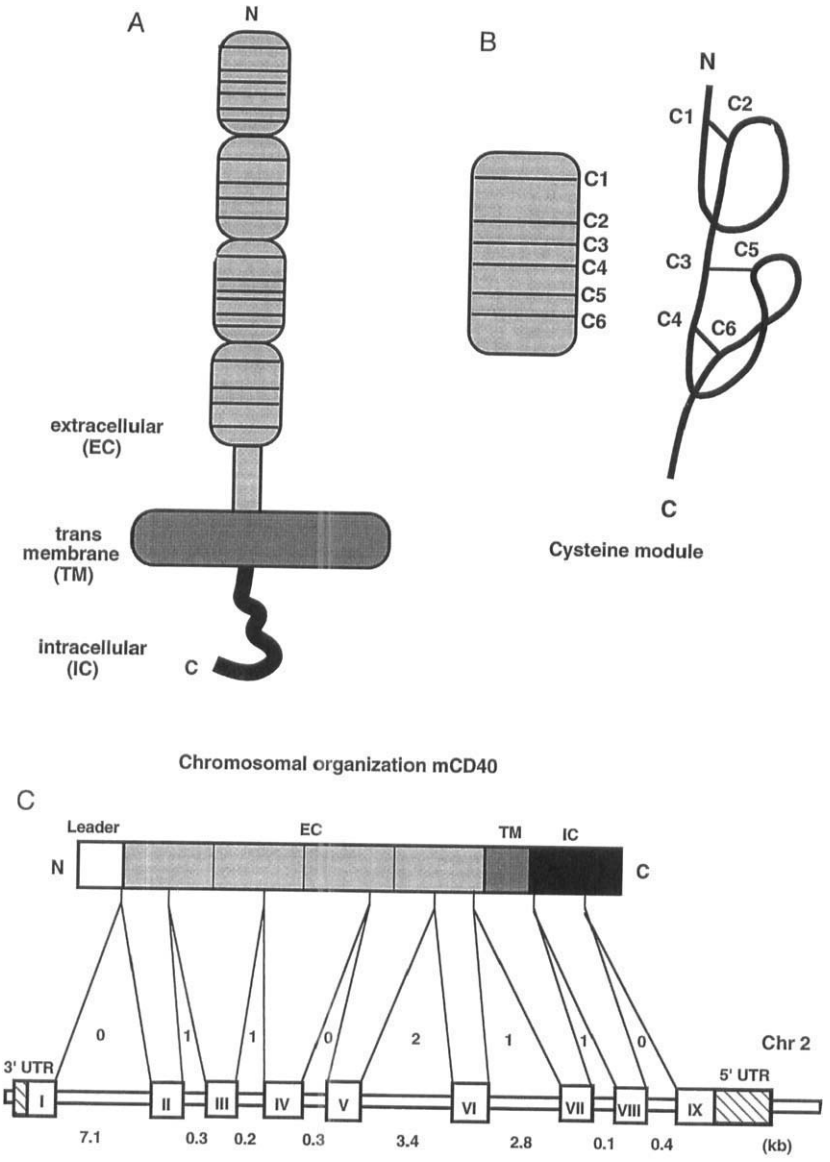


FIG. 1. Structural features of CD40, including protein structure (A), cysteine module (B), and chromosomal organization (C). Places where the coding sequence is interrupted by introns are indicated, as well as the phase of this splicing (0, intercutting between codons; 1, intercutting after first base of triplet; 2, intercutting after second base of triplet). The sizes (in kilobases) of the respective introns are indicated on the bottom. See text for more details

chain of CD40 which does not betray a close relationship to any other characterized molecule.

The murine cDNA was cloned from LPS + IL-4-stimulated murine B cells by cross-hybridization with the human cDNA probe (Torres and Clark, 1992). The mouse CD40 protein is composed of 305 AA with a 193-AA extracellular region including a 21-AA leader sequence, a 22-AA transmembrane domain, and a 90-AA intracellular region. Human and murine CD40 molecules share 62% amino acid identity in the complete coding sequence and 78% identity in the intracellular extensions. The last 32 C-terminal AA of human CD40 are completely conserved in the mouse sequence. In addition 22/22 extracellular cysteine residues are conserved, suggesting that both mouse and human CD40 fold into the same protein domains. The mouse CD40 gene is expressed as two mRNA species of 1.7 and 1.4 kb. These two forms are generated by alternative polyadenylation using two different polyadenylation sites in the 3′ untranslated sequence and thus result in the same coding sequence. Activation of mouse B cells seems to give a specific up-regulation of the smaller 1.4-kb transcript (Torres and Clark, 1992).

The CD40 murine gene consists of nine exons and spans a total region of 16.3 kb genomic DNA (Grimaldi *et al.*, 1992). The schematic representation of the mouse CD40 gene organization, shown in Fig. 1, clearly indicates that the cysteine repeat domains are not coded for by separate exons. In contrast, cysteine repeats are mostly divided by introns at positions which are conserved by other members of the TNF-R family (Birkeland *et al.*, 1995). This is especially true for the type 1 introns (intercutting codons after the first nucleotide base) in the first two N-terminal cysteine repeats. For murine CD40, the signal peptide, the transmembrane region, and the cytoplasmic tail are coded for by exons separate from the cysteine repeats, but this is not a general rule for all TNFR family members.

The mouse CD40 gene is located on the distal region of chromosome 2 which is syntenic to human chromosome 20q11-q13 (Grimaldi *et al.*, 1992). Accordingly, the human CD40 gene was mapped to chromosome 20 by using human-rodent somatic cell hybrids (Ramesh *et al.*, 1993b) and to 20 q11-20q13-2 by *in situ* hybridization (Lafage-Pochitaloff *et al.*, 1994). Deletions of this part of chromosome 20 are observed frequently in myeloid malignancies, but the significance of this observation remains to be established.

B. Relationship with Other TNF-R Superfamily Members

As stated above, CD40 was found to be homologous to the previously described p75 low-affinity nerve growth factor receptor. The p75 LNGFR can therefore be seen as the founding father of a superfamily of receptor-

like molecules that share a common binding domain composed of tandemly repeated cysteine-rich modules (Mallett and Barclay, 1991). However, since interaction of LNGFR with its natural ligands is not typical for the rest of the receptor family, nowadays the family is more appropriately named after two later additions of this family: the TNF-R superfamily. The family is composed of at least 10 genes described in human and mouse (LNGFR, TNF-R1, TNF-R2, TNF-Rrp, CD40, CD27, CD30, CD95, Ox40, 4-1BB), as well as some viral and fungal homologs. All the human and mouse genes code for type I transmembrane proteins, consisting of an N-terminal signal peptide, which directs this end of the molecule to the outside of the cell, followed by an extracellular region, a single membrane-spanning region, and an intracellular region. Although it would be outside the scope of this chapter to discuss all these molecules in great detail, we will try to give a brief summary of some of their characteristics, and, where possible, refer to recent reviews on these molecules. These results are schematically drawn in Fig. 2 and gathered in Table 1.

LNGFR has been cloned using human cosmid clones which encoded LNGFR expression when transfected in mouse L cell fibroblasts (Chao *et al.*, 1986). Human LNGFR codes for a 399-AA mature protein consisting of 222 AA extracellular, 22 AA transmembrane, and a 155-AA intracellular region (Johnson *et al.*, 1986). The human gene is localized at chromosome 17q12-q22 (Huebner *et al.*, 1986). LNGFR acts principally to recruit a number of neurotrophin ligands, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and NT4, to the cell surface, aiding in the formation of the signaling complex formed by dimeric *trk* receptors that possess intracellular tyrosine kinase domains (Raffioni *et al.*, 1993). Mutations preventing binding of NGF to LNGFR have no deleterious effect on the biological activity of NGF, questioning the role of this receptor in signal transduction (Ibanez *et al.*, 1992).

TNF-R1 (CD120a) and TNF-R2 (CD120b) are two widely expressed receptor molecules, also referred to as p55-TNF-R and p75-TNF-R, respectively, which can both bind two different ligands: tumor necrosis factor-α (TNF-α, also known as cachectin) and lymphotoxin-α (LT-α, also known as TNF-β) (Tartaglia and Goeddel, 1992). The two cytokines TNF-α and LT-α are also widely expressed and have pleiotropic activities (Beutler and Cerami, 1989; Paul and Ruddle, 1988). Human TNFR1 was cloned after purification of the protein from HL60 cells as well as from the serum of cancer patients, followed by partial protein sequencing and the generation of degenerate primers (Loetscher *et al.*, 1990; Schall *et al.*, 1990). The predicted mature protein consists of 415 AA (172 AA extracellular, 23 AA transmembrane, 220 AA intracellular) and is coded for by a single mRNA species of 3 kb. Human TNFR2 was cloned by direct expres-

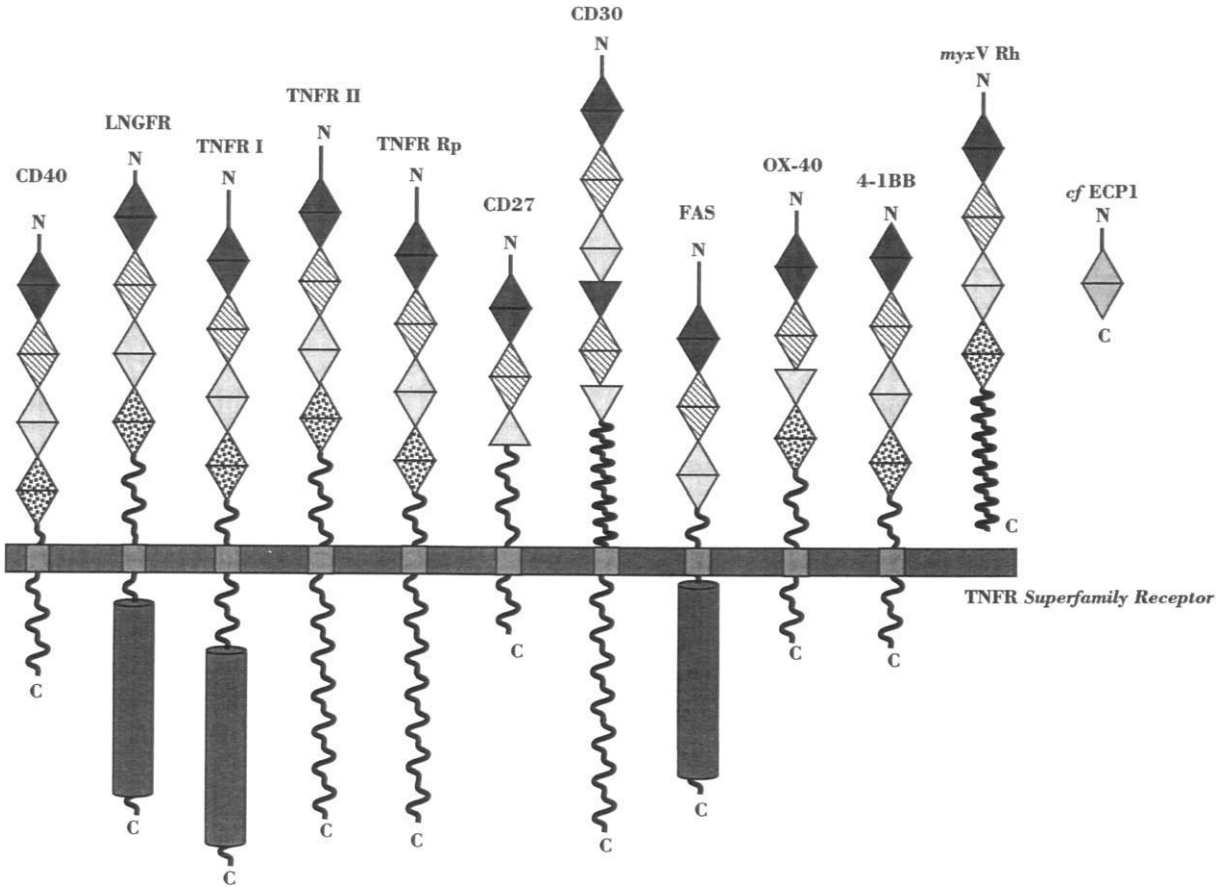


FIG. 2. Schematic representation of the tumor necrosis factor receptor superfamily. See text and Table 1 for details.

TABLE I

TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY

	CD40	LNGFR	TNF-R1	TNF-R2	TNF-Rrp	CD27	CD30	Fas	Ox40	4-1BB
Size (AA)	277	399	415	440	408	240	577	319	249	238
Extra	193	222	172	236		171	365	155	185	169
TM	22	22	23	30		21	24	19	27	27
Intra	62	155	220	174		48	188	145	37	42
mRNA(kb)	1.5	3.8	3	4.5		1.3	3.8/2.6	2.7/1.9	1.4	4.4/1.8
Exons	9 (m)	6 (h)	10 (h)			6 (h)		9 (h)	7 (m)	10 (m)
chr (h)	20q11-q13	17q12-q22	12p13	1p36	12p13	12p13	1p36	10q24	1p36	
chr (m)	2	1	6	4				19	4	4

Note. Data given are on human (h) proteins/genes, except for chromosomal organization and location, which also include mouse (m) data.

sion cloning from a human lung fibroblast library by expression screening with radiolabeled TNF-α (Smith *et al.*, 1991b). The predicted mature protein consists of 440 AA (236 AA extracellular, 30 AA transmembrane, and 174 AA intracellular) and is coded for by a single mRNA species of 4.5 kb. Mouse TNF-R1 and TNF-R2 have been cloned by cross-hybridization with human cDNA probes (Goodwin *et al.*, 1991; Lewis *et al.*, 1991). The human gene for TNF-R1 is located on chromosome 12p13, spans about 13 kb, and consists of 10 exons, which, as for mouse CD40, are interrupting the cysteine repeat domains (Fuchs *et al.*, 1992). For the mouse, the TNF-R1 gene is located to the syntenic region of chromosome 6. The human gene for TNF-R2 has been localized on chromosome 1p36 (Kemper *et al.*, 1991).

TNF-Rrp is a TNF-R-related protein which has been cloned as an open reading frame from a human chromosome 12 specific library of heteronuclear cDNAs (Baens *et al.*, 1993). The gene organization is similar to that of TNF-R1, and spans 9 kb of chromosome 12p13. The protein consists of 408 AA, and has been shown to be a specific receptor for membrane-bound LT-$\alpha\beta$ heterotrimers (Crowe *et al.*, 1994). The importance of this receptor is demonstrated by gene inactivation of LT-α, which prevents the generation of LT$\alpha\beta$ heterotrimers and results in abnormal development of peripheral lymphoid organs and complete absence of lymph nodes and Peyer's patches (De Togni *et al.*, 1994).

CD27 is an activation molecule expressed as a disulfide-linked homodimer on subsets of T and B lymphocytes (Hintzen *et al.*, 1994). A human cDNA encoding CD27 was cloned from PHA-stimulated PBMC by expression cloning (Camerini *et al.*, 1991). Mature CD27 consists of 240 AA (171 extracellular, 21 transmembrane, and 48 intracellular). Northern blot analysis shows a single mRNA species of 1.3 kb. CD27 is localized at chromosome 12p13 and consists of six exons spanning approximately 7 kb (Loenen *et al.*, 1992b). Mouse CD27 cDNA was cloned from a thymocyte cDNA library by hybridization with a partial genomic mouse clone which had been isolated by cross-hybridization with the human probe (Gravestein *et al.*, 1993).

CD30 is an activation molecule on T cells and B cells, initially detected on Reed-Sternberg cells of patients with Hodgkin's disease (Del Prete *et al.*, 1995; Falini *et al.*, 1995). The CD30 human cDNA was cloned by expression from the HTLV$^+$ T cell line HUT102 (Durkop *et al.*, 1992). The mature CD30 protein consists of 577 AA (365 AA extracellular, 24 AA transmembrane, and 188 AA intracellular) forming six cysteine-rich modules. The extracellular region contains two large homologous domains, probably caused by gene duplication, as reflected by the fact that mouse CD30 only contains one such domain (three cysteine modules) (Bowen,

1993). Northern blot analysis shows a major 3.8-kb and a minor 2.6-kb band of human CD30 mRNA (Durkop *et al.*, 1992). The human CD30 gene has been localized on chromosome 1p36 (Fonatsch *et al.*, 1992).

CD95 (Fas/Apo), in this chapter referred to as Fas, is a widely distributed molecule, which, upon crosslinking, induces apoptosis of the expressing cells (Nagata and Golstein, 1995). This receptor is involved in activation-induced apoptosis during the induction of peripheral tolerance and in cytotoxicity. Human Fas has been cloned from a T lymphoma cDNA library by direct expression cloning (Itoh *et al.*, 1991). The mature protein consists of 319 AA (155 AA extracellular, 19 AA transmembrane, and 145 AA intracellular). Northern blot analysis shows two major mRNA species of 2.7 and 1.9 kb, which are probably the result of alternative polyadenylation. The same gene was cloned after purification and partial amino acid sequencing of the Apo-1 antigen (Oehm *et al.*, 1992). Significant homology in the intracellular region of Fas and TNF-R1 was observed, often referred to as "death domain" because of the important role of this region in the induction of apoptosis (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Mouse Fas was cloned from a macrophage cDNA library by cross-hybridization with the human probe (Watanabe-Fukunaga *et al.*, 1992b). The mature protein consists of 306 AA, and Northern blot analysis shows a single mRNA species of 2.1 kb. The mouse gene is located on chromosome 19, and was shown to be identical to the *lpr* gene, whose mutations lead to lymphoproliferation and autoimmune phenomena (Watanabe-Fukunaga *et al.*, 1992a). The human gene is located at chromosome 10q24 (Inazawa *et al.*, 1992; Lichter *et al.*, 1992) and consists of nine exons spanning a region of 25 kb (Behrmann *et al.*, 1994; Cheng *et al.*, 1995b). Recently, three patients with lymphoproliferative syndrome were demonstrated to have mutations in their Fas gene (Rieux-Laucat *et al.*, 1995).

Ox40 was initially identified by expression cloning of a marker of activated rat T cells (Mallett *et al.*, 1990). The mouse Ox40 gene was cloned by PCR using primers designed with the rat sequence (Calderhead *et al.*, 1993). This allowed the cloning of human Ox40 by cross-hybridization with the mouse Ox40 probe (Baum *et al.*, 1994). The same human protein was independently cloned by expression cloning of the human ACT35 antigen, defined as a T cell activation marker found on the $HTLV^+$ T cell line HUT102 (Latza *et al.*, 1994). The mature human Ox40 protein consists of 249 AA (185 AA extracellular, 27 AA transmembrane, and 37 AA intracellular). Northern blot analysis shows a single mRNA species of 1.4 kb, predominantly expressed in $CD4^+$ T cells. The human Ox40 gene is located at chromosome 1p36. The mouse Ox40 gene is located at chromosome 4, which is syntenic to human 1p36, and consists of seven exons spanning 3 kb (Birkeland *et al.*, 1995).

4-1BB was isolated as a mouse inducible T cell gene by differential screening (Kwon and Weissman, 1989). Genomic organization of mouse 4-1BB shows that it spans 13 kb, located at chromosome 4, and consists of 10 exons including 2 exons in the 5′ nontranslated region (Kwon *et al.*, 1994). Human 4-1BB was cloned from PHA + PMA-stimulated T cells by cross-hybridization with the mouse probe (Alderson *et al.*, 1994). The same gene was independently cloned as a gene induced by lymphocyte activation (ILA) (Schwarz *et al.*, 1993). The mature protein consists of 238 AA (169 AA extracellular, 27 AA transmembrane, and 42 AA intracellular) and is coded for by multiple mRNA species of 4.4, 4.0, and 1.8 kb. The 4-1BB mRNA is broadly expressed, including in activated T cells, B cells, monocytes, and nonlymphoid cells (Schwarz *et al.*, 1995).

Viral homologs of the TNF-Rs were first described by Smith *et al.* (1990) who noticed a significant resemblance between the extracellular domains of several TNF-R superfamily members and the protein product of the transcriptionally active open reading frame T2 in the Shope Fibroma virus genome (SFV-T2). This poxvirus protein is a secreted receptor for both TNF-α and LT (Smith *et al.*, 1991a) and reveals a novel aspect of viral subversion of the host immune system (see Gooding, 1992, for review). A close homolog of SFV-T2 has been characterized from Myxoma virus (MYX-T2) (Upton *et al.*, 1991). The Vaccinia virus genome contains an open reading frame, SalF19R, that appears to encode a fragment of a poxvirus T2-like protein, and can be extended by jumping reading frames (Howard *et al.*, 1991). More recent homologs of this poxvirus branch of the TNF-R superfamily include the Variola virus G4R (Shchelkunov *et al.*, 1993) and the Cowpox virus crmB (Hu and Pickup, 1991) gene products.

ECP1 is a fungal pathogenic protein isolated from *Cladosporium fulvum*, a tomato pathogen (Van den Ackerveken *et al.*, 1993). ECP1 is a secreted protein of 65 residues that appears to contain a single characteristic TNF-R-like cysteine repeat of 46 AA length, and is suggested to play a role in suppressing the tomato plant defense response by binding plant cytokine-like molecules secreted in the face of fungal attack (Bazan, 1993).

It can be concluded that the TNF-R family consists of different receptor molecules which share homologies in their structural properties. Most of the receptors are widely distributed and seem to have overlapping activities in the process of lymphocyte activation, survival, or death. Although they might have a single ancestor gene, the genes are now widely dispersed through the genome. Nevertheless some genes are still clustered on defined regions of the chromosomes: human chromosome 12p13 (TNF-R1, TNF-Rrp, and CD27), human chromosome 1p36 (TNF-R2, CD30, and Ox40), and mouse chromosome 4 (TNF-R2, Ox40, and 4-1BB). The fact that CD40 and Fas are the only genes identified on their respective chromo-

somes leaves the possibility that other members of this intriguing family might exist.

C. Cells Expressing CD40

1. B Lymphocytes

CD40 has been considered early as a pan B cell antigen (Ling *et al.*, 1987). In human fetal and adult bone marrow, CD40 is detectable on the majority of B cell precursors (BCP), which express CD19 and CD10 but lack surface Ig (Law *et al.*, 1990; Moreau *et al.*, 1993; Saeland *et al.*, 1992; Uckun *et al.*, 1990). CD40 is acquired early in B cell ontogeny, as it is present on BCP expressing the CD34 progenitor cell antigen (Saeland *et al.*, 1992) and has been reported on fetal liver $CD19^+$ cells prior to rearrangements at the IgH locus (pro-B cells) (Uckun *et al.*, 1990).

CD40 is detected on all B cells isolated from adult and cord blood, tonsils, and spleens. It is expressed on resting naive $sIgD^+$ $sIgM^+$ B cells in the primary follicles and the mantle zone of secondary follicles within the peripheral lymphoid organs (Banchereau *et al.*, 1994). The density of CD40 is identical on naive B cells, centroblasts, and centrocytes, which compose the germinal centers of secondary follicles, and on the $CD38^-$ $sIgD^-$ memory B cells (Liu *et al.*, 1995).

Plasmablasts isolated from tonsils express CD40 (Merville *et al.*, 1995a; Pellat-Deceunynck *et al.*, 1994), whereas roughly half of the antibody-secreting cells circulating in the blood 8 days after vaccination appear to have lost CD40 (Quiding-Järbrink *et al.*, 1995). The fully differentiated plasma cells of mucosal lamina propria and bone marrow do not express CD40.

Polyclonal activators, such as anti-IgM, anti-CD20, or anti-Bgp95 antibodies or phorbol esters, slightly up-regulate CD40 expression on B cells (Ledbetter *et al.*, 1987b; Stamenkovic *et al.*, 1989). Cytokines like IFN-γ (Stamenkovic *et al.*, 1989) and IL-4 (Vallé *et al.*, 1989) are able to increase CD40 levels on B cells. In addition, the EBV gene LMP1 is able to up-regulate CD40 expression in some B cell lines (Wang *et al.*, 1990).

2. Malignant B Lymphocytes

The fact that CD40 is expressed at all different stages of B cell development is also reflected by the expression of CD40 on most of their malignant counterparts.

CD40 is expressed on malignant BCP in B lineage acute lymphoblastic leukemias (B-ALL) at various maturation stages, ranging from pro-B cells to pre-B cells expressing cytoplasmic μ chain (Uckun *et al.*, 1990, 1991). Twenty-eight to 44% of B-ALL cases have been shown to express CD40 (Law *et al.*, 1990; Uckun *et al.*, 1990). Among the positive B-ALL, CD40

is present only on a proportion of the leukemic cells, a feature which has been associated with clonogenic capacity (Uckun *et al.*, 1990).

Virtually all cases of malignancies of mature B lymphocytes, including B-chronic lymphocytic leukemia (B-CLL), non-Hodgkin's lymphoma (NHL), and hairy cell leukemia (HCL) express CD40 on their B cell surface (Beiske *et al.*, 1988; Crawford and Catovsky, 1993; Fluckiger *et al.*, 1992; Funakoshi *et al.*, 1994; Kluin-Nelemans *et al.*, 1994). Burkitt lymphoma cell lines and EBV-transformed B cell lines all display CD40 (Paulie *et al.*, 1985).

Results on the expression of CD40 on multiple myeloma (MM) have been controversial. Initial studies suggested that CD40 expression was negative on 16 samples from MM patients (Jackson *et al.*, 1988), and only weakly positive on one out of four myeloma cell lines (RPMI 8226) (Hamilton *et al.*, 1991). Later studies, maybe because of better staining methods, showed CD40 staining on malignant B cells of a great majority of MM cells freshly isolated from patients (Pellat-Deceunynck *et al.*, 1994; Tong *et al.*, 1994; Urashima *et al.*, 1995; Westendorf *et al.*, 1994). Indeed, high-level CD40 expression has been claimed to correlate with progressive myeloma (Pellat-Deceunynck *et al.*, 1994). The heterogeneity of CD40 expression on MM cells may indeed reflect the fact that CD40 expression gets lost during terminal B cell differentiation.

3. *Reed Sternberg Cells*

Reed Sternberg (RS) cells are typical cells observed in Hodgkin's disease, which may be of diverse origin (Haluska *et al.*, 1994). Recent experiments have suggested that at least in some cases, RS cells might be of B cell origin as demonstrated by a monoclonal rearranged Ig gene (Tamaru *et al.*, 1994). While RS cells have been traditionally characterized by expression of CD30, another member of the TNF-R superfamily (Durkop *et al.*, 1992), CD40 has also been detected on these cells (Kennedy *et al.*, 1989). Staining of paraffin sections of lymph nodes of patients with Hodgkin's disease showed CD40 staining in 26/37 or 139/139 cases (Carbone *et al.*, 1995; O'Grady *et al.*, 1994). CD40 expression was confirmed on isolated primary or cultured RS cells (Gruss *et al.*, 1994).

4. *Hematopoietic Progenitors*

CD40 is expressed on all cord blood $CD34^+$ progenitors, which lack a $CD19^+$ $CD10^+$ subset, and on the majority of bone marrow $CD34^+$ cells (Saeland *et al.*, 1992). CD40 expression is more heterogeneous on $CD34^+$ progenitors than on mature B cells. CD40 expression is lost during myeloid development after culturing $CD34^+$ cells in the presence of IL-3 (Saeland *et al.*, 1992).

5. *T Lymphocytes*

CD40 expression was observed on rare $CD4^+$ and $CD8^+$ T cell clones (Ware *et al.*, 1991), as well as on HTLV I-transformed T cells lines of human and baboon origin (Dunlap *et al.*, 1989; Indzhiia *et al.*, 1992). CD40 expression has been detected on T cells isolated from rheumatoid arthritis patients (Potocnik *et al.*, 1990). Both on human $\alpha\beta TCR^+$ T cells and on mouse $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ T cells, the expression of CD40 has been suggested by functional experiments that show CD40-L to be a growth factor for activated T cells (Armitage *et al.*, 1993b; Fanslow *et al.*, 1994; Ramsdell *et al.*, 1994a). There are no data available on mRNA expression, but CD40 expression was demonstrated by FACS analysis and immunoprecipitation. Apart from T cell-expressed CD40, the molecule can also be demonstrated as a cell-bound soluble molecule. Staining with a polyclonal anti-CD40 antibody detected soluble CD40 bound to CD40-L expressed on activated T cells, after interaction with B cells or dendritic cells (van Kooten *et al.*, 1994; Caux *et al.*, 1994).

6. *Monocytes*

Primary human monocytes freshly isolated or cultured for 48 hr show low but detectable CD40 surface protein (Alderson *et al.*, 1993). Strong expression is observed on cultured plastic adherent human monocytes (Wagner *et al.*, 1994). GM-CSF, IL-3, and IFN-γ strongly up-regulate the CD40 expression, as demonstrated both by surface expression and by elevated mRNA levels (Alderson *et al.*, 1993).

7. *Dendritic Cells*

Immunohistological analysis of tonsil and spleen sections has shown very high levels of CD40 on interdigitating dendritic cells in the T-cell-rich areas of secondary lymphoid organs (Ling *et al.*, 1987). These cells derive from skin/mucosal Langerhans cells which only weakly express CD40 (Romani *et al.*, 1989). Note that the use of trypsin to isolate Langerhans cells from skin results in a strong decrease of CD40 expression (Peguet-Navarro *et al.*, submitted for publication). Following culture, Langerhans cells express CD40 at high levels. Dendritic cells can be generated *in vitro* by culturing $CD34^+$ hematopoietic progenitor cells in the presence of GM-CSF/IL-3 and TNF-α (Caux *et al.*, 1992). These cells, which express CD1a and CD40 at high levels, include both Langerhans cells with Birbeck granules and Factor XIIIa-positive dermal dendrocytes. On these cells, expression of CD40 was shown to be up-regulated, after crosslinking with CD40-L-transfected cells (Caux *et al.*, 1994). Finally, dendritic cells isolated from peripheral blood also express CD40 (Freudenthal and Steinman, 1990; Pinchuk *et al.*, 1994; Zhou and Tedder, 1995), and may represent Langerhans cells homing toward secondary lymphoid organs.

8. *Follicular Dendritic Cells*

CD40 is expressed on follicular dendritic cells (FDC) of secondary lymphoid organs as shown by immunohistologic analysis on tissue sections (Banchereau *et al.*, 1994). CD40 expression is also shown by flow cytometry on freshly isolated FDC (Kim *et al.*, 1994; Schriever *et al.*, 1989), on FDC-like cell lines cultured in the presence of GM-CSF (Clark *et al.*, 1992), on FDC-like cell lines grown without GM-CSF (Kim *et al.*, 1994), and on EBV-transformed FDC-like cell lines (Lindhout *et al.*, 1994).

9. *Epithelial Cells*

The CD40 antigen was initially identified with the mAb S2C6 which was generated from mice immunized with a urinary bladder carcinoma (Koho *et al.*, 1984). Subsequently, the CD40 antigen has been identified on carcinomas of other origins such as colon, prostate, breast, and lung, as well as on melanomas (Ledbetter *et al.*, 1987a; Stamenkovic *et al.*, 1989). Concerning normal epithelia, immunohistochemical staining showed expression of CD40 on the basal (proliferating) epithelial layer of nasopharynx, tonsil, and ectocervix (Young *et al.*, 1989).

CD40 is expressed in the CD45-negative stromal cell population of human thymus (Galy and Spits, 1992). Immunohistology shows CD40 expression on cortical and medullary thymic epithelial cells, as well as thymic interdigitating cells and B cells. Expression of CD40 is specifically maintained on cultured thymic epithelial cells but not on thymic fibroblasts. CD40 was also demonstrated on cloned thymic epithelial cells derived from cortical epithelium (Fernandez *et al.*, 1994). IL-1α, TNF-α, and IFN-γ significantly upregulate CD40 levels and 1.4 kb CD40 transcripts on cultured epithelial cells (Galy and Spits, 1992).

Recently, we found that cultured proximal tubular epithelial cells (PTEC) from human kidneys also express CD40 (van Kooten *et al.*, manuscript in preparation). Interestingly, PTEC, which together with thymus and intestinal epithelial cells are from mesoderm and not ectoderm origin, can be distinguished by their spontaneous expression of MHC class II (Unanue and Allen, 1986). An analysis of the regulation of CD40 expression and function on these cells is currently underway.

10. *Endothelial Cells*

Endothelial cells of microvessels in the skin intensely express CD40 (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995). In agreement, cultured human umbilical vein endothelial cells (HUVEC) constitutively express the CD40 antigen. The expression of CD40 is significantly up-regulated by IL-1α, TNF-α, IFN-γ, and IFN-β, but not by IL-4 (Karmann *et al.*, 1995).

11. Basophils

Basophils isolated from the blood of chronic myeloid leukemia patients have been reported to express CD40, as shown by binding of mAb B-E10 (Valent *et al.*, 1990). In contrast there was no detectable expression on mast cells.

12. Synoviocytes and Fibroblasts

CD40 is expressed on human synoviocytes and skin fibroblasts as shown by FACS analysis of freshly isolated cells (Rissoan *et al.*, submitted for publication). Northern blot analysis shows a normal size CD40 mRNA signal of 1.5 kb. Immunohistochemistry shows a distinct CD40 staining of the synovial lining cells of synovial tissue. Cultured cells show enhanced CD40 expression in response of IFN-γ, and to a lesser extent TNF-α, but not with IL-1β, IL-4, IL-6, or GM-CSF.

13. Other Cell Types

Immunohistological analysis performed with the anti-CD40 mAb G28.5 on many different tissues (Möller and Mielke, 1989) has also indicated staining of various tissues, but the CD40-expressing cells have not always been identified: smooth muscle cells (+/−), cardiac myocytes (+), epidermis (weakly +), gastrointestinal mucosa (+), gallbladder mucosa (+), bronchus mucosa (+), salivary gland ductal and acinar cells (+), pancreas ductal cells, mammary ductal and acinar cells (+/−), sweat gland (weakly +), prostata gland cells (weakly +), thyroid gland (+), and parathyroid gland (+).

In summary, CD40 is expressed on cells with high proliferation potential, such as hematopoietic progenitors, B lymphocytes, and epithelial cells, as well as cells able to present antigen such as dendritic cells, activated monocytes, B lymphocytes, and follicular dendritic cells (Fig. 3). This suggests that the physiological role of CD40 may be broader than initially suggested by its pan-B cell expression.

D. Generation of Soluble Forms of TNFR Superfamily Members

The release of soluble forms of receptors, which normally function as transmembrane molecules, is now seen as a general concept (Bazil, 1995; Heaney and Golde, 1993). The generation of soluble molecules can take place via two general mechanisms: either through alternative splicing of the gene product or through proteolysis of the transmembrane protein. The receptor cleavage may serve two purposes: (i) upon cleavage from the membrane, a receptor will not transmit further signals; (ii) since most soluble products retain their ligand binding activity, the soluble receptor

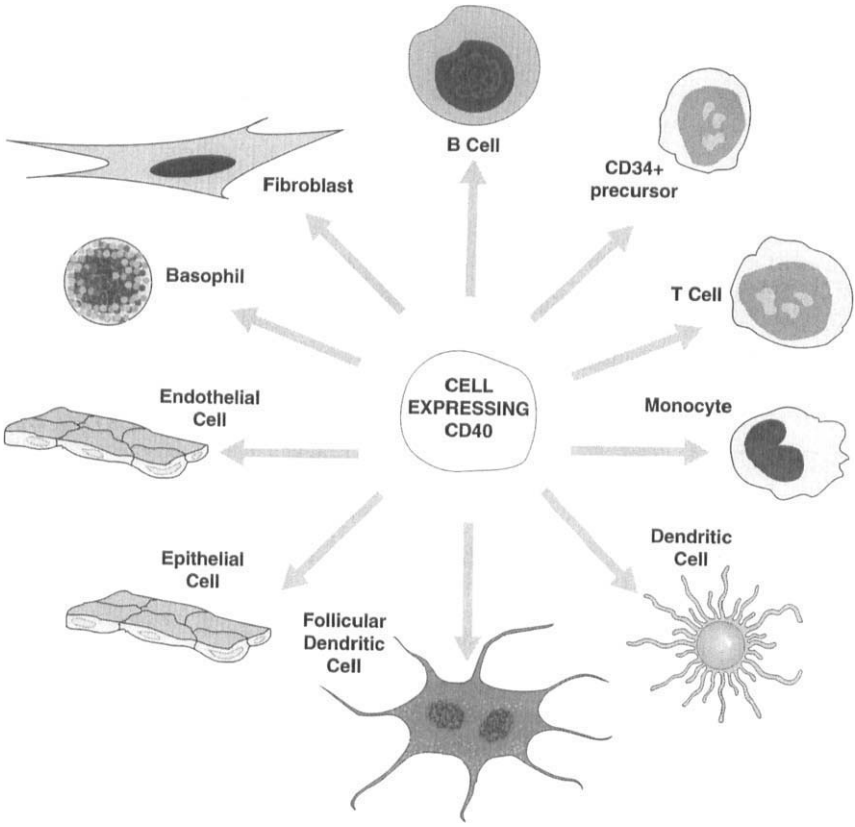


FIG. 3. Cells expressing CD40.

may prevent available ligand to signal functional transmembrane receptors. Therefore, these soluble receptors might have important regulatory roles.

Numerous EBV-transformed B cell lines release soluble CD40 (sCD40) spontaneously, as detected with a specific ELISA (Björck *et al.*, 1994; Grammer *et al.*, 1995; van Kooten *et al.*, 1994). Supernatants of *Staphylococcus aureus* strain Cowan I-activated normal B cells cultured in the presence of IL-4 or IL-2 also contain sCD40. Although the mechanisms of sCD40 release have not been studied yet, it is possible that these molecules originate from proteolytic cleavage since there are no indications of alternatively spliced CD40 mRNA (Stamenkovic *et al.*, 1989). sCD40 of EBV cell line supernatants retains its binding capacity since it binds to CD40-L expressed on activated T cells (Grammer *et al.*, 1995; van Kooten

et al., 1994), as well as to a recombinant CD40-L–CD8 chimeric molecule, when the sCD40 is immobilized on the plastic matrix (Björck *et al.*, 1994).

The release of soluble receptor molecules seems to be a general property of the members of the TNF-R superfamily members, inasmuch as sTNF-R1 and sTNF-R2 (Engelmann *et al.*, 1990; Porteu and Nathan, 1990), sLNGFR (DiStefano and Johnson, 1988), sCD27 (Hintzen *et al.*, 1991), sCD30 (Josimovic-Alasevic *et al.*, 1989), sFas (Cheng *et al.*, 1994; Knipping *et al.*, 1995), and s4-1BB (Goodwin *et al.*, 1993b) have been identified. sTNF-R (Loetscher *et al.*, 1990; Porteu and Nathan, 1990; Schall *et al.*, 1990) and sCD27 (Loenen *et al.*, 1992a) have been shown to be generated by proteolysis of the membrane molecule, whereas s4-1BB appears to be the result of an alternative splice variant, lacking the transmembrane region (Goodwin *et al.*, 1993b). Furthermore, multiple splice variants of the Fas molecule are described, explaining the generation of sFas molecules (Cascino *et al.*, 1995; Cheng *et al.*, 1994). The soluble Fas molecule is able to block the induction of apoptosis, further underlining the possible functional importance of this family of soluble receptor molecules.

III. CD40-L as a Member of the TNF Superfamily

A. Cloning and Structure of CD40-L

Murine EL-4 thymoma cells have been used as a model system for T–B cell interactions, since they can induce the proliferation of resting human B lymphocytes (Zubler *et al.*, 1987). A soluble form of CD40 able to inhibit CD40-mediated responses (Fanslow *et al.*, 1992) offered the opportunity to examine the existence of a putative CD40-ligand on EL-4 cells.

A cDNA coding for CD40-L (Armitage *et al.*, 1992a) was isolated from the EL-4 line following enrichment of cells binding to a CD40-Fc fusion protein. The murine cDNA predicts a polypeptide of 260 AA consisting of a 22-AA cytoplasmic domain, a 24-AA transmembrane domain, and a 214-AA extracellular domain with four cysteines. Murine CD40-L is a type II membrane protein which lacks a N-terminal signal peptide and presents an extracellular carboxy terminus.

A human CD40-L cDNA has been isolated by screening stimulated human blood T cell libraries with the murine CD40-L probe (Cocks *et al.*, 1993; Gauchat *et al.*, 1993a; Hollenbaugh *et al.*, 1992; Spriggs *et al.*, 1992). Another group had independently isolated a TNF-related activation protein (TRAP) from activated human T cells which turned out to be the human homolog to murine CD40-L (Graf *et al.*, 1992). The cDNA for human CD40-L encodes a polypeptide of 261 AA consisting of a 22-AA cytoplasmic domain, a 24-AA transmembrane domain, and a 215-AA

extracellular domain with five cysteines (Fig. 4). The murine and human CD40-L display a conserved N-linked glycosylation site in the extracellular domain and the human CD40-L displays an additional, but probably not utilized, glycosylation site in the cytoplasmic domain. The two sequences exhibit 78% AA identity. There is 75% identity in the extracellular domain, 96% between the transmembrane regions, and 81% between the cytoplasmic domains. The gene for CD40-L is located at the X chromosome, position Xq26.3-Xq27.1 (Graf *et al.*, 1992), which led to the identification of CD40-L mutations in patients suffering from X-linked hyper-IgM syndrome (see Section VI). The human CD40-L gene spans 12–13 kb of chromosomal DNA and consists of five exons (Villa *et al.*, 1994). The first exon codes for the intracellular, transmembrane, and a small portion of the extracellular region, whereas exons II–V code for the rest of the extracellular domain (Fig. 4).

Northern blot analysis of activated T cells demonstrates the presence of two mRNA species of 2.1 and 1.4 kb (Cocks *et al.*, 1993; Spriggs *et al.*, 1992) that differ in the length of their 3′ untranslated ends. CD40-L mRNA has been detected in activated $CD4^+$ and $CD8^+$ T cells. RT-PCR analysis also indicates the presence of CD40-L in NK cells and purified monocytes (Cocks *et al.*, 1993). Northern blot analysis of the B cell line Daudi and the histiocytic lymphoma U937 have shown specific hybridization signals at 3.7 and 1.7 kb, suggesting expression of CD40-L-related molecules in B cells and monocytes (Graf *et al.*, 1992). However, the nature of these molecules has not yet been determined. The positive CD40-L mRNA signal in mast cells, basophils, and eosinophils indeed corresponds to CD40-L inasmuch as these cells bind CD40 and antibodies to CD40-L (Gauchat *et al.*, 1995; Gauchat *et al.*, 1993b). Finally, a recent study showed the presence of low levels of 1.8–2.1 kb CD40-L mRNA in activated B cells and B cell lines (Grammer *et al.*, 1995).

B. Cells Expressing CD40-L

Fusion proteins made from the extracellular domain of human CD40 and the Fc region of human IgG1 have not only been used to clone and identify CD40-L, but also to study the regulation of CD40-L expression. CD40-L is expressed on activated mature T cells but not on resting T cells (Hermann *et al.*, 1993; Lane *et al.*, 1992; Noelle *et al.*, 1992b). CD40-L can be induced on TH0, TH1, and TH2 cells (Roy *et al.*, 1993) and is primarily restricted to $CD4^+$ cells, although a small population of $CD8^+$ T cells also expresses CD40-L. Strong CD40-L staining is demonstrated on $CD8^+$ mouse (Cronin *et al.*, 1995) and human T cell clones (Hermann *et al.*, submitted for publication). CD40-L is also expressed on activated $CD4^-$ $CD8^-$ $\gamma\delta TCR^+$ T cells, but with lower expression levels than for

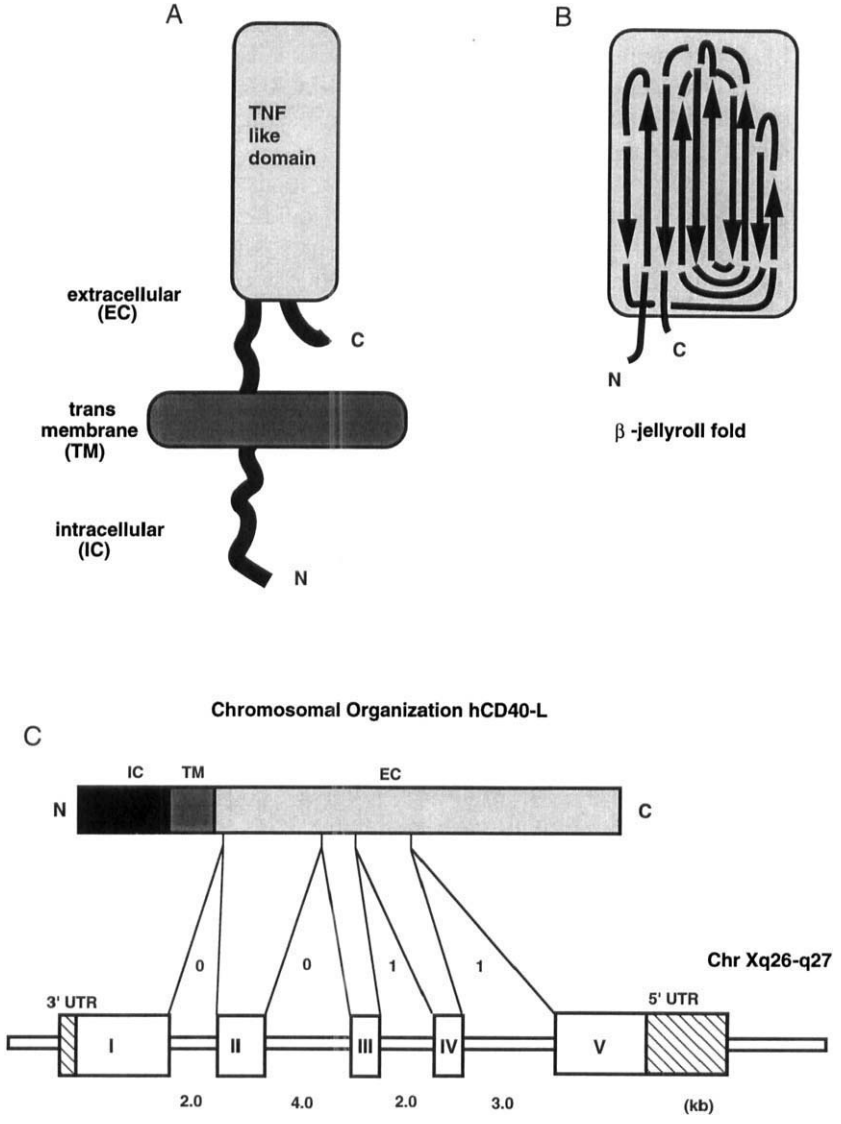


FIG. 4. Structural features of CD40-L, including protein structure (A), jellyroll fold (B), and chromosomal organization (C). Places where the coding sequence is interrupted by introns are indicated, as well as the phase of this splicing (0, intercutting between codons; 1, intercutting after base of triplet; 2, intercutting after second base of triplet). The sizes (in kilobases) of the respective introns are indicated on the bottom. See text for more details.

$\alpha\beta$TCR$^+$ T cells (Horner *et al.*, 1995). Little information is available about CD40-L expression on activated immature T cells/thymocytes. Studies performed with the monoclonal antibody 5C8, specific for T-BAM/CD40-L, confirm most of the data observed with the CD40 chimeric molecules (Lederman *et al.*, 1992a).

Immunohistochemistry demonstrates that human CD40-L is expressed on CD3$^+$ CD4$^+$ T lymphocytes of the germinal center light zone of secondary follicles in all peripheral lymphoid tissues and the interfollicular T-cell-rich areas (Lederman *et al.*, 1992a). This is confirmed in a recent study in which CD40-L positive cells were located mainly in the outer zone of germinal centers and margins of the T zones (Casamayor-Palleja *et al.*, 1995). CD40-L positivity can be identified on a rare population of T cells in the medulla and cortex of normal thymus (Lederman *et al.*, 1992a).

Immunohistochemistry on murine spleen isolated 3 to 4 days following immunization with the thymus-dependent antigen KLH demonstrates an increase of CD4$^+$ CD40-L$^+$ T cells in and around the terminal arterioles, and on the periphery of the outer periarteriolar lymphoid sheath. Double-immunohistochemical analysis reveals that the B cells producing the specific antibodies are juxtaposed to CD40-L$^+$ T cells (Van den Eertwegh *et al.*, 1993). The latter study failed to show the presence of CD40-L$^+$ T cells in germinal centers. This difference with human data may be linked to differences between species or the reagents used.

Expression of CD40-L has been detected on cells other than T lymphocytes. Lung and skin mast cells and blood basophils also express CD40-L as detected by a CD40–Fc chimeric molecule (Gauchat *et al.*, 1993b). This is also true for the immature mast cell line HMC-1 and the immature basophilic cell line KU812 when stimulated by a combination of PMA and ionomycin. The same group also showed staining of CD40-L on activated blood eosinophils or the eosinophilic cell line EOL-3 (Gauchat *et al.*, 1995). Cells could be activated by PMA + ionomycin, FMLP, or PMA + IL-5. Interestingly, a strong constitutive staining was observed on eosinophils of a patient with hyper-eosinophilia. In all of these cases it was shown that the CD40-L molecule was functional by the ability to induce B cells to secrete IgE. Finally, purified human B cells or B cell lines, stimulated with a combination of PMA and ionomycin, express CD40-L as shown by staining with a CD40–Fc chimeric molecule (Grammer *et al.*, 1995).

C. Regulation of CD40-L Expression

The expression of CD40-L on activated T cells is only transient and seems to be tightly regulated. CD40-L expression can be seen as early as 5–15 min after activation of CD4$^+$ CD45RO$^+$ T cells isolated from human tonsils (Casamayor-Palleja *et al.*, 1995). Such surface expression does not

require *de novo* protein synthesis and probably consists of preformed CD40-L. A second wave of CD40-L expression, which can be detected on all $CD4^+$ T cells, occurs early (1–2 hr) after activation. Expression is maximal after 6–8 hr, followed by a gradual loss of CD40-L expression, in a way which is influenced by the mode of T cell activation (Castle *et al.*, 1993). Apart from this intrinsic transient expression, additional mechanisms operate to ensure that CD40-L expression remains restricted in time. The functional expression of CD40-L, as detected with the CD40 chimera, is inhibited after coculture with B cells (Hermann *et al.*, 1993). This effect can be explained both by a down-regulation of CD40-L mRNA and by the release of sCD40 which binds to CD40-L (van Kooten *et al.*, 1994). In addition CD40–CD40-L interaction leads to receptor-mediated endocytosis of CD40-L, followed by its lysosomal degradation (Yellin *et al.*, 1994). Finally, the proteolytic cleavage of CD40-L, resulting in a soluble molecule (Graf *et al.*, 1995), could also prevent a protracted CD40–CD40-L interaction.

Multiple modes of polyclonal T cell activation have been shown to induce CD40-L expression *in vitro*, including PHA, PMA plus ionomycin, and anti-CD3 and anti-CD2 antibodies (Armitage *et al.*, 1992b; Hermann *et al.*, 1993; Kwekkeboom *et al.*, 1994; Lane *et al.*, 1992; Noelle *et al.*, 1992b). Signal transduction for CD40-L expression requires activation of PKC and a rise in intracellular calcium (Fuleihan *et al.*, 1994). In addition, CD28 engagement further augments anti-CD3-induced CD40-L expression (de Boer *et al.*, 1993; Klaus *et al.*, 1994b). Pretreatment of T cells with cyclosporin A prevents induction of CD40-L expression. As a result, PBMC of transplant patients receiving cyclosporin A fail to express CD40-L upon stimulation (Fuleihan *et al.*, 1994). Addition of IFN-γ or IFN-α inhibits the expression of CD40-L on IL-4-activated T cells (Gauchat *et al.*, 1993a; Roy *et al.*, 1993). This explains the inhibitory effect of these cytokines on the IgE production by activated MNC, but not by B cells activated with CD40 antibodies (Pène *et al.*, 1988b; Rousset *et al.*, 1991; Zhang *et al.*, 1991).

D. Pathophysiological Expression of CD40-L

In view of the important role of CD40-L in the induction and regulation of humoral immune responses (see Sections VI and VII), much attention has been focused on expression of CD40-L in situations where humoral immune responses are defective. The best example, patients suffering from the X-linked hyper-IgM syndrome, will be dealt with in Section VI.

In the first months of their life, newborns are not able to mount an efficient humoral immune response and show no/low isotype switching. Interestingly, cord blood-derived PBL or T cells display greatly diminished

or absent expression of CD40-L following activation with PMA ionomycin (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995; Fuleihan *et al.*, 1994; Nonoyama *et al.*, 1995). This was observed both at the level of mRNA and for surface expression. However, priming of cord blood cells with PHA + IL-2 (Brugnoni *et al.*, 1994; Durandy *et al.*, 1995) or Con A + PMA + IL-2 (Nonoyama *et al.*, 1995) induced cells to mature so that they can express normal levels of CD40-L upon restimulation. During ontogeny E^+ cells of 19- to 28-week fetal blood samples can be induced to express high levels of CD40-L following activation (Durandy *et al.*, 1995).

Patients suffering from common variable immunodeficiency (CVI) represent a heterogeneous group characterized by hypogammaglobulinemia and altered B cell differentiation. Northern blot analysis of PMA/PHA-stimulated PBL of 31 CVI patients showed a decreased mean expression of CD40-L mRNA (Farrington *et al.*, 1994). The patients with the lowest CD40-L mRNA levels also showed decreased or absent CD40-L on cell surface. Lower CD40-L mRNA levels correlated with lower IL-2 mRNA levels, while levels of other cytokines (IL-4, IL-6, IFN-γ) were not affected.

E. Relationship with TNF Superfamily Members

The initial characterization of the mouse CD40-L did not easily yield its chain similarity to the TNF superfamily (Armitage *et al.*, 1992a). More detailed analyses showed that a ~200-AA domain, which formed the major extracellular domain of CD40-L, could be aligned with available TNF-α and LT sequences in a structurally sound manner (Farrah and Smith, 1992). The region in question comprises the bioactive, receptor-binding, globular portion of TNF-like molecules that folds, by example of TNF-α (Eck *et al.*, 1992; Jones *et al.*, 1989) and LT (Banner *et al.*, 1993; Eck *et al.*, 1992), into a barrel-like structure reminiscent of viral capsid proteins. This distinctive TNF fold consists of two packed sheets of eight major antiparallel β strands linked in a "β jellyroll" topology with an N-terminal loop insertion that contains two additional, short β strands (Eck and Sprang, 1989; Eck *et al.*, 1992; Jones *et al.*, 1992). These structural characteristics of TNF-like molecules have been reviewed extensively (Bazan, 1993; Smith *et al.*, 1994).

The natural ligands for different members of the TNF-R family, forming the TNF superfamily, will be described briefly hereafter (Fig. 5 and Table II). All proteins are produced as type II proteins, with the exception of LT-α, which is produced as a soluble molecule. However, LT-α can exist in a cell-bound form by association with p33, now identified as LT-β.

TNF-α and LT-α are two pleiotropic cytokines which have been extensively reviewed (Beutler and Cerami, 1989; Paul and Ruddle, 1988). TNF-α was cloned after purification and partial amino acid sequencing (Pennica

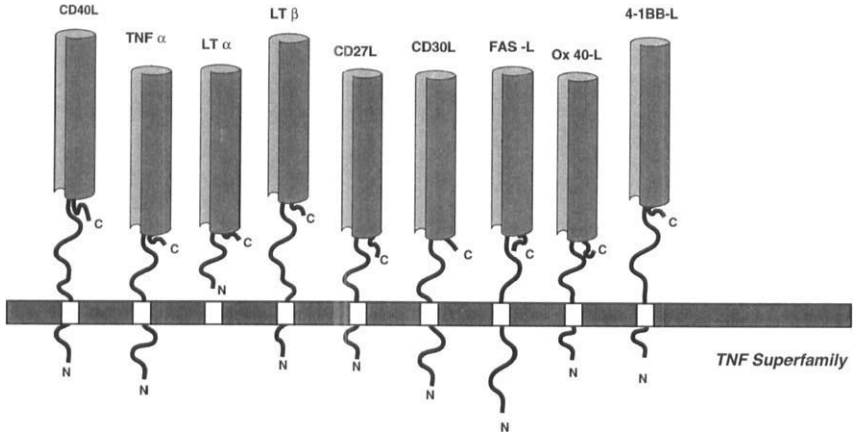


FIG. 5. Schematic representation of the tumor necrosis factor superfamily. See text and Table II for details.

et al., 1984; Wang *et al.*, 1985). The protein consists of 233 AA, including a very long 76-AA leader sequence. In fact, it was not directly appreciated that this region contains a transmembrane region which allows surface expression of TNF-α (Kriegler *et al.*, 1988). LT-α was also cloned after purification and consists of 205 AA, including a 34-AA signal peptide (Gray *et al.*, 1984). Both TNF-α and LT-α gene are located on human chromosome 6 and mouse chromosome 17, within the MHC class I locus (Muller *et al.*, 1987; Spies *et al.*, 1986).

LT-β was cloned after isolation of the p33 protein (Browning *et al.*, 1993). The protein consists of 240–244 AA (the N terminal sequence showing some variations), with 15–19 AA intracellular, 30 AA transmembrane, and 195 AA extracellular. The gene is located on human chromosome 6, very close to TNF-α and LT-α, and like these two genes is formed by four exons.

CD27-L was identified by a CD27–Fc chimeric molecule and directly cloned by expression from an EBV-transformed B cell line (Goodwin *et al.*, 1993a). The protein consists of 193 AA (20 AA intracellular, 18 AA transmembrane, and 155 AA extracellular) and is coded for by a single mRNA species of 1.2 kb. The gene is located on chromosome 19p13. The same gene was independently cloned by expression of the CD70 antigen, a recently clustered activation molecule (Bowman *et al.*, 1994).

CD30-L was cloned by cross-hybridization with a mouse CD30-L probe which was identified with a human CD30–Fc chimeric protein (Smith *et al.*, 1993a). Human CD30-L consists of 234 AA (40 AA intracellular,

TABLE II

TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY

	CD40L	TNF-α	LT-α	LT-β	CD27-L	CD30-L	Fas-L	Ox40-L	4-1BB-L
Size (AA)	261	233	171	240	193	234	281	183	254
Extra	215	177	171	195	155	172	179	133	206
TM	24	24		30	22	22	22	27	23
Intra	22	32		15	20	40	80	23	25
mRNA (kb)	2.1/1.4	1.8	1.4	0.9/1.0	1.2	3.5	2.0	3.5/1.3	5/2/1.5
Exons	5	4	4	4			4		
chr (h)	Xq26-q27	6	6	6	19p13	9q32-q34	1q23	1q25	19p13
chr (m)	X	17	17	17			1	1	17

Note. Data given are on human (h) proteins/genes, except for chromosomal organization and location, which also include mouse (m) data.

22 AA transmembrane, and 172 AA extracellular) and is coded for by a single mRNA species of 3.5 kb. The gene is localized on human chromosome 9q32 -9q34 and mouse chromosome 4.

Fas-L was first cloned from a mouse/rat T cell hybridoma using a murine Fas–Fc chimeric protein, resulting in the rat Fas-L (Suda *et al.*, 1993). Mouse Fas-L was cloned by cross-hybridization, and the gene is located on chromosome 1 (Lynch *et al.*, 1994; Takahashi *et al.*, 1994a). Point mutations in the Fas-L were identified in mice with the *gld* phenotype, suffering from generalized lymphoproliferation. The human gene was cloned from a genomic library by cross-hybridization with the rat Fas-L probe (Takahashi *et al.*, 1994b). The human protein consists of 281 AA (80 AA intracellular, 22 AA transmembrane, and 179 AA extracellular). The Fas-L gene is formed by four exons and is located on chromosome 1q23.

Ox40-L was cloned, from human and mouse, by the use of Ox40–Fc chimeric proteins (Baum *et al.*, 1994; Godfrey *et al.*, 1994). The human gene is identical to the previously cloned gp34, a gene transcriptionally activated by $p40^{tax}$ of HTLV1 (Miura *et al.*, 1991). The human protein consists of 183 AA (23 AA intracellular, 27 AA transmembrane, and 133 AA extracellular). The murine gene is on chromosome 1, linked to *gld*, whereas the human gene is located on chromosome 1q25 (Baum *et al.*, 1994).

4-1BB-L was cloned, from mouse and human, using 4-1BB–Fc chimeric molecules (Alderson *et al.*, 1994; Goodwin *et al.*, 1993b). The human protein consists of 254 AA (25 AA intracellular, 23 AA transmembrane, and 206 AA extracellular). The human gene is located at chromosome 19p13, whereas the mouse gene is located on chromosome 17. These different gene locations and the low homology between the mouse and human sequences suggest that these might be alternative ligands. This would fit with the promiscuity of the 4-1BB receptor, which has also been demonstrated to bind extracellular matrix proteins (Chalupny *et al.*, 1992).

As mentioned for the members of the TNF-R superfamily, the genes from the TNF superfamily are not entirely dispersed throughout the genome. A limited grouping of genes is possible in both human and mouse: human chromosome 6 and mouse chromosome 17 (TNF-α, LT-α, LT-β), human chromosome 1q23-25 and mouse chromosome 1 (Fas-L, Ox40-L), and human chromosome 19p13 (CD27-L, 4-1BB-L). Whether these linked genes also have similar biological activities, apart from the TNF-α and LT-α genes, is not yet clear.

F. Generation of Soluble Forms of TNF Superfamily Members

With the exception of LT-α, TNF superfamily members are produced as type II transmembrane proteins. TNF-α itself is cleaved from the mem-

brane, thereby yielding a circulating bioactive cytokine. The enzyme involved in this cleavage is an uncharacterized metalloproteinase, probably a Zn^{2+}-dependent endopeptidase, whose activity can be blocked by specific metalloproteinase inhibitors such as Gl129471 and BB-2284 (Gearing *et al.*, 1994; McGeehan *et al.*, 1994). The inhibition of membrane TNF-α proteolysis has important clinical implications, since it prevents the generation of circulating TNF-α and thereby reduces the mortality in an *in vivo* model for sepsis (Mohler *et al.*, 1994).

A soluble human CD40-L molecule was found in supernatants of transfected cells or activated T cells (Graf *et al.*, 1995). The existence of such a molecule was already suggested by the demonstration of an active CD40-binding protein in the supernatant of the EL-4 mouse T cell line (Armitage *et al.*, 1992b). The human sCD40-L, isolated from activated T cells, is an 18-kDa protein, which is probably generated by proteolytic cleavage (Graf *et al.*, 1995). This soluble protein contains the complete TNF-like domain and starts at residue Met^{113}, as shown by N-terminal amino acid sequencing. Expression of full-length CD40-L cDNA in *Escherichia coli* resulted in the generation of full-length protein (29 kDa) as well as soluble fragments of 18 and 14 kDa (Mazzei *et al.*, 1995). From these fragments, only the 18-kDa form, starting at residue Glu^{108}, was shown to be biologically active. The biological activity of the natural sCD40-L has to be further confirmed.

In a similar way, Fas-L was demonstrated to exist in a soluble form (Tanaka *et al.*, 1995). Finally, the generation of soluble Ox40-L is most likely, because the antibody used to clone the gp34 (human Ox40-L) was raised against a soluble fraction of HTLV-1 transformed T cells (Miura *et al.*, 1991). The pathophysiological role of these soluble ligands remains to be established.

IV. Signal Transduction through CD40

A. General Concepts for TNF-R Superfamily Members

The TNF-R superfamily is defined by structural homologies in the extracellular, ligand-binding domains of these receptors. In the same way, the TNF superfamily is also defined by structural homologies. The structural models predict that TNF-α, LT-α, and CD40-L are capable of trimerization (Eck and Sprang, 1989; Eck *et al.*, 1992; Jones *et al.*, 1989; Peitsch and Jongeneel, 1993). As a consequence, ligand–receptor interaction also leads to trimerization of the receptors, as demonstrated by the X-ray crystal structure of LT-α and TNF-R1 (Banner *et al.*, 1993).

Inasmuch as the TNF-R superfamily members are characterized by overlapping biological activities in lymphocyte activation, survival, and death, these may use common pathways of signal transduction. Yet there are no obvious homologies between the intracellular regions of these receptors.

There might be short stretches of sequence similarities, but the significance of these is not known yet. A possible exception is the "death domain" observed in Fas, TNF-R1, and LNGFR intracellular regions (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993) (Fig. 6). This stretch of about 80 AA is essential for transmission of the apoptosis signal by both Fas and TNF-R1, as demonstrated by mutational analysis and by naturally occurring mouse mutants, i.e., the *lpr*^{cg} mutation (Watanabe-Fukunaga *et al.*, 1992a).

Despite the lack of strong intracellular homologies, it can be assumed that different receptors use at least partially similar second messenger signals. Much research has focused on the signal-transducing pathways of TNF-Rs (Heller and Krönke, 1994; Jaattela, 1991; Vilcek and Lee, 1991), including general pathways such as Ca^{2+} flux, PKC, protein tyrosine kinases, but also the ceramide second messenger system which might be more specific for TNF-R signaling (Cleveland and Ihle, 1995; Kolesnick and Golde, 1994). In the present chapter, we concentrate on CD40 signal transduction as one prototype for TNF-R superfamily members.

B. CD40 Signal Transduction

The importance of the CD40 intracellular domain in signal transduction has been directly inferred by the deleterious nature of a Thr234 → Ala

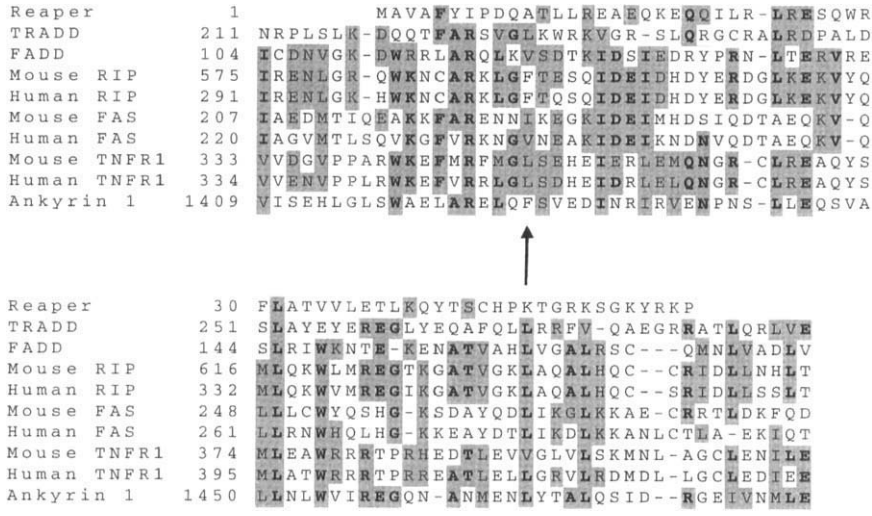


FIG. 6. Sequence homology in the death domains. Alignment for each protein begins at the indicated residue. Residues identical in three or more proteins are shown in bold, and those residues considered conserved in three or more proteins are shaded. Similar amino acids were defined as follows: A, G; S, T; E, D; R, K, H; Q, N; V, I, L, M; Y, F; W, P, C. Arrows indicates position of the *lpr* mutation in murine Fas (after Cleveland and Ihle, 1995). See text for references on analyzed proteins.

mutation (Inui *et al.*, 1990). As the CD40 cytoplasmic segment does not contain any enzymatic domain, it is likely that the CD40-mediated protein tyrosine kinase activity occurs through activation of separate kinases. This way of coupling cell surface receptors to intracellular signaling pathways seems to be a general concept which is also used by most cytokine receptors (Taniguchi, 1995). Association and activation of different signaling pathways will be dependent on the activation status and the cell type used, e.g., crosslinking of CD45 inhibits proliferation of small tonsillar B cells (naive and memory cells), but not of large tonsillar B cells (germinal center cells) activated by CD40 crosslinking (Gruber *et al.*, 1989). Therefore, most results on signal transduction cannot be extrapolated to all B cells, and might explain the sometimes controversial data.

Crosslinking of CD40 on nonresting human B lymphocytes induces tyrosine phosphorylation of four distinct substrates (Uckun *et al.*, 1991). Activation of protein tyrosine kinases (PTK) appears important for the transduction of CD40 signals because PTK inhibitors inhibit B cell aggregation (Kansas and Tedder, 1991) as well as CD40-mediated rescue of germinal center B cells (Knox and Gordon, 1993). Engagement of CD40 on the Daudi cell line induces activation of the src type kinase *lyn*, following its increased phosphorylation, but does not affect the phosphorylation or the activity of *fyn*, *fgr*, or *lck* kinases (Ren *et al.*, 1994). This is in contrast to another study in which a tyrosine phosphorylation of the PTKs *lyn* and *fyn* as well as *syk* was shown (Faris *et al.*, 1994). This latter study also suggests that *atk/btk* might be involved in CD40 signaling, since *atk/btk*-deficient EBV-transformed B cells derived from XLA patients display altered CD40 responses. Studies with XID B cells from CBA/N mice, the mouse equivalent to human XLA, demonstrated conflicting results. While one study identified a deficient CD40 signaling in XID B cells (Hasbold and Klaus, 1994), another study found a normal CD40 signal transduction but an altered CD38 transduction pathway (Santos-Argumedo *et al.*, 1995).

CD40 engagement turns on phosphorylation of the 85-kDa subunit of phosphatidyl inositol 3 kinase (PI3-K), while it does not affect its 110-kDa subunit. This results in increased activity of PI3-K, an enzyme which catalyzes the phosphorylation of phosphoinositols on the 3′ moiety and which plays a key role in mitogenesis. Finally, CD40 ligation induces within 1 min increased phosphorylation of PLC-γ2 (phospholipase C). Phosphorylation of PLC-γ2 is consistent with anti-CD40-induced IP3 production (Uckun *et al.*, 1991).

Using membranes of activated T cells, CD40-dependent B cell activation is mediated by tyrosine phosphorylation and protein kinase A (PKA)-dependent mechanisms (rise in cAMP), but is independent of protein kinase C (PKC) signaling (Kato *et al.*, 1994; Marshall *et al.*, 1994). There are

clear differences in the intracellular pathways triggered by either antigen receptor occupancy or CD40 engagement. In particular, gene inactivation in mice of the *vav* gene, a tyrosine phosphorylation substrate, results in a defective antigen receptor triggering (both B and T cells), but normal CD40-mediated signal transduction (Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995).

After early biochemical changes, these signals are translated via yet unresolved mechanisms into the activation of specific transcription factors which drive further gene activations. The last 2 years have witnessed the early attempts made at characterizing the transcription factors involved in CD40 signaling. Crosslinking of CD40 on both tonsillar B cells and B cell lines results in activation of NF-kB and NF-kB-like transcription factors (Berberich *et al.*, 1994; Francis *et al.*, 1995; Lalmanach-Girard *et al.*, 1993). Gel retardation assays showed the presence of, at least, the NF-kB family members p50, p65 (relA), and c-Rel. Activation was dependent of tyrosine kinases but independent of PKC. In addition, CD40 activation results in nuclear expression of AP1 (Francis *et al.*, 1995) and NF-AT (Choi *et al.*, 1994; Francis *et al.*, 1995; Klaus *et al.*, 1994a). In keeping with this, CD40 signaling differs from BCR triggering, as shown by CsA/FK506-sensitive NF-AT induction following anti-Ig triggering and CsA/FK506-resistant NF-AT induction following CD40 ligation (Klaus *et al.*, 1994a).

C. A New Family of Signal-Transducing-Associated Molecules

Within the above mentioned studies, the direct link between the CD40 surface receptor and signal transduction has remained unresolved. Recently, three independent groups (Cheng *et al.*, 1995a; Hu *et al.*, 1994; Mosialos *et al.*, 1995) have identified, using the yeast two-hybrid system (Fields and Song, 1989), a protein which can directly associate with the cytoplasmic tail of CD40. This protein, called CRAF1 (CD40 receptor-associated factor 1) or LAP1 (LMP1-associated protein 1), is a novel protein containing five zinc fingers, a zinc RING finger, and a C-terminal domain which is homologous with the TNF-R-associated proteins TRAF1 and TRAF2 (Rothe *et al.*, 1994) (Fig. 7). Binding studies showed that the CRAF-C domain is responsible for the interaction with the cytoplasmic tail of CD40. CRAF1 binding appears specific for CD40 as it does not bind to cytoplasmic tails of TNF-R1, TNF-RII, or Fas (Cheng *et al.*, 1995a; Hu *et al.*, 1994). In contrast, LAP1 binding appears not to be restricted to CD40, in that it also binds to TNF-RII and LT-βR, whereas TNF-RI and Fas binding are negative. These opposing data remain to be clarified. Interestingly, CRAF1 is unable to bind to a mutant CD40, having a point mutation at position Thr 234 $\rightarrow$ Ala (Hu *et al.*, 1994). This explains the defective signal transduction of this mutant (Inui *et al.*, 1990) and demon-

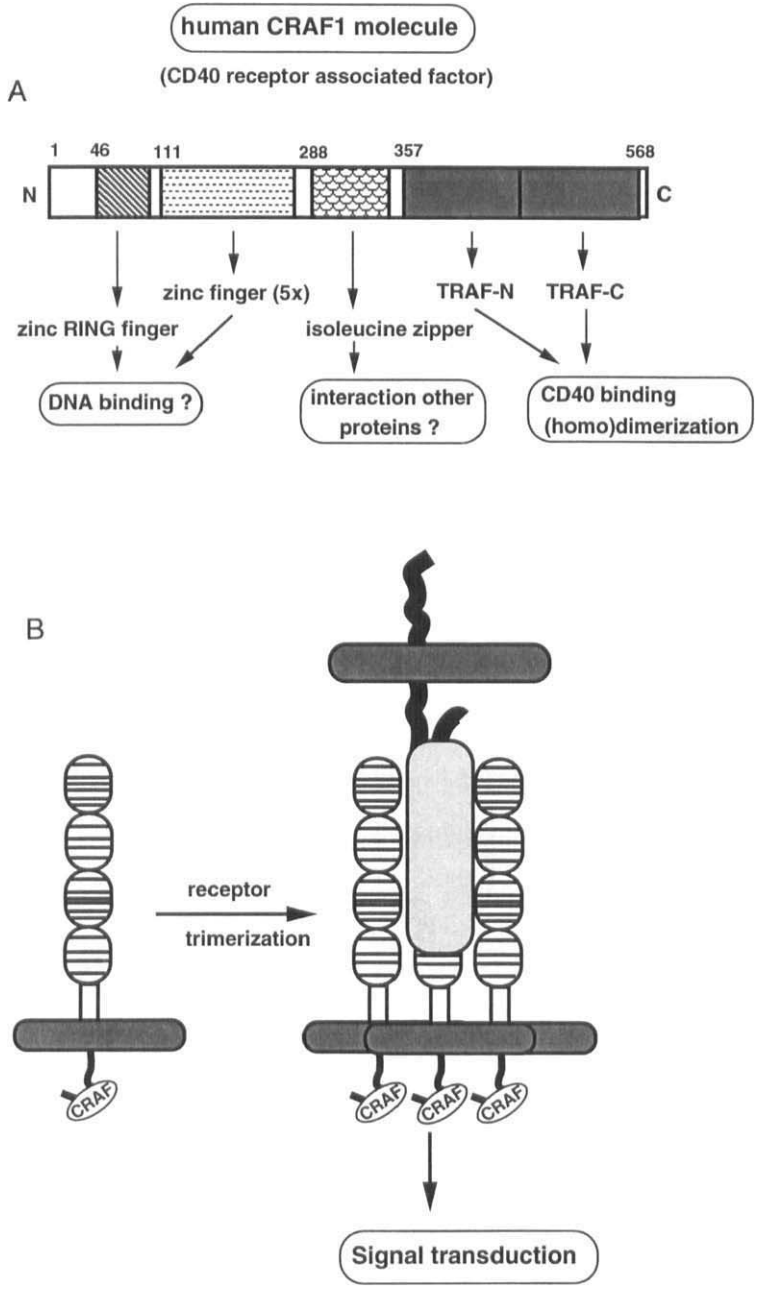


FIG. 7. Transduction of CD40 signals. (A) Schematic representation of the CRAF-1 protein. (B) Schematic representation of CD40 trimerization.

strates the importance of CRAF1 association for CD40-mediated signals. The conservation of this Thr residue in other TNFR family members (CD27, 4-1BB, Fas) might have important implications for their signal transducing pathways. The fact that CRAF1 is expressed in all human tissues tested (Mosialos *et al.*, 1995) makes it tempting to speculate that it associates with receptors other than CD40.

While two groups cloned the CRAF1 molecule using the CD40 intracellular tail, one (Mosialos *et al.*, 1995) cloned the same protein by screening for proteins interacting with the EBV-transforming gene product LMP1 (latent infection membrane protein-1). Physiologically, TRAF- or CRAF-mediated signals will be launched by the crosslinking of surface receptors, inducing homo- or heterodimerization of TRAF or CRAF. After EBV transformation, it is likely that the oligomerization of these molecules is obtained by the spontaneous aggregation capacity of the LMP1 molecule. Thus EBV can potentially utilize or interfere with CD40, TNF-R, or LT-βR signaling pathways.

Again using the two hybrid system, Fas was demonstrated to associate with several proteins. In particular it associates with the FAP-1 protein, which is a protein tyrosine phosphatase of 2500 AA that can bind to the inner leaflet of the plasma membrane without traversing it (Sato *et al.*, 1995). Overexpression of this protein renders cells resistant to Fas-induced apoptosis, and cells that spontaneously express high levels of FAP-1 consistently are relatively resistant to Fas-mediated apoptosis. FAP-1 binds to the 15 carboxy-terminal AA of Fas, a region which has been demonstrated to be a negative regulator of Fas activity, because apoptosis increases after deletion of this fragment (Itoh and Nagata, 1993).

The two-hybrid system furthermore allowed the identification of the Fas binding protein MORT-1 (Boldin *et al.*, 1995). This protein appears to contribute to the apoptotic mechanism, since overexpression of MORT-1 results in increased cell death without the need for receptor ligation. The MORT-1 protein shows no obvious homology with the CRAF or TRAP proteins, but surprisingly contains a region homologous with the death domain. MORT-1 binds to human and mouse Fas, but not to a Fas mutant which has lost its function (lpr^{cg}), or to TNF-R1, TNF-R2, or CD40. In fact, the MORT-1 protein, also identified as FADD (Chinnaiyan *et al.*, 1995), seems to be part of a larger family of Fas-associated proteins including RIP (Stanger *et al.*, 1995) and TRADD (Hsu *et al.*, 1995). All these proteins are characterized by a death domain sequence, forming a death domain family involved in cell death, including TNF-R1, Fas, MORT-1/FADD, RIP, TRADD, reaper (a Drosophila protein), and ankyrin-1 (Cleveland and Ihle, 1995) (Fig. 6).

Thus, for Fas signal transduction the final outcome of receptor crosslinking depends on the balance of positive and negative regulatory proteins associated with the intracellular region. Regarding CD40 signal transduction, we will likely end up with the identification of multiple CD40-binding proteins.

In conclusion numerous proteins associate with the intracellular regions of TNF-R superfamily members. Deciphering the contribution of these proteins in the functions of these receptors will represent a major challenge.

V. Functional Consequences of *in Vitro* CD40 Engagement

Human mature B cells have been the main cell type for which the functional consequences of CD40 ligation have been analyzed. CD40 ligation affects B cells directly (Section V,A) and also alters their response to other signals (Section V,B). The effects of CD40 activation on cell types other than mature B cells have recently been the object of much attention and are discussed in Section V,C.

A. Activation of Mature B Lymphocytes

In vitro activation of human B lymphocytes through CD40 crosslinking has been very extensively studied. These functional effects have been reviewed earlier, and readers are invited to read the following papers for more detailed information (Armitage and Alderson, 1995; Banchereau *et al.*, 1994; Banchereau and Rousset, 1992; Clark and Ledbetter, 1994; Durie *et al.*, 1994b; Hollenbaugh *et al.*, 1994). Although initially thought to be a T-cell-independent way of B cell activation, it is now clear that *in vitro* CD40-mediated B cell activation is mimicking the interaction between B cells and activated T cells. Since these interactions between T and B cells take place within secondary lymphoid organs at defined locations and with a precise timing during T-cell-dependent immune responses, much insight has arisen from the definition of B cell subpopulations and their distinct compartmentalization (Lagresle *et al.*, 1993; Liu *et al.*, 1995; Pascual *et al.*, 1994). The *in vitro* data on B cell activation summarized hereafter should therefore be seen in the context of the *in vivo* development of T-cell-dependent humoral immune responses (see Sections VI and VII). For the sake of clarity, the *in vitro* effects of CD40 ligation will be reviewed by separate topics including activation, proliferation, and differentiation.

1. Activation of B Lymphocytes

Resting B cells cultured in the presence of anti-CD40 antibody increase their size (Vallé *et al.*, 1989) and form homotypic aggregates (Barrett *et al.*, 1991; Björck *et al.*, 1991; Gordon *et al.*, 1988; Kansas and Tedder,

1991; Kansas *et al.*, 1991). Activation of B lymphocytes through CD40 also stimulates their adhesiveness to endothelial cells in a VLA-4-dependent fashion (Flores-Romo *et al.*, 1993). Triggering B cell CD40 results in an increased expression of CD23 (Lederman *et al.*, 1992b), B7-1/CD80 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994), B7-2/CD86 (Liu *et al.*, 1995; Roy *et al.*, 1995), and Fas (Garrone *et al.*, 1995; Schattner *et al.*, 1995). The significance of these phenotypic changes will be discussed below (Section V,B).

Crosslinking of CD40 induces B cells to produce numerous cytokines, including IL-1β, IL-6, IL-10, TNF-α, LT-α, and GM-CSF (Boussiotis *et al.*, 1994; Burdin *et al.*, 1993, 1995; Clark and Shu, 1990; Worm and Geha, 1994). The produced cytokines act as autocrine growth and differentiation factors inasmuch as neutralizing antibodies against TNF-α (Boussiotis *et al.*, 1994) or against IL-6 and IL-10 (Burdin *et al.*, 1995) are shown to inhibit the proliferation and Ig production of CD40-activated B cells, respectively.

Soluble anti-CD40 (Liu *et al.*, 1989) and CD40-L-transfected cells (Holder *et al.*, 1993) are able to prevent/slow down the apoptotic death of germinal center B cells. In contrast to follicular dendritic cells (FDC), CD40 activation is not able to down-regulate the endonuclease activity in germinal center B cells (Lindhout *et al.*, 1995), suggesting alternative mechanisms for FDC-induced rescue. In line with these results, the rapid apoptosis of tonsillar plasma cells, which do express CD40, can be rescued by interaction with bone marrow fibroblasts or synoviocytes, but not by CD40 crosslinking (Merville *et al.*, 1995a). Yet CD40 activation is able to rescue the apoptosis of B cell lines induced by B cell receptor engagement, both through bcl-2-dependent and -independent mechanisms (Gregory *et al.*, 1991; Lederman *et al.*, 1994; Parry *et al.*, 1994; Sumimoto *et al.*, 1994; Tsubata *et al.*, 1993; Valentine and Licciardi, 1992). For the WEHI-231 immature B cell lymphoma, the CD40-mediated rescue is mediated via up-regulation of Bcl-x_L, another member of the bcl-2 family (Choi *et al.*, 1995).

2. *Proliferation of B Lymphocytes*

Anti-CD40 antibodies have been isolated according to their ability to costimulate with either anti-IgM antibodies or phorbol esters for the proliferation of purified B cells (Clark and Ledbetter, 1986; Gordon *et al.*, 1987; Ledbetter *et al.*, 1987b; Vallé *et al.*, 1989). Culture of resting B cells in the CD40 system (anti-CD40 antibody presented by fibroblastic mouse L cells transfected with FcγRII/CD32) or on CD40-L-transfected cells results in strong and long-lasting proliferation (Banchereau *et al.*, 1991; Cocks *et al.*, 1993; Gauchat *et al.*, 1993a; Grabstein *et al.*, 1993; Hollenbaugh *et al.*, 1992; Spriggs *et al.*, 1992). These culture conditions allow the prolifera-

tion of various B cell subpopulations including naive $sIgD^+$ $sIgM^+$ B cells, germinal center and memory B cells, and $CD5^+$ and $CD5^-$ B cells (Arpin *et al.*, 1995; Defrance *et al.*, 1992b). Certain soluble anti-CD40 antibodies, soluble trimeric forms of CD40-L, and soluble CD8–CD40-L chimeric proteins can also induce DNA replication of B cells.

Addition of IL-4 to CD40-activated B cell cultures results in their sustained proliferation (Armitage *et al.*, 1993a; Defrance *et al.*, 1992b; Rousset *et al.*, 1991; Spriggs *et al.*, 1992) and allows the generation of factor-dependent long-term normal B cell lines (Banchereau *et al.*, 1991). B cell clones of several hundred cells can be obtained under these conditions. IL-13, a cytokine which shares homology with IL-4 (McKenzie *et al.*, 1993; Minty *et al.*, 1993) can also induce a strong and long-lasting proliferation of B cells stimulated in the CD40 system or with CD40-L-transfected cells (Cocks *et al.*, 1993). Cells cultured in the CD40 system with IL-4 express CD19, CD20, CD40, sIg, high levels of CD23, and HLA class II antigens, and a significant proportion of cells still expresses sIgD (Galibert *et al.*, 1994).

Both viral and human IL-10 enhance the proliferation of B cells cultured in the CD40 system or with CD40-L-transfected cells, as determined both by tritiated thymidine incorporation and by increased viable cell numbers (Armitage *et al.*, 1993a; Rousset *et al.*, 1992, 1995). IL-10 appears to be almost as efficient as IL-4 during the first week of culture but proliferation slows down thereafter. The combination of IL-4 and IL-10 results in additive proliferation. IL-2 poorly enhances the proliferation of CD40-activated B cells. IL-10 up-regulates the expression of CD25/Tac on anti-CD40-activated B cells and accordingly addition of IL-2 strongly enhances B cell proliferation (Fluckiger *et al.*, 1993). B cells cultured in IL-10 differ microscopically from those cultured in IL-4 in that loose aggregates are observed early on, which then yield cultures mostly composed of single large cells.

3. *Differentiation of B Lymphocytes*

CD40 activation of human and murine B cells results in the production of marginal amounts of immunoglobulins (Armitage *et al.*, 1993a; Grabstein *et al.*, 1993; Maliszewski *et al.*, 1993; Rousset *et al.*, 1991). However, CD40-activated B cells cultured with SAC particles produce large amounts of IgM, IgG, and IgA without IgE (Defrance *et al.*, 1992a). Naive $sIgD^+$ $sIgM^+$ B cells secrete only IgM, whereas $sIgD^-$ $sIgM^{+/-}$ memory B cells secrete IgG and IgA and lower amounts of IgM.

Addition of IL-4 or IL-13 to CD40-activated B cells results in a slight increase in the production of IgM and IgG and in the secretion of IgE following isotype switching (Armitage *et al.*, 1993a; Jabara *et al.*, 1990;

Punnonen *et al.*, 1993b; Rousset *et al.*, 1991). Stimulation of blood B cells with soluble anti-CD40 antibody induces germline transcription of all heavy chain isotypes except $\gamma 4$ and ε (Jumper *et al.*, 1994). The inhibitory effects of interferons on IL-4-induced IgE production by mononuclear cells (Pène *et al.*, 1988a) may be due to down-regulation of CD40-L on IL-4-activated T cells inasmuch as IFNs do not inhibit the IL-4-induced IgE production of CD40-activated B cells (Gauchat *et al.*, 1993a; Rousset *et al.*, 1991; Roy *et al.*, 1993).

Addition of IL-10 to CD40-activated B lymphocytes results in the production of considerable amounts of IgM, IgG, and IgA without any IgE as a result of a differentiation into plasma blasts (Armitage *et al.*, 1993a; Rousset *et al.*, 1992, 1995). IL-10 induces CD40-activated total tonsil B cells to secrete IgG1, IgG2, and IgG3. CD40-activated naive $sIgM^+$ $sIgD^+$ B cells produce mainly IgM, but also substantial amounts of IgG1 and IgG3, indicating that IL-10 can also act as a switching factor (Brière *et al.*, 1994). CD40-activated naive $sIgD^+$, $sIgM^+$ B cells cultured with IL-10 also produce low levels of IgA and addition of TGF-β induces large amounts of IgA, while inhibiting IgM and IgG production (Defrance *et al.*, 1992a). TGF-β is likely to represent an IgA-switch factor both in human and in mouse (Coffman *et al.*, 1989; Lebman *et al.*, 1990), as strengthened by the finding that TGF-β is able to produce α_1 and α_2 germline transcripts in activated human B cells (Islam *et al.*, 1991). Finally, the combination of CD40 activation and a T cell supernatant induces the production of IgG2 by naive IgD^+ B cells, suggesting the presence of an IgG2 switching factor (Servet-Delprat *et al.*, 1995). Thus, the engagement of CD40 on B cells seems to turn on the isotype switching machinery, the specificity of which is subsequently provided by cytokines.

For the terminal differentiation of B lymphocytes, additional CD40-independent cellular interactions are necessary. *In vitro* studies have shown that those signals can be given by bone marrow stromal cells or synoviocytes (Dechanet *et al.*, 1995; Merville *et al.*, 1995b; Roldan and Brieva, 1991). Two-step cultures of CD40-activated germinal center B cells have shown that withdrawal of the CD40-L stimulation leads to differentiation of activated B cells into plasma cells, whereas prolonged CD40-L stimulation results in the generation of memory B cells (Arpin *et al.*, 1995).

The pleiotropic effects of CD40 engagement on B cell physiology are summarized in Fig. 8.

B. CD40 Activation Interferes with Other Signaling Pathways

The interaction of T and B cells during the development of an immune response is a concerted action of several molecular interactions (Parker, 1993, for review), with CD40–CD40-L being one of the most promi-

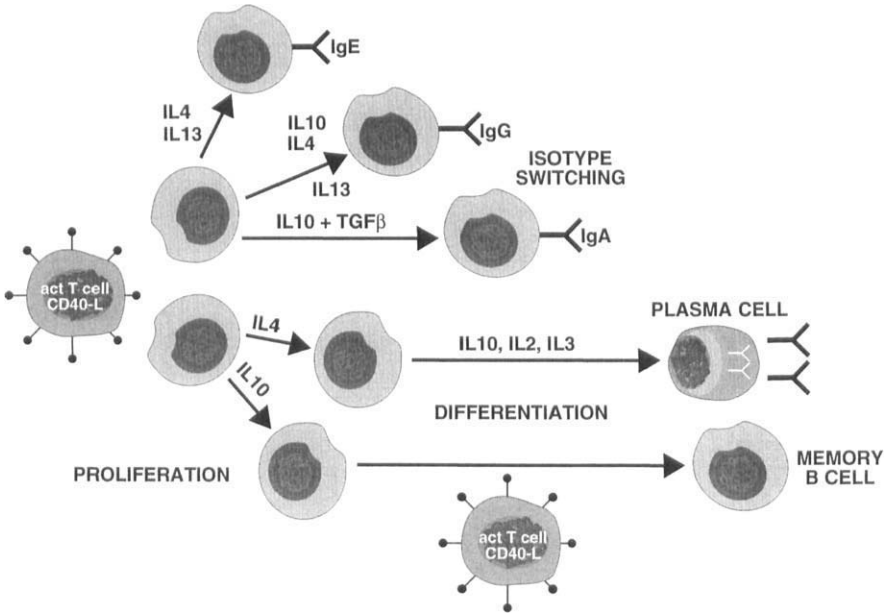


FIG. 8. Functional consequences of triggering B lymphocyte CD40.

nent. It is therefore of major interest to evaluate the influence of CD40–CD40-L interactions on these other signaling pathways.

1. CD28–CD80/CD86 Pathway

The interaction of CD28 and/or CTLA4 (constitutive and inducible T cell antigens, respectively) with B7-1/CD80 and/or B7-2/CD86 (costimulatory molecules being present on antigen-presenting cells) plays an essential role in T cell activation and determines whether T cells enter into either an effector state or an anergic state (Guinan *et al.*, 1994; Schwartz, 1992). Activation of CD40 on both normal or B-CLL B cells induces an enhanced expression of CD80 (Ranheim and Kipps, 1993; Yellin *et al.*, 1994), rendering them efficient APCs. In fact, in human and mouse, both CD80 and CD86 are up-regulated after CD40 activation, with a stronger expression on memory B cells when compared to naive B cells (Liu *et al.*, 1995; Roy *et al.*, 1995). This effect is not restricted to B cells, since crosslinking CD40 on dendritic cells, professional antigen-presenting cells (Steinman, 1991), also results in strong up-regulation of CD80 and CD86 (Caux *et al.*, 1994; Pinchuk *et al.*, 1994). On the other hand, crosslinking of T cell CD28 by CD80/CD86 enhances the expression of CD40-L (de Boer *et al.*, 1993;

Klaus *et al.*, 1994b), thus indicating the presence of a positive activation loop involving the B cell/APC CD40 and the T cell CD28.

2. *Fas–Fas-L Pathway*

The interaction of Fas with Fas-L plays a pivotal role in the elimination of both T and B lymphocytes via apoptosis (Nagata and Golstein, 1995). While Fas is expressed on all germinal center B cells (Möller *et al.*, 1993), it is absent on naive B cells and only weakly expressed on memory B cells. Importantly, activation of resting (naive and memory) B cells via the CD40 molecule induces a rapid expression of Fas (Garrone *et al.*, 1995; Schattner *et al.*, 1995). These cells become sensitive to Fas-induced apoptosis with a delayed kinetics, suggesting a functional germinal center phenotype (Lagresle *et al.*, 1995). Since Fas-L is induced during T cell activation (Ramsdell *et al.*, 1994b; Suda *et al.*, 1995), probably next to CD40-L, the delayed induction of apoptosis by Fas ligation might represent a way to limit the size of a specific B cell clone that is generated during T–B interactions. In a mouse model, it is shown that both CD40-L and anti-IgM induce Fas expression, but only CD40-activated B cells are sensitive to Fas-dependent TH1-cell-mediated cytotoxicity (Rothstein *et al.*, 1995). The combination of CD40 and anti-IgM activation inhibits this susceptibility to cytotoxicity, providing an additional mechanism that eliminates CD40-activated bystander B cells, but ensures survival of antigen-specific B cells.

3. *B Cell Receptor Pathway*

During the activation and selection of B cells, engagement of the B cell receptor complex by antigen plays a critical role. Simultaneous crosslinking of BCR (with anti-Ig antibodies) and CD40 was shown to synergize for strong DNA synthesis thus allowing the identification of anti-CD40 antibodies (Clark and Ledbetter, 1986; Gordon *et al.*, 1987; Ledbetter *et al.*, 1987b; Vallé *et al.*, 1989). This double activation also induces B cells to express a phenotype comparable to that of germinal center B cells; the B cell subpopulation that undergoes selection processes (Galibert *et al.*, 1996). Cotriggering of CD40 and BCR results in the induction of CD38, Fas, and carboxypeptidase M, a recently described germinal center marker. Consistent with a germinal center phenotype, CD40 plus BCR-stimulated cells exhibited reduced viability when cultured in the absence of stimuli. In addition, prolonged cotriggering of CD40 and BCR (via crosslinked anti-Igβ/CD79b or a combination of anti-κ and anti-λ) results in a rapid loss of viable cells after 5 days of culture (van Kooten *et al.*, 1995, submitted). In fact, it could have been expected that the dual Antigen Receptor-CD40 triggering would elicit B cells with a germinal center phenotype inasmuch

as the generation of germinal centers *in vivo* is contingent both on T cells and antigen. The data suggest that germinal center B cells pass through a stage where protracted BCR crosslinking results in their elimination. This process may represent a safeguard to remove autoreactive B cells which have been generated by somatic hypermutation. Such a mechanism has been shown to operate *in vivo* in mouse models where administration of high doses of antigen results in prompt and massive apoptosis of antigen-specific germinal center B cells (Pulendran *et al.*, 1995; Shokat and Goodnow, 1995). However, this process may be prevented/delayed when appropriate T cell signals are present inasmuch as anergic self-reactive B cells, in the transgenic HEL-anti-HEL mouse model, can be activated *in vitro* by a combination of CD40-L and IL-4 (Cooke *et al.*, 1994; Eris *et al.*, 1994).

C. CD40 Activation of Cell Types Other Than Mature B Lymphocytes

1. B Cell Precursors

Soluble anti-CD40 antibodies neither stimulate the proliferation of normal B cell precursors (BCP) nor alter the effect of known growth signals for such cells (Law *et al.*, 1990; Saeland *et al.*, 1993). However, $CD19^+$ $CD10^+$ BCP can grow in the CD40 system provided IL-3 is added (Saeland *et al.*, 1993). This proliferation is further potentiated by IL-7 and IL-10. At variance with mature B cells, IL-4 does not induce proliferation of BCP cultured in the CD40 system. BCP can also be induced to proliferate by triggering their CD40 with CD40-L present on activated $CD4^+$ T cell clones (Renard *et al.*, 1994). Activation of BCP through CD40, either in the CD40 system or in the presence of T cells, does not consistently promote differentiation into mature slg^+ B cells. A small proportion of immature $slgM^+$ cells emerge in CD40-dependent cultures, but cells bearing other isotypes are not observed (Saeland *et al.*, 1993). In contrast, pre-B cells have been demonstrated to undergo isotype switch and Ig production in the presence of IL-4 and membranes of activated T cells (Punnonen *et al.*, 1993a). Triggering of CD40 can induce high-level surface membrane expression of CD23 on BCP (Saeland *et al.*, 1993).

2. Malignant B Cells

In most cases, activation of malignant B cells has been shown to parallel the activation of normal mature B cells, resulting in B cell proliferation and/or differentiation, dependent on the added cytokine (Crawford and Catovsky, 1993; Fluckiger *et al.*, 1992; Johnson *et al.*, 1993; Kluin-Nelemans *et al.*, 1994). An important practical application of these results may be the use of this activation procedure in cytogenetic analysis of B cell malignancies. In fact, CD40 activation of hairy cell leukemia B cells has allowed

cytogenetic analysis in 42/43 tested cases (Kluin-Nelemans *et al.*, 1994). Regarding multiple myeloma, CD40 crosslinking was found to enhance IL-6 production as well as proliferation, possibly suggesting a role for CD40–CD40-L interactions in the growth of these cells (Tong *et al.*, 1994; Urashima *et al.*, 1995; Westendorf *et al.*, 1994).

In contrast to these studies, CD40 crosslinking of two B cell lines derived from patients with diffuse large-cell lymphomas and of two EBV-induced B cell lymphoma cell lines was found to inhibit cell proliferation (Funakoshi *et al.*, 1994). A similar inhibitory effect of CD40 engagement could be demonstrated *in vivo* in SCID mice bearing these tumor cell lines. The alteration in signal transduction that results in such a negative signal remains to be identified. It is tempting to speculate that CD40 may occasionally associate with proteins that normally transduce the Fas apoptotic signal (see Section IV,C), as suggested by the low homology of the CD40 intracellular region with the death domain (Itoh *et al.*, 1991; Oehm *et al.*, 1992).

3. *Reed Sternberg Cells*

Reed Sternberg cells, characteristic of Hodgkin's lymphomas, can bind to CD40-L-expressing cells. Furthermore, RS cells display enhanced clonogenic capacity and colony cell survival after engagement of CD40 by soluble CD40-L (Carbone *et al.*, 1995). An earlier study showed that crosslinking RS cells of CD40 on results in an enhanced production of IL-6, IL-8, TNF-α, and LT-α, as well as an up-regulation of the costimulatory molecules ICAM-1, and B7-1, though no mitogenic activity could be seen (Gruss *et al.*, 1994).

4. *Hematopoietic Progenitors*

CD40 activation of $CD34^+$ progenitors, isolated either from bone marrow or from cord blood, results in enhanced proliferation as shown by increased [^{3}H]thymidine incorporation and viable cell numbers (Flores-Romo, submitted for publication). More strikingly, this activation results in a differentiation of the progenitors into dendritic cells, as shown by morphology, phenotype, and functional antigen-presenting capacities. Whether these CD40-L-generated dendritic cells differ from those generated in TNF-α plus GM-CSF (Caux *et al.*, 1992) or those generated in IL-4 plus GM-CSF (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994) is presently under investigation.

5. *T Cells*

Human resting T lymphocytes activated by a soluble trimeric CD40-L or by CD40-L-transfected cells express activation molecules such as CD25/

Tac, CD69, and CD40-L (Armitage *et al.*, 1993b). Crosslinking of CD40 on activated T cells results in enhanced proliferation and secretion of IFN-γ, TNF-α, and IL-2. Both $\alpha\beta$ and $\gamma\delta$ mouse T cells are stimulated in a similar way, resulting in enhanced proliferation, induction of activation markers (CD25, CD69), and secretion of cytokines (IL-2, IFN-γ, and TNF-α) (Fanslow *et al.*, 1994; Ramsdell *et al.*, 1994a).

6. *Monocytes*

Monocytes stimulated by CD40-L-transfected cells secrete low amounts of IL-6 and IL-8 (Alderson *et al.*, 1993). Addition of GM-CSF, IL-3, or IFN-γ, which up-regulate their CD40 expression, further boosts CD40-L-induced secretion of IL-6 and IL-8 and allows secretion of TNF-α. The functional CD40 expression on monocytes is now widely documented and crosslinking results in enhanced production of IL-1 (Wagner *et al.*, 1994), TNF-α, IL-1, IL-6, IL-8, IL-10, MIP-1α (Caux *et al.*, 1994), and IL-12 (Shu *et al.*, 1995). Crosslinking of monocyte CD40 further turns on their tumoricidal activity against a melanoma cell line (Alderson *et al.*, 1993). Finally, the inhibition by anti-CD40 antibodies of T-cell-induced nitric oxide production by mouse macrophages (Tian *et al.*, 1995) suggests that CD40 ligation activates NO synthesis.

7. *Dendritic Cells*

CD40 is strongly expressed on dendritic cells (DC) isolated from different tissues (peripheral blood, skin, tonsil, spleen, and thymus) as well as on dendritic cells generated *in vitro* by culturing $CD34^+$ progenitors in the presence of GM-CSF and TNF-α (or IL-4). When considering the latter cell population, CD40 crosslinking has been shown to result in major functional alterations, including (i) an enhanced survival which is further stimulated by addition of GM-CSF; (ii) morphological changes characterized by a very dense and uniform system of dendrites at the membrane surface; (iii) important phenotypical changes, with a down-regulation of CD1a and a strong up-regulation of CD58 (LFA-3), CD80 (B7-1), CD86 (B7-2), HLA-DR, $-$DP, $-$DQ, CD40, and CD25; (iv) an enhanced production of IL-8, TNF-α and MIP-1α (Caux *et al.*, 1994). Interestingly, freshly isolated human epidermal Langerhans cells also respond to CD40 engagement by enhanced viability and up-regulation of CD54 and CD86 (Peguet-Navarro *et al.*, submitted for publication). Peripheral blood-derived DC also respond to CD40 activation with an up-regulation of CD80 expression (Pinchuk *et al.*, 1994). Furthermore, during an allo-response between peripheral blood DC and allogeneic T cells, DC display increased CD40 expression (Zhou and Tedder, 1995). The triggering of CD40 on dendritic cells contributes to the subsequent T cell proliferation,

inasmuch as the latter event is significantly inhibited by addition of an antibody against CD40 (Zhou and Tedder, 1995).

8. *Follicular Dendritic Cells*

Interaction between activated T cells and follicular dendritic cell (FDC)-like cell lines results in enhanced growth of FDC-like cells, as well as increased expression of ICAM-1 (Kim *et al.*, 1994). The blocking of these effects with a CD40–Fc chimeric molecule suggests a functional expression of CD40 on these FDC-like cells.

9. *Epithelial Cells*

Thymic epithelial cells are induced to secrete GM-CSF following triggering with soluble anti-CD40 in conjunction with IL-1 or IFN-γ (Galy and Spits, 1992). This effect is observed early (24 hr) after triggering and occurs in the absence of cell proliferation. Also, crosslinking of CD40 on proximal tubular epithelial cells (PTEC) results in induction of cytokine production, notably IL-6, IL-8, and MCP-1 (Van Kooten *et al.*, manuscript in preparation).

10. *Endothelial Cells*

Crosslinking of CD40 on cultured human umbilical vein endothelial cells (HUVEC) results in a marked up-regulation of ICAM-1 (CD54), VCAM-1 (CD106), and E-selectin (CD62E), thus resulting in increased ability to bind leukocytes (Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995).

11. *Synoviocytes/Fibroblasts*

Crosslinking of CD40 expressed on skin fibroblasts and synoviocytes results in an enhanced proliferation of these cells as shown by [^{3}H]thymidine incorporation, viable cell numbers, and cell cycle analysis (Rissoan *et al.*, submitted for publication). The proliferation is further enhanced by addition of IFN-γ to cultures, likely because IFN-γ increases fibroblast CD40 expression. CD40 ligation also results in increased production of GM-CSF and MIP-1α by synoviocytes.

Thus, as summarized in Fig. 9, CD40 appears to be functional on multiple cell types.

VI. *In Vivo* Role of CD40–CD40-L Interactions

A. Mutated CD40-L in the X-Linked Hyper-IgM Syndrome

The X-linked hyper-IgM syndrome (HIGM), one of the immunodeficiencies mapped to loci distributed throughout the X chromosome, has been shown to be caused by mutations in the CD40-L (Callard *et al.*,

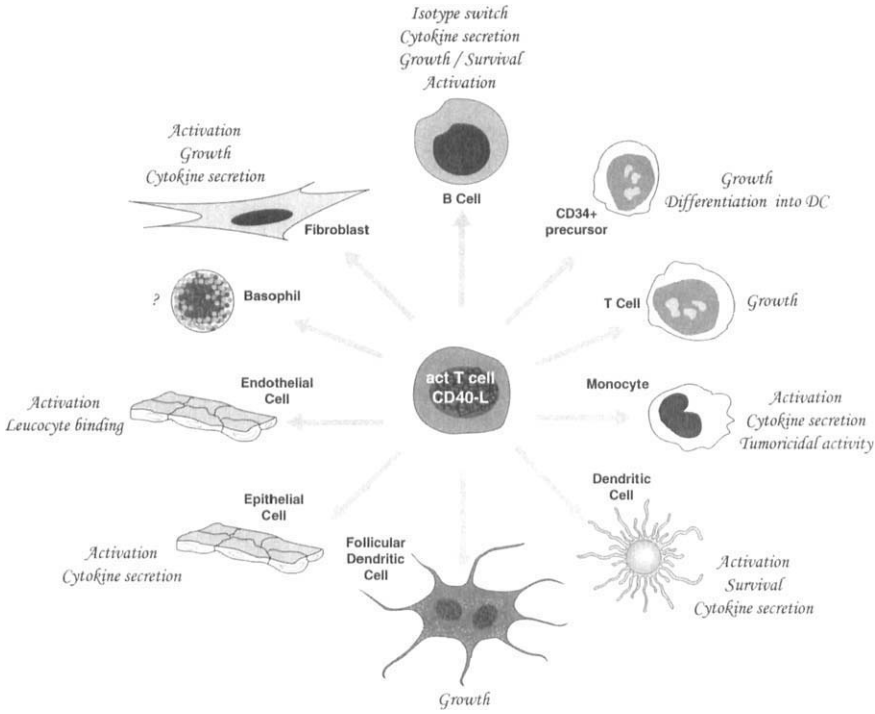


FIG. 9. Functional consequences of CD40 triggering on different cell types. Activation includes increase in cell size and alteration of phenotype.

1993; Kroczek *et al.*, 1994). Other immunodeficiencies include X-linked agammaglobulinemia, X-linked severe combined immunodeficiency, Wiskott–Aldrich syndrome, and X-linked lymphoproliferative syndrome (Conley, 1992; see Smith *et al.*, 1993b, for review). Males affected with hyper-IgM syndrome are susceptible to bacterial infections often encountered in B cell deficiencies, but also suffer from neutropenia as well as *Pneumocystis carinii* pneumonia and *Cryptosporium* intestinal infections, diseases often observed in T cell deficiencies (Notarangelo *et al.*, 1992). These patients do not make antibodies to exogenous antigens but make a variety of autoantibodies. Their serum has slightly or vastly elevated concentrations of polyclonal IgM and IgD, but no detectable IgA or IgE and very low levels of IgG. The secondary lymphoid organs of these patients display no germinal centers (Facchetti *et al.*, 1995) although they have normal levels of circulating B cells and plasma cells producing IgM and IgD in lymphoid tissues and the gastrointestinal tract. Immunization of these patients with bacteriophage Ø 174, a thymus-dependent antigen,

results in a poor humoral immune response that is restricted to the IgM isotype (Ochs *et al.*, 1971). Most of these features are consistent with a defect in the generation of memory B cells.

The gene mutated in the hyper-IgM syndrome was mapped to chromosomal region Xq24-27 (Padayachee *et al.*, 1992). The localization of the CD40-L gene in region Xq26-3-Xq27-1 (Allen *et al.*, 1993; Aruffo *et al.*, 1993; Graf *et al.*, 1992) led to the demonstration that the defect in the hyper-IgM syndrome is due to point mutations or deletions in the gene encoding the CD40-L (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993 Fuleihan *et al.*, 1993; Hill and Chapel, 1993; Korthäuer *et al.*, 1993). Today, mRNA transcripts of at least 20 patients have been sequenced. Determination of the CD40-L gene organization demonstrated that a substantial amount of mutations can be attributed to splice site mutations (Villa *et al.*, 1994). The CD40-L genomic sequence furthermore allowed identification of a highly polymorphic dinucleotide (CA) repeat in the 3′ untranslated region, which can be used for prenatal diagnostics (DiSanto *et al.*, 1994). X-inactivation studies in healthy carriers showed that the X chromosome with the mutated CD40-L sequence was not selectively inactivated, suggesting that a small population of CD40-L$^+$ cells is enough to sustain B cell memory.

Activated T cells from these patients do not bind CD40 fusion proteins, although cells from some of these patients stain with polyclonal antibody specific for the CD40-L. This suggests either expression of the truncated CD40-L on the cell surface or expression of a conformationally altered protein unable to bind CD40. Several expressed mutated CD40-L molecules were found incapable of activating B lymphocytes from normal individuals (Allen *et al.*, 1993; Aruffo *et al.*, 1993), whereas the B lymphocytes from hyper-IgM patients can be stimulated to proliferate and secrete Igs either with activated T cells (Mayer *et al.*, 1986) or with anti-CD40 antibodies and cytokines (Allen *et al.*, 1993; Aruffo *et al.*, 1993; Durandy *et al.*, 1993; Fuleihan *et al.*, 1993; Korthäuer *et al.*, 1993; Ramesh *et al.*, 1993a). In one report, B cells from HIGM patients were not able to produce IgG or IgA antibodies after *in vitro* activation with anti-CD40 and IL-10 (Saiki *et al.*, 1995). T cells from HIGM patients can induce naive B cells to produce low levels of IgM, perhaps reflecting the intact primary immune response of these patients (Klaus *et al.*, 1994b; Nishioka and Lipsky, 1994; Renard *et al.*, 1994). Interestingly, such CD40-L-negative T cell clones were found able to induce memory B cells to proliferate and to secrete IgG and IgA, further confirming the critical role of CD40 in isotype switching rather than B cell differentiation (F. Brière, personal communication; Lane *et al.*, 1995). Surprisingly, 4/12 T cell clones generated from a HIGM patient were able to induce naive B cells to secrete IgE, suggesting

the possible involvement in IgE switch of T cell molecules other than CD40-L (Life *et al.*, 1994).

Somatic hypermutation, a process that allows the introduction of point mutations in the Ig variable genes, and which takes place within the dark zone of germinal centers (Berek and Ziegner, 1993), represents the basic mechanism for affinity maturation of antibodies. Analysis of somatic mutations in V_{H6} genes in six different HIGM patients indicated, in five cases, Ig genes with no, or background levels, of somatic mutations (Chu *et al.*, 1995; Razanajaona *et al.*, submitted for publication), thus suggesting that CD40–CD40-L interactions play a role in the induction of somatic mutations. Yet CD40 activation seems not to be the molecular trigger of somatic mutations inasmuch as an *in vitro* activation of naive B cells by a combination of anti-CD40 and IL-4 does not result in the induction of somatic mutations, although proliferation and isotype switch can be documented (Galibert *et al.*, 1995). Surprisingly, one HIGM patient was shown to display Ig genes with almost normal levels of somatic mutations (Razanajaona *et al.*, submitted for publication). The explanation for this apparent discrepancy may reside in the nature of the CD40-L mutation. Indeed, this patient CD40-L, the only reported one with a mutation in the transmembrane region, can be expressed very transiently on activated T cells, conferring them partial functional activity. Therefore, the requirement for a fully functional CD40–CD40-L interaction does not appear necessary for somatic mutations to occur, while it does for the generation of a complete B cell memory response (see Sections VIB and VII).

Patients with clinical symptoms of the hyper-IgM syndrome, but not associated to chromosome X, have been reported. Two independent studies have now shown that this non-X-linked HIGM might be caused by an intrinsic B cell defect (Callard *et al.*, 1994; Conley *et al.*, 1994). In fact, these patients have normal expression of CD40, but display a defect in CD40-mediated signaling, whose molecular basis remains to be elucidated.

B. Animal Models with Disrupted CD40–CD40-L Interactions

The *in vivo* significance of CD40–CD40-L interactions for the development of humoral immune responses has now been validated in several animal models, including *in vivo* treatment with CD40–CD40-L antagonists, transgenic mice, and "knockout" mice. The model best mimicking the hyper-IgM syndrome, the human "CD40-L knockout," is the CD40-L KO mice (Renshaw *et al.*, 1994; Xu *et al.*, 1994). Such mice display decreased IgM responses to thymus-dependent antigens, no antigen-specific IgG1, and no development of germinal centers, in spite of normal numbers of T and B lymphocytes. As expected, these mice respond normally to T-independent antigens with increased IgM and IgG3 levels.

B cell responses of CD40-L KO mice are normal after *in vitro* activation (Renshaw *et al.*, 1994). A similar phenotype, resembling the HIGM syndrome, is observed in mice where the CD40 gene is inactivated (Castigli *et al.*, 1994; Kawabe *et al.*, 1994). All these animals are mostly investigated for T–B cell responses. However, with the recent insight in the much broader expression of CD40, it will be of interest to further study these animals for other defects, especially during specific situations of infections and other disease states. In this context, it is found that basal levels of granulopoiesis are normal in CD40 KO mice, whereas reactive granulopoiesis appears to be diminished (Kawabe *et al.*, 1994).

Apart from gene inactivation, several studies have investigated the *in vivo* role of CD40–CD40-L interactions by administration of either anti-CD40-L (anti-gp39) or sCD40-Ig or by construction of sCD40-Ig transgenic mice. Treatment with a high-affinity blocking anti-CD40-L antibody results in a complete blocking of functional T–B interactions, with no secondary T-dependent immune responses, no germinal center formation, and no generation of memory B cells (Foy *et al.*, 1994, 1993; Van den Eertwegh *et al.*, 1993). In contrast, injection of sCD40-Ig, a relatively low-affinity reagent, results in the prevention of B cell memory, although germinal centers can still be induced (Gray *et al.*, 1994). In accordance, mice transgenic for sCD40-Ig display no phenotype of functional disruption of T–B interactions (Lane *et al.*, 1994). Surprisingly, mice transgenic for sCTLA4-Ig did show such a HIGM-like phenotype with the lack of memory B cell formation and the absence of germinal centers (Lane *et al.*, 1994).

These data suggest that the generation of B cell memory, which includes germinal center formation, isotype switch, affinity maturation, and B cell differentiation, needs an optimal CD40–CD40-L interaction. However, a partial CD40–CD40-L interaction does allow the generation of germinal centers, but not the development of complete B cell memory (Fig. 10). This fits with the clinical status of a HIGM patient with a partially functional CD40-L and virtually normal levels of somatic hypermutation, a process most likely taking place in germinal centers (Razanajaona *et al.*, submitted for publication).

The numerous infections observed in patients with nonfunctional CD40-L suggest that CD40 crosslinking agents may prove useful in such cases. Accordingly, administration of CD40-L-transfected vaccinia virus induces an antiviral state *in vivo* (Ruby *et al.*, 1995). Importantly, this effect can be observed in animals that lack T and B cells (SCID mice, nude mice), thus indicating the activation of functional CD40 on other cell types.

C. Role of CD40–CD40-L in Other Disease States

The important physiological role of CD40–CD40-L interactions suggests that interfering with this pathway might have beneficial effects in disease

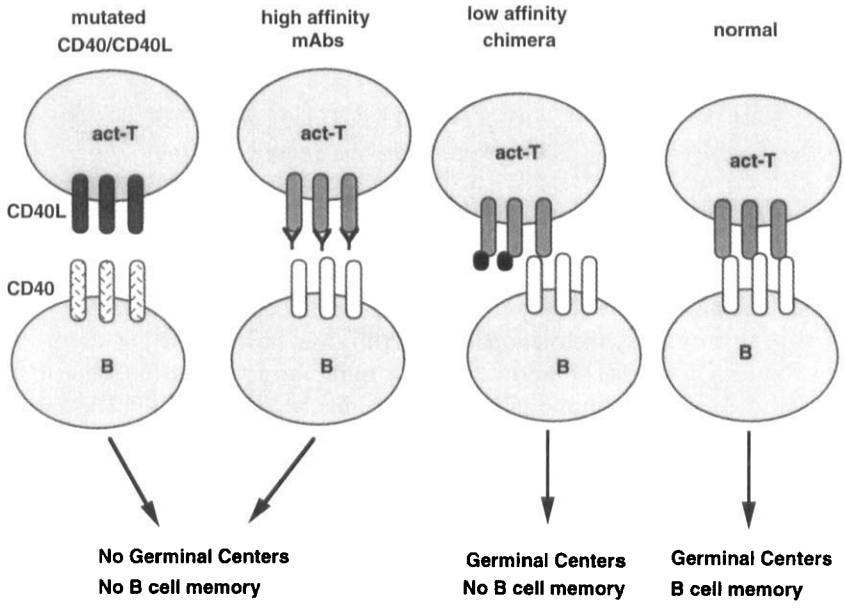


FIG. 10. *In vivo* consequence of partial or complete inhibition of CD40–CD40-L interactions.

states where aberrant immune responses (e.g., autoimmunity) take place. Because of the broad expression of CD40, an excessive CD40–CD40-L interaction can also lead to activation of cellular targets other than B cells. The availability of high-affinity blocking antibodies against mouse CD40-L (anti-gp39) has made it possible to test the involvement of CD40–CD40-L in several disease models.

Arthritis induced in mice by immunization with type II collagen is blocked by injection of anti-CD40-L as reflected by a diminished joint inflammation, lower serum Ab titers to collagen, a lower infiltration of inflammatory cells, and a reduced erosion of cartilage and bone (Durie *et al.*, 1993). The expression of CD40 on human synoviocytes isolated from rheumatoid arthritis joints (Rissoan *et al.*, submitted for publication) could also represent a target that contributes to the observed protection.

Development of glomerulonephritis is one of the hallmarks of lupus prone mice, such as (SWR × NZB)F1 mice whose T cells show increased CD40-L expression (Mohan *et al.*, 1995). Three injections of anti-CD40-L antibody to mice in a prenephritic lupus stage resulted in delayed and reduced incidence of glomerulonephritis. Interestingly, this treatment appears not to affect the development of pathogenic Th cells, but to functionally block the generation of pathogenic B cells.

Administration of anti-CD40-L has also been shown to inhibit graft versus host disease in a mouse model where parental lymphocytes are transferred into an F1 host (Durie *et al.*, 1994a). The anti-CD40-L inhibits both the donor allospecific Th cell response against host B cells, and the allo-CTL response, indicating that cell-mediated immunity is also altered.

VII. Role of CD40–CD40-L in B Cell Immunopoiesis: A Synthesis

The key role of CD40 for T-cell-dependent B cell activation *in vitro* is firmly supported by the *in vivo* finding that mutations in CD40-L gene result in hyper-IgM syndrome. Based on the kinetics and *in vivo* expression of CD40 and CD40-L, a model for the timing and sites of CD40–CD40-L interactions during antigen-dependent, T-cell-dependent immune responses is presented, based on earlier versions (Banchereau *et al.*, 1994; MacLennan, 1994). The processes are divided in two parts: an extrafollicular reaction and a germinal center reaction (Fig. 11).

A. Extrafollicular Reaction

At sites of tissue injury, pathogens/antigens are captured by CD40-low Langerhans cells (dendritic cells of the skin), which consequently migrate to secondary lymphoid organs (Steinman, 1991). Upon arrival, antigen-loaded CD40-high dendritic cells (now called interdigitating dendritic cells, IDC) move into the paracortical T-cell-rich areas. IDC process the antigen and present the peptides bound to MHC class II antigens to naive or memory antigen-specific T cells. It is presently not known whether IDC could also present nonprocessed antigen to B cells, though it is tempting to speculate that antigen-loaded IDC contribute to the selection of antigen-specific naive B cells. The interaction between IDC and T cells results in a specific T cell activation with secretion of cytokines and the expression of activation antigens, including CD40-L (Bogen *et al.*, 1993). Subsequently, CD40-L-expressing T cells can directly modulate IDC cytokine production and phenotype (strong up-regulation of CD80 and CD86), which in turn potentiates T cell activation. At the same time, a cognate T–B cell interaction directly activates B cells to proliferate and differentiate. In this way, naive B cells generate plasma blast cells that produce IgM antibodies, characteristic of primary responses. At this stage, CD40–CD40-L interactions are not mandatory to initiate this primary B cell reaction, inasmuch as humans and mice displaying disrupted CD40–CD40-L interactions are able to mount primary immune responses (Section VI). The molecules involved in CD40-independent activation of B cells have not yet been identified, but may involve other members of the TNF/TNF-R superfamilies such as TNF-α (Aversa *et al.*, 1993; Macchia *et al.*, 1993). The extrafol-

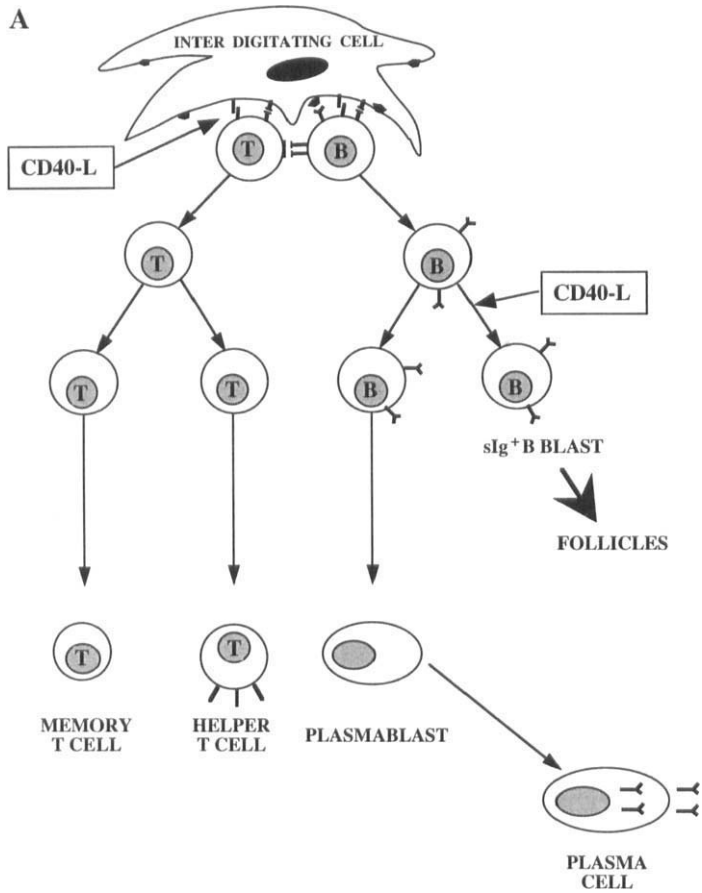
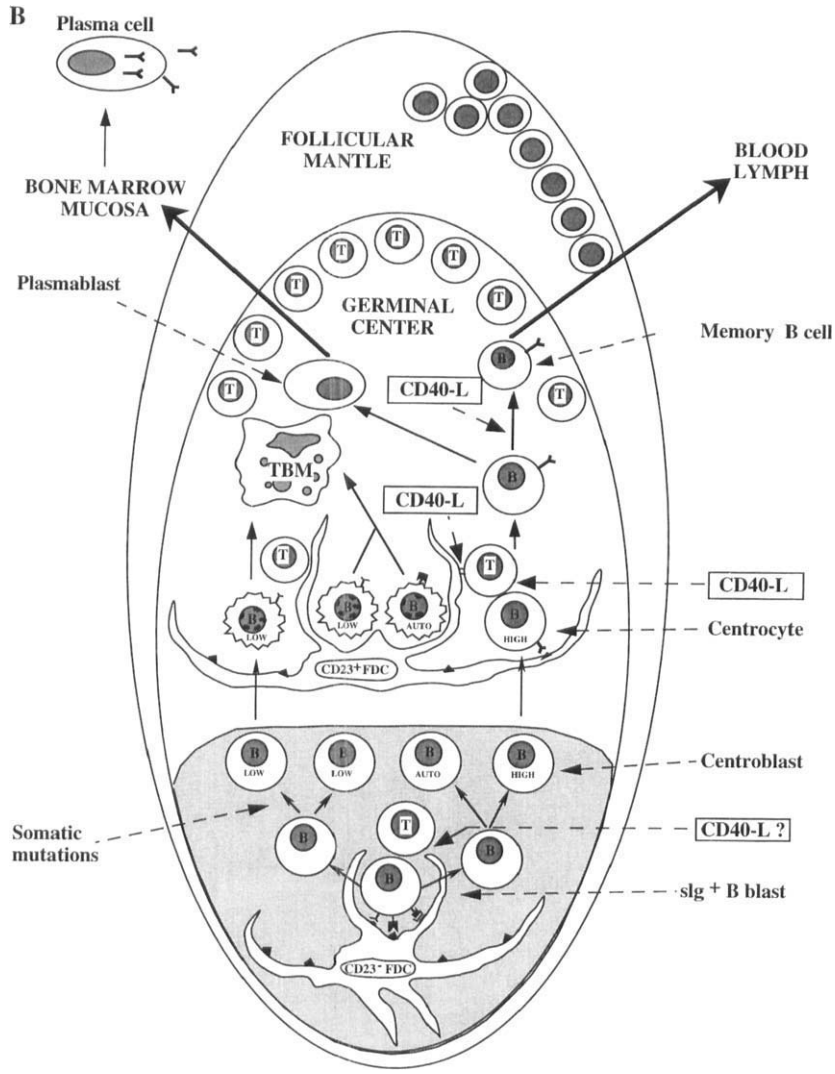


FIG. 11. Schematic representation of antigen-induced, T-cell-dependent B cell immunopoiesis in secondary lymphoid organs. (A) Extrafollicular reaction: At the antigen/pathogen port of entry (mucosa, epidermis), antigen-presenting cells, such as dendritic cells and Langerhans cells, capture the antigen and then migrate via the afferent lymphatics into the T-cell-rich areas of regional lymph nodes where they interact and interdigitate with T and B lymphocytes. Meanwhile the antigen is endocytosed, processed, and reexpressed in the form of peptides associated to class II molecules, complexes that will be further recognized by T cells. Antigen deposited on the dendritic cell surface possibly in the form of immune complexes is also presented to the B cells which can endocytose, process, and present it. Tight interactions occur between T cells, antigen-presenting cells, and B cells that involve several surface antigens. Activated T cells express CD40-L which can further signal the dendritic cells to express higher levels of accessory molecules such as CD80, CD86, and CD56. The activated T cell may also deliver a CD40-L signal to the B cell though this is not mandatory. The activated T cells release cytokines which permit autocrine proliferation of T cells and their differentiation into effector helper T cells. Furthermore, released cytokines also permit the proliferation of B cells and their differentiation into plasmablasts, migrating to medullary cords where they differentiate into short-lived plasma cells producing antigen-specific antibodies. These antibodies form, with free antigen, immune complexes that deposit on follicular dendritic cells. Some of the activated B cell blasts migrate into the follicles. This step neccessitates the B cells to receive a CD40 signal. We propose in fact that a triad, composed of the antigen-specific B cell, the antigen-specific T cell, and the antigen-presenting cell, migrates into the follicles. (B) Ger-



minal center reaction: The B blasts enter into intense proliferation thus generating the centroblasts that finally compose the germinal center dark zone. The centroblasts undergo somatic mutations, and then differentiate into centrocytes that form the light zone and undergo selection processes. If the generated centrocytes display receptors of high affinity, they will retrieve antigen from the immune complexes bound to FDC, process it, and present it to the antigen-specific T cells. The activated T cells will permit, in a CD40-L-dependent fashion, the expansion of the B cells and their isotype switch. The progeny of these B cells will become memory B cells, if CD40-L triggering is provided, or plasma blasts that will emigrate from the germinal center to colonize either bone marrow or mucosal lamina propria when they will terminally differentiate into long-lived plasma cells. In the absence of any external signals, the centrocytes that will express low-affinity antigen receptor will enter into apoptosis. Likewise the centrocytes that display antigen receptors with affinity for autoantigen enter into apoptosis following protracted antigen receptor triggering.

licular reaction also results in the generation of T and B cell blasts, which migrate into the primary follicles and play a key role in the generation of germinal centers (Jacob and Kelsoe, 1992). The absence of germinal centers in HIGM patients and CD40–CD40-L KO mice indicates a crucial role for CD40–CD40-L interactions in the launching of the germinal center reaction.

B. Germinal Center Reaction

The B cell blasts that have been generated during the extrafollicular reaction and that have migrated into the primary follicles undergo an extensive proliferation. These cells, called centroblasts, form the dark zone of the germinal center and undergo high-rate somatic mutation in their Ig V region genes (Berek and Ziegner, 1993). Signals inducing this high-rate proliferation and introduction of somatic mutations are presently not known and their identification represents a kind of "holy grail" for B cell immunologists. We presently believe that, in this event, CD40–CD40-L interactions are necessary but not sufficient, although this remains to be formally demonstrated.

After this intensive proliferation, cells go out of cycle (and are now called centrocytes), and accumulate to form the light zone of germinal centers, where they undergo affinity selection.

Three scenarios may happen according to whether somatic mutations have (i) increased the affinity of the antigen receptor for the elicitating antigen, (ii) decreased or left unaltered the affinity of the antigen receptor for the elicitating antigen, (iii) changed the specificity of the antigen receptor into an autoreactive receptor.

(i) The generated centrocytes display antigen receptors that have acquired a higher affinity for the elicitating antigen ("B high" in Fig. 11B). The centrocytes with high-affinity BCR will retrieve antigen from the immune complexes bound to FDC. The FDC/B cell interaction provides a survival signal to the antigen-specific B cell which is delivered through the triggering of the specific antigen receptor and the engagement of adhesion molecules such as VCAM-1 and LFA 1. B cells process the retrieved antigen and then present it to the antigen-specific T cells (Gray *et al.*, 1991; Kosco *et al.*, 1989).

This T cell/B cell dialog permits the expansion of the uniquely produced centrocyte that express high-affinity BCR. This dialog involves CD40–CD40-L interactions as shown by the presence of CD40-L-positive T cells within germinal centers (Casamayor-Palleja *et al.*, 1995; Lederman *et al.*, 1992a). The importance of these CD40–CD40-L interactions has also been demonstrated *in vivo* by the administration of soluble CD40 (a weak antagonist to CD40-L) which results in the blocking of the generation

of B cell memory while the formation of germinal centers is not affected (Gray *et al.*, 1994). The CD40–CD40-L interaction is most likely to be crucial at that level in turning on the isotype switch machinery whose specificity is given by cytokines such as IL-4/IL-13, IL-10 or TGF-β. In fact, it is economic for the immune system to set up isotype switch after selection of high affinity mutants rather than at the same time (e.g., in the dark zone) or even earlier (e.g., during the extrafollicular reaction). Furthermore, germinal center $CD4^+$ T cells isolated according to CD57 expression display IL-4 and IL-10 which represent switch factors toward IgG4, IgE, IgG1, IgG3, and IgA (Butch *et al.*, 1993).

At this stage, interactions between T cells and FDCs are likely to occur. In particular, FDCs may be induced to proliferate as indicated by the considerably denser FDC network in secondary follicles by contrast to primary follicles and by the existence of $Ki67^+$ FDCs in secondary follicles. FDC proliferation may indeed be triggered in response to T cell CD40-L signaling inasmuch as FDC-like cell lines proliferate in response to T cells in a CD40-dependent fashion and as fibroblasts are induced to proliferate in response to CD40 ligation (Rissoan *et al.*, submitted for publication).

In all cases the high-affinity B cell clone expansion will be limited because the prolonged contact between the antigen-specific T cell and the antigen specific B cell results in cell death as a consequence of protracted Fas/Fas-L interactions (Garrone *et al.*, 1995) (Fig. 12). In fact, such as mechanism prevents the possibility that immune responses may be dominated by only a few B cell clones that would prevent the building of a repertoire vast enough to match the extreme diversity of potentially harmful antigens. Yet it is interesting to stress that BCR-triggered B cells are less sensitive to Fas/Fas-L-dependent B cell death than B cells that have not engaged their BCR (Rothstein *et al.*, 1995; Garrone, unpublished observations). This would give antigen-triggered B cells a survival advantage compared to non-antigen-triggered B cells that may interact with the activated T cells in a noncognate fashion.

(ii) Centrocytes display low-affinity antigen receptors against immunizing antigens ("B low" in Fig. 11B). Such cells will receive no signals either from their BCR or from their CD40 antigen, as no antigen-specific T cell will be encountered. We propose that these B cells will be eliminated by entering into apoptosis either spontaneously or following noncognate interaction with activated T cells. These cells may express Fas-L and thus may represent an ultimate selection gate to kill nonselected B cells in a Fas/Fas-L-dependent interaction.

(iii) Centrocytes display antigen receptors that acquired an affinity for autoantigens ("B auto" in Fig. 11B). A very large repertoire of autoantigens is likely to be present within the germinal center microenvironment. The

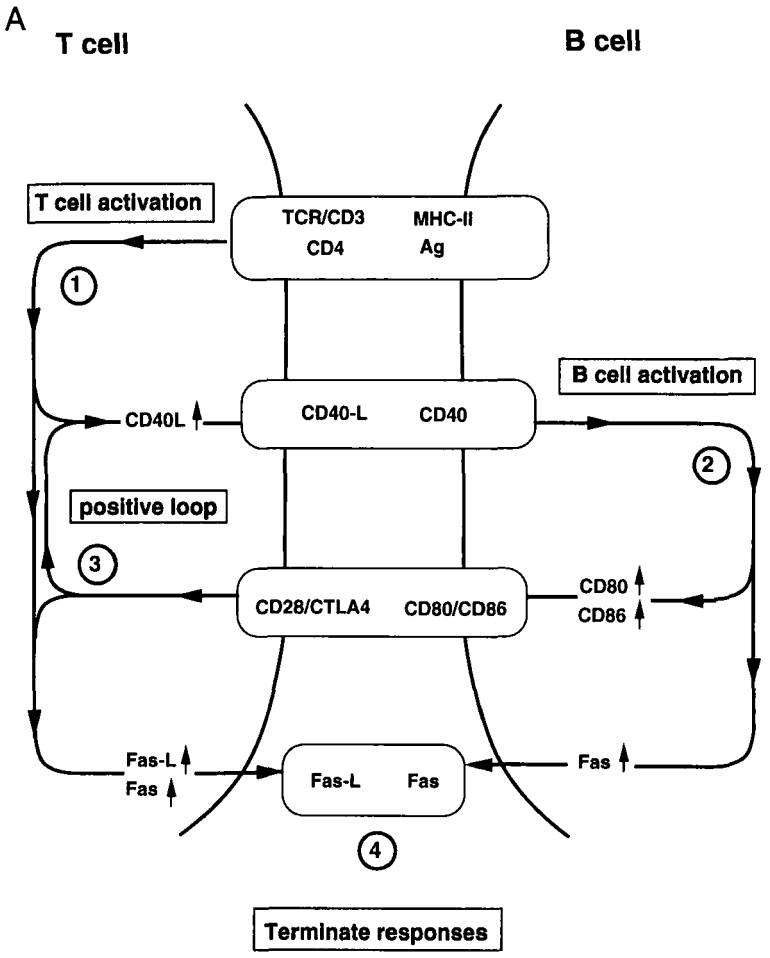


FIG. 12. Crosstalk between CD40/CD40-L, CD80–CD86/CD28–CTLA4, and Fas/Fas-L. (A) Sequential interactions during T–B cell interactions which have been discussed in Section V,B. (1) Cognate T–B cell interaction results in T cell activation, including up-regulation of CD40-L, Fas, and Fas-L. (2) B cells become activated via CD40–CD40-L interactions, resulting in up-regulation of CD80, CD86, and Fas. (3) Further T cell activation is induced via CD80–CD86 vs CD28–CTLA4 interactions, resulting in a positive activation loop. (4) The expresssion of Fas and Fas-L, which have been induced during both B and T cell activation, results in a termination of both activation processes. (B) Model showing that the induction of Fas expression on B cells after CD40 activation limits the size of the generated B cell clone (see Section VII,B).

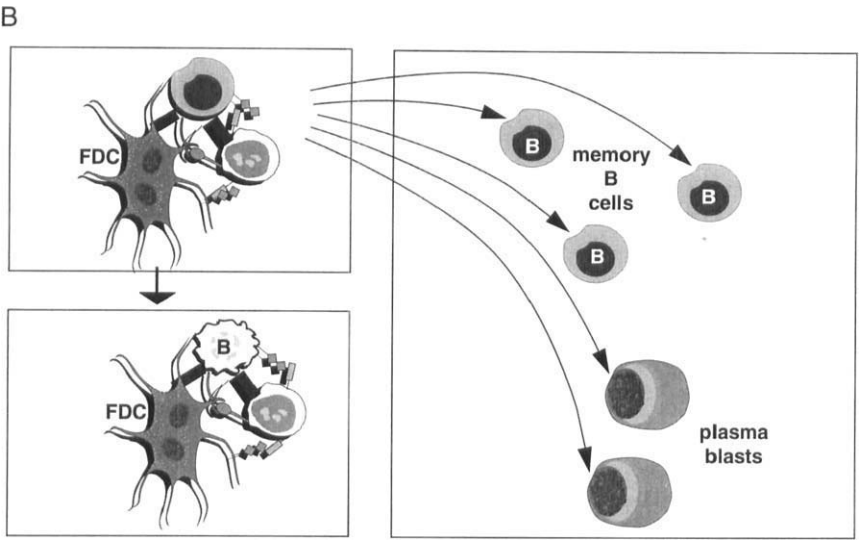


FIG. 12. (*Continued*)

centrocytes that have acquired BCR binding to such antigens will undergo protracted BCR triggering in the absence of specific T cell help (autoreactive T cells are normally deleted in the thymus). As demonstrated *in vitro*, these cells will enter apoptosis (Van Kooten *et al.*, 1995, submitted). In keeping with this, recent *in vivo* studies have indeed demonstrated that injection of high doses of soluble antigen induces apoptosis (and phagocytosis by tingible body macrophages) of antigen-specific B cells within germinal centers (Pulendran *et al.*, 1995; Shokat and Goodnow, 1995). It is not clear yet whether the autoantigen will be presented specifically by FDC or any other cell type. The B cells that will escape this selection stage, e.g., by interacting in a noncognate fashion with activated T cells that express CD40-L and secrete cytokines, will be rescued and will thus represent the source of high-affinity autoantibodies. Such an event, however, is unlikely to result in autoimmunity as long as autoreactive T cells, necessary for launching a secondary humoral response, do not escape thymic selection.

When a B cell clone has passed the different selection criteria, a further development leads to either direct effector cells (antibody-secreting plasma cells) or memory cells that will act promptly in case of an encounter with the same pathogen/antigen. At this decision point, CD40–CD40-L interactions appear to play an important role. *In vitro* studies have shown

that proliferating centrocytes will mature into memory B cells in response to prolonged CD40 triggering and into plasma blasts when CD40 signaling is limited and IL-10 available (Arpin *et al.*, 1995; Dechanet *et al.*, 1995; Merville *et al.*, 1995b; Rousset *et al.*, 1995).

VIII. Perspectives

As reviewed herein, the past few years have witnessed considerable progress in our knowledge of the CD40 and CD40-L molecules. Major developments have been obtained on (i) the expression and functional role of CD40 in cells other than B cells, (ii) the *in vivo* significance of CD40–CD40-L interactions, and (iii) the signal transduction machinery activated following crosslinking of the CD40 antigen.

Concerning CD40, it is very well possible that it may bind to other natural ligands inasmuch as other members of the TNF-R superfamily bind several ligands, as exemplified by the two TNF-Rs, the LNGFR, and 4-1BB. Interestingly, 4-1BB binds both a TNF-like molecule (Goodwin *et al.*, 1993b) and extracellular matrix proteins (Chalupny *et al.*, 1992). The use of these latter ligands may be particularly relevant for CD40-positive cells such as epithelial cells, endothelial cells, and fibroblasts which are in close contact to extracellular matrix. It is tempting to speculate that these cells may encounter CD40-L-positive T cells only in pathological circumstances (T cell infiltrated, fibrosis, arthritis, cancers, etc.). A thorough investigation of the available CD40 and CD40-L KO mice might give further insight into the role of CD40 on nonlymphoid cells and the possible presence of alternative CD40 ligands.

The apparently crucial role of CD40 triggering in B cell immunopoiesis, including isotype switch and B cell memory formation, is well established. However, major questions remain to be addressed regarding antigen-dependent B cell responses: (i) what are the CD40–CD40-L independent interactions leading to activation of naive B cells? (ii) What is the mechanism (CD40-L independent?) underlying the massive proliferation of centroblasts in germinal center dark zones? (iii) What activation signals (CD40-L independent?) result in the induction of the somatic mutation machinery?

Of the TNF and TNF-R superfamilies, TNF-R–TNF, Fas–Fas-L, and CD40–CD40-L have been best studied. However, all family members have been shown to be involved in activation, proliferation, or survival of lymphocytes. In this context, the B cell growth and differentiation obtained by crosslinking B cell Ox40 ligand (Stüber *et al.*, 1995) may indeed explain some of the questions raised above.

We expect the next few years to bring a wealth of information concerning the pathways activated by CD40. Presently the TNF-Rs and Fas have represented the most thoroughly analyzed members of this superfamily, but the information generated will swiftly translate into CD40. This understanding may ultimately permit the design of new chemical entities that will interrupt CD40-activated signal transduction pathways. The available *in vivo* information about altered CD40–CD40-L interactions clearly suggests that interrupting these pathways may be of great interest for the management of several pathological conditions, such as autoimmunity and graft versus host disease.

Acknowledgments

The authors thank Daniel Lepot for his invaluable help in drawing the figures and Sandrine Bonnet-Arnaud for her precious secretarial assistance. This review was made possible because of the scientific contributions of our many collaborators including Dominique Blanchard, Francine Brière, Pierre Garrone, Serge Lebecque, Yong-Jun Liu, Leopoldo Flores-Romo, Diane Razanajaona, Françoise Rousset, Sem Saeland and Nicolas Burdin, Laurent Galibert, Patrice Hermann, and Marie-Clotilde Rissoan. The support of Jacques Chiller is greatly appreciated.

References

Alderson, M. R., Armitage, R. J., Tough, T. W., Strockbine, L., Fanslow, W. C., and Spriggs, M. K. (1993). CD40 expression by human monocytes: Regulation by cytokines and activation of monocytes by the ligand for CD40. *J. Exp. Med.* **178,** 669–674.

Alderson, M. R., Smith, C. A., Tough, T. W., Davis-Smith, T., Armitage, R. J., Falk, B., Roux, E., Baker, E., Sutherland, G. R., and Din, W. S. (1994). Molecular and biological characterization of human 4-1BB and its ligand. *Eur. J. Immunol.* **24,** 2219–2227.

Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Disteche, C. M., Simoneaux, D. K., Fanslow, W. C., Belmont, J., and Spriggs, M. K. (1993). CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* **259,** 990–993.

Armitage, R. J., and Alderson, M. R. (1995). B-cell stimulation. *Curr. Op. Immunol.* **7,** 243–247.

Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D., and Spriggs, M. K. (1992a). Molecular and biological characterization of a murine ligand for CD40. *Nature* **357,** 80–82.

Armitage, R. J., Macduff, B. M., Spriggs, M. K., and Fanslow, W. C. (1993a). Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. *J. Immunol.* **150,** 3671–3680.

Armitage, R. J., Sato, T. A., Macduff, B. M., Clifford, K. N., Alpert, A. R., Smith, C. A., and Fanslow, W. C. (1992b). Identification of a source of biologically active CD40 ligand. *Eur. J. Immunol.* **22,** 2071–2076.

Armitage, R. J., Tough, T. W., Macduff, B. M., Fanslow, W. C., Spriggs, M. K., Ramsdell, F., and Alderson, M. R. (1993b). CD40 ligand is a T-cell growth factor. *Eur. J. Immunol.* **23,** 2326–2331.

Arpin, C., Dechanet, J., van Kooten, C., Merville, P., Grouard, G., Brière, F., Banchereau, J., and Liu, Y.-J. (1995). *In vitro* generation of memory B cells and plasma cells. *Science* **268,** 720–722.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L. S., Stenkamp, R., Neubauer, M., Roberts, R. L., Noelle, R. J., Ledbetter, J. A., Francke, U., and Ochs, H. D. (1993). The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* **72,** 291–300.

Aversa, G., Punnonen, J., and de Vries, J. E. (1993). The 26-kD transmembrane form of tumor necrosis factor α on activated $CD4^+$ T cell clones provides a costimulatory signal for human B cell activation. *J. Exp. Med.* **177,** 1575–1585.

Baens, M., Chaffanet, M., Cassiman, J. J., van den Berghe, H., and Marynen, P. (1993). Construction and evaluation of a hncDNA library of human 12p transcribed sequences derived from a somatic cell hybrid. *Genomics* **16,** 214–218.

Banchereau, J., Bazan, F., Blanchard, D., Brière, F., Galizzi, J.-P., van Kooten C., Liu, Y.-J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annu. Rev. Immunol.* **12,** 881–922.

Banchereau, J., de Paoli, P., Vallé, A., Garcia, E., and Rousset, F. (1991). Long term human B cell lines dependent on interleukin 4 and antibody to CD40. *Science* **251,** 70–72.

Banchereau, J., and Rousset, F. (1991). Growing human B lymphocytes in the CD40 system. *Nature* **353,** 678–679.

Banchereau, J., and Rousset, F. (1992). Human B lymphocytes: Phenotype, proliferation and differentiation. *Adv. Immunol.* **52,** 125–251.

Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H., and Lesslauer, W. (1993). Crystal structure of the soluble human 55 kd TNF receptor-human TNFβ complex: Implications for TNF receptor activation. *Cell* **73,** 431–445.

Barrett, T. B., Shu, G., and Clark, E. A. (1991). CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. *J. Immunol.* **146,** 1722–1729.

Baum, P. R., Gayle, R. B., Ramsdell, F., Srinivasan, S., Sorensen, R. A., Watson, M. L., Seldin, M. F., Baker, E., Sutherland, G. R., and Clifford, K. N. (1994). Molecular characterization of murine and human OX40/OX40 ligand systems: Identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J.* **13,** 3992–4001.

Bazan, F. (1993). Emerging families of cytokines and receptor. The structure of a TNF receptor-ligand complex shows at the molecular level how members of two growing families of cytokines and receptors interact. *Curr. Biol.* **3,** 603–606.

Bazil, V. (1995). Physiological enzymatic cleavage of leukocyte membrane molecules. *Immunol. Today* **16,** 135–139.

Behrmann, I., Walczak, H., and Krammer, P. H. (1994). Structure of the human APO-1 gene. *Eur. J. Immunol.* **24,** 3057–3062.

Beiske, K., Clark, E. A., Holte, H., Ledbetter, J. A., Smeland, E. B., and Godal, T. (1988). Triggering of neoplastic B cells via surface IgM and the cell surface antigens CD20 and CDw40. Responses differ form normal blood B cells and are restricted to certain morphologic subsets. *Int. J. Cancer* **42,** 521–528.

Berberich, I., Shu, G. L., and Clark, E. A. (1994). Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J. Immunol.* **153,** 4357–4366.

Berek, C., and Ziegner, M. (1993). The maturation of the immune response. *Immunol. Today* **14,** 400–404. [Published erratum appears in *Immunol. Today* **14,** 479 (1993).]

Beutler, B., and Cerami, A. (1989). The biology of cachectin/TNF. A primary mediator of the host response. *Annu. Rev. Immunol.* **7,** 625–655.

Birkeland, M. L., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Barclay, A. N. (1995). Gene structure and chromosomal localization of the mouse homologue of rat OX40 protein. *Eur. J. Immunol.* **25,** 926–930.

Björck, P., Axelsson, B., and Paulie, S. (1991). Expression of CD40 and CD43 during activation of human B lymphocytes. *Scand. J. Immunol.* **33,** 211–218.

Björck, P., Braesch-Andersen, S., and Paulie, S. (1994). Antibodies to distinct epitopes on the CD40 molecule co-operate in stimulation and can be used for the detection of soluble CD40. *Immunology* **83,** 430–437.

Bogen, S. A., Fogelman, I., and Abbas, A. K. (1993). Analysis of IL-2, IL-4, and IFN-γ-producing cells in situ during immune responses to protein antigens. *J. Immunol.* **150,** 4197–4205.

Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* **270,** 7795–7798.

Bonnefoy, J. Y., and Noelle, R. J. (1994). The CD40/CD40L interaction. All things to all immunologists. *Res. Immunol.* **145,** 199–249.

Boussiotis, V. A., Nadler, L. M., Strominger, J. L., and Goldfeld, A. E. (1994). Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. *Proc. Natl. Acad. Sci. USA* **91,** 7007–7011.

Bowen, M. (1993). "Functional characterization of CD30 on the large granular lymphoma cell line YT, and the cloning of a murine CD30 cDNA homologue." Thesis, University of Miami.

Bowman, M. R., Crimmins, M. A., Yetz-Aldape, J., Kriz, R., Kelleher, K., and Herrmann, S. (1994). The cloning of CD70 and its identification as the ligand for CD27. *J. Immunol.* **152,** 1756–1761.

Braesch-Andersen, S., Paulie, S., Aspenström, P., Koho, H., and Perlmann, P. (1989). Biochemical characteristics of the human B-cell and carcinoma antigen CD40. *In* "Leukocyte Typing IV. White Cell Differentiation Antigens" (W. Knapp, B. Dörken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. K. von dem Borne, Eds.), pp. 96–97. Oxford University Press, Oxford.

Brière, F., Servet-Delprat, C., Bridon, J.-M., Saint-Rémy, J.-M., and Banchereau, J. (1994). Human interleukin 10 induces naive sIgD$^+$ B cells to secrete IgG_1 and IgG_3. *J. Exp. Med.* **179,** 757–762.

Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brine-Greco, B., Foley, S. F., and Ware, C. F. (1993). Lymphotoxin β, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* **72,** 847–856.

Brugnoni, D., Airo, P., Graf, D., Marconi, M., Lebowitz, M., Plebani, A., Gilliani, S., Malacarne, F., Cattaneo, R., Ugazio, A. G., *et al.* (1994). Ineffective expression of CD40 ligand on cord blood T cells may contribute to poor immunoglobulin production in the newborn. *Eur. J. Immunol.* **24,** 1919–1924.

Burdin, N., Péronne, C., Banchereau, J., and Rousset, F. (1993). Epstein-Barr virus-transformation induces B lymphocytes to produce human interleukin-10. *J. Exp. Med.* **177,** 295–304.

Burdin, N., van Kooten, C., Galibert, L., Abrams, J. S., Wijdenes, J., Banchereau, J., and Rousset, F. (1995). Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. *J. Immunol.* **154,** 2533–2544.

Butch, A. W., Chung, G.-H., Hoffmann, J. W., and Nahm, M. H. (1993). Cytokine expression by germinal center cells. *J. Immunol.* **150,** 39–47.

Calderhead, D. M., Buhlmann, J. E., van den Eertwegh, A. J., Claassen, E., Noelle, R. J., and Fell, H. P. (1993). Cloning of mouse Ox40: A T cell activation marker that may mediate T-B cell interactions. *J. Immunol.* **151,** 5261–5271.

Callard, R. E., Armitage, R. J., Fanslow, W. C., and Spriggs, M. K. (1993). CD40 ligand and its role in X-linked hyper-IgM syndrome. *Immunol. Today* **14,** 559–564.

Callard, R. E., Smith, S. H., Herbert, J., Morgan, G., Padayachee, M., Lederman, S., Chess, L., Kroczek, R. A., Fanslow, W. C., and Armitage, R. J. (1994). CD40 ligand (CD40L) expression and B cell function in agammaglobulinaemia with normal or elevated levels of IgM (HIM): A comparison of X-linked, autosomal recessive and non-X linked forms of the disease, and obligate carriers. *J. Immunol.* **153,** 3295–3306.

Camerini, D., Walz, G., Loenen, W. A. M., Borst, J., and Seed, B. (1991). The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J. Immunol.* **147,** 3165–3169.

Carbone, A., Gloghini, A., Gattei, V., Aldinucci, D., Degan, M., P., D. P., Zagonel, V., and Pinto, A. (1995). Expression of functional CD40 antigen on Reed-Sternberg cells and Hodgkin's disease cell lines. *Blood* **85,** 780–789.

Casamayor-Pelleja, M., Khan, M., and MacLennan., I.-C. M. (1995). A subset of $CD4^+$ memory T cells contains preformed CD40 Ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J. Exp. Med.* **181,** 1293–1301.

Cascino, I., Fiucci, G., Papoff, G., and Ruberti, G. (1995). Three functional soluble forms of the human apoptosis-inducing fas molecule are produced by alternative splicing. *J. Immunol.* **154,** 2706–2713.

Castigli, E., Alt, F. W., Davidson, L., Bottaro, A., Mizoguchi, E., Bhan, A. K., and Geha, R. S. (1994). CD40-deficient mice generated by recombination-activating gene-2-deficient blastocyst complementation. *Proc. Natl. Acad. Sci. USA* **91,** 12135–12139.

Castle, B. E., Kishimoto, K., Stearns, C., Brown, M. L., and Kehry, M. R. (1993). Regulation of expression of the ligand for CD40 on T helper lymphocytes. *J. Immunol.* **151,** 1777–1788.

Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. (1992). GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature* **360,** 258–261.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., van Kooten, C., Durand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* **180,** 1263–1272.

Chalupny, N. J., Peach, R., Hollenbaugh, D., Ledbetter, J. A., Farr, A. G., and Aruffo, A. (1992). T-cell activation molecule 4-1BB binds to extracellular matrix proteins. *Proc. Natl. Acad. Sci. USA* **89,** 10360–10364.

Chao, M. V., Bothwell, M. A., Ross, A. H., Koprowski, H., Lanahan, A. A., Buck, C. R., and Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. *Science* **232,** 518–521.

Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S., and Baltimore, D. (1995a). Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* **267,** 1494–1498.

Cheng, J., Liu, C., Koopman, W. J., and Mountz, J. D. (1995b). Characterization of human Fas gene. Exon/intron organization and promoter region. *J. Immunol.* **154,** 1239–1245.

Cheng, J., Zhou, T., Liu, C., Shapiro, J. P., Brauer, M. J., Kiefer, M. C., Barr, P. J., and Mountz, J. D. (1994). Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* **263,** 1759–1762.

Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81,** 505–512.

Choi, M. S. K., Boise, L. H., Gottschalk, A. R., Quintans, J., Thompson, C. B., and Klaus, G. G. B. (1995). The role of bcl-xl in CD40-mediated rescue from anti-μ-induced apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol.* **25,** 1352–1357.

Choi, M. S. K., Brines, R. D., Holman, M. J., and Klaus, G. G. B. (1994). Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. *Immunity* **1,** 179–187.

Chu, Y. W., Marin, E., Fuleihan, R., Ramesh, N., Rosen, F. S., Geha, R. S., and Insel, R. A. (1995). Somatic mutation of human immunoglobulin V genes in the X-linked hyperIgM syndrome. *J. Clin. Invest.* **95,** 1389–1393.

Clark, E. A., Grabstein, K. H., and Shu, G. L. (1992). Cultured human follicular dendritic cells. Growth characteristics and interactions with B lymphocytes. *J. Immunol.* **148,** 3327–3335.

Clark, E. A., and Ledbetter, J. A. (1986). Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc. Natl. Acad. Sci. USA* **83,** 4494–4498.

Clark, E. A., and Ledbetter, J. A. (1994). How B and T cells talk to each other. *Nature* **367,** 425–428.

Clark, E. A., and Shu, G. (1990). Association between IL-6 and CD40 signaling. IL-6 induces phosphorylation of CD40 receptors. *J. Immunol.* **145,** 1400–1406.

Cleveland, J. L., and Ihle, J. N. (1995). Contenders in FasL/TNF death signaling. *Cell* **81,** 479–482.

Cocks, B. G., de Waal-Malefyt, R., Galizzi, J.-P., de Vries, J. E., and Aversa, G. (1993). IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int. Immunol.* **5,** 657–663.

Coffman, R. L., Lebman, D. A., and Shrader, B. (1989). Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* **170,** 1039–1045.

Conley, M. E. (1992). Molecular approaches to analysis of X-linked immunodeficiencies. *Annu. Rev. Immunol.* **10,** 215–238.

Conley, M. E., Larche, M., Bonagura, V. R., Lawton, A. R., Buckley, R. H., Fu, S. M., Coustan Smith, E., Herrod, H. G., and Campana, D. (1994). Hyper IgM syndrome associated with defective CD40-mediated B cell activation [see comments]. *J. Clin. Invest.* **94,** 1404–1409.

Cooke, M. P., Heath, A. W., Shokat, K. M., Zeng, Y., Finkelman, F. D., Linsley, P. S., Howard, M., and Goodnow, C. C. (1994). Immunoglobulin signal transduction guides the specificity of B cell-T interactions and is blocked in tolerant self-reactive B cells. *J. Exp. Med.* **179,** 425–438.

Crawford, D. H., and Catovsky, D. (1993). In vitro activation of leukaemic B cells by interleukin-4 and antibodies to CD40. *Immunology* **80,** 40–44.

Cronin, D. C., Stack, R., and Fitch, F. W. (1995). IL-4-producing $CD8^+$ T cell clones can provide B cell help. *J. Immunol.* **154,** 3118–3127.

Crowe, P. D., Van Arsdale, T. L., Walter, B. N., Ware, C. F., Hession, C., Ehrenfels, B., Browning, J. L., Din, W. S., Goodwin, R. G., and Smith, C. A. (1994). A lymphotoxin-beta-specific receptor. *Science* **264,** 707–710.

de Boer, M., Kasran, A., Kwekkeboom, J., Walter, H., Vandenberghe, P., and Ceuppens, J. L. (1993). Ligation of B7 with CD28/CTLA-4 on T cells results in CD40 ligand expression, interleukin-4 secretion and efficient help for antibody production by B cells. *Eur. J. Immunol.* **23,** 3120–3125.

Dechanet, J., Merville, P., Durand, I., Banchereau, J., and Miossec, P. (1995). The ability of synoviocytes to support terminal differentiation of activated B cells may explain plasma cell accumulation in rheumatoid synovium. *J. Clin. Invest.* **95,** 456–463.

Defrance, T., Vanbervliet, B., Brière, F., Durand, I., Rousset, F., and Banchereau, J. (1992a). Interleukin 10 and Transforming Growth Factor β cooperate to induce anti-CD40-activated naive human B cells to secrete Immunoglobulin A. *J. Exp. Med.* **175,** 671–682.

Defrance, T., Vanbervliet, B., Durand, I., Briolay, J., and Banchereau, J. (1992b). Proliferation and differentiation of human CD5$^+$ and CD5$^-$ B cell subsets activated through their antigen receptors or CD40 antigens. *Eur. J. Immunol.* **22,** 2831–2839.

Del Prete, G., Maggi, E., Pizzolo, G., and Romagnani, S. (1995). CD30, Th2 cytokines and HIV infection: A complex and fascinating link. *Immunol. Today* **16,** 76–80.

De Togni, P., Goellner, J., Ruddle, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., and Schoenberger, J. (1994). Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* **264,** 703–707.

DiSanto, J. P., Bonnefoy, J. Y., Gauchat, J. F., Fischer, A., and de Saint Basile, G. (1993). CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* **361,** 541–543.

DiSanto, J. P., Markiewicz, S., Gauchat, J. F., Bonnefoy, J. Y., Fischer, A., and de Saint Basile, G. (1994). Brief report: Prenatal diagnosis of X-linked hyper-IgM syndrome. *N. Engl. J. Med.* **330,** 969–973.

DiStefano, P. S., and Johnson, E. M. (1988). Identification of a truncated form of the nerve growth factor receptor. *Proc. Natl. Acad. Sci. USA* **85,** 270–274.

Dunlap, N. E., Berry, A. K., Gore, I., Decker, W. D., Snyder, T. L., Shaw, G. M., Hanh, B. H., and Tildern, A. B. (1989). Expression of activation antigens on HTLV-1 and THLV-II cell lines. *In* "Leukocyte Typing IV. White Cell Differentiation Antigens" (W. Knapp *et al.,* Eds.), pp. 487–488. Oxford University Press, Oxford.

Durandy, A., De Saint Basile, G., Lisowska-Grospierre, B., Gauchat, J. F., Forveille, M., Kroczek, R. A., Bonnefoy, J. Y., and Fischer, A. (1995). Undetectable CD40 ligand expression on T cells and low B cell responses to CD40 binding agonists in human newborns. *J. Immunol.* **154,** 1560–1568.

Durandy, A. Schiff, C., Bonnefoy, J.-Y., Forveille, M., Rousset, F., Mazzei, G., Milili, M., and Fischer, A. (1993). Induction by anti-CD40 antibody or soluble CD40 ligand and cytokines of IgG, IgA and IgE production by B cells from patients with X-linked hyper IgM syndrome. *Eur. J. Immunol.* **23,** 2294–2299.

Durie, F. H., Aruffo, A., Ledbetter, J., Crassi, K. M., Green, W. R., and Fast, L. D. (1994a). Antibody to the ligand of CD40, gp39, blocks the occurrence of the acute and chronic forms of graft-vs-host disease. *J. Clin. Invest.* **94,** 1333–1338.

Durie, F. H., Fava, R. A., Foy, T. M., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. *Science* **261,** 1328–1330.

Durie, F. H., Foy, T. M., Masters, S. R., Laman, J. D., and Noelle, R. J. (1994b). The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol. Today* **15,** 406–410.

Durkop, H., Latza, U., Hummel, M., Eitelbach, F., Seed, B., and Stein, H. (1992). Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for Hodgkin's disease. *Cell* **68,** 421–427.

Eck, M. J., and Sprang, S. R. (1989). The structure of tumor necrosis factor α at 2.6 A resolution. Implications for receptor binding. *J. Biol. Chem.* **264,** 17595–17606.

Eck, M. J., Ultsch, M., Rinderknecht, E., de Vos, A. M., and Sprang, S. R. (1992). The structure of human lymphotoxin (tumor necrosis factor-B) at 1.9-A. *J. Biol. Chem.* **267,** 2119–2122.

Engelmann, H., Holtmann, H., Brakebush, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O., and Wallach, D. (1990). Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem.* **265,** 14497–14504.

Eris, J. M., Basten, A., Brink, R., Doherty, K., Kehry, M. R., and Hodgkin, P. D. (1994). Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent

T-cell signals but are defective in antigen-receptor-mediated functions. *Proc. Natl. Acad. Sci. USA* **91,** 4392–4396.

Facchetti, F., Appiani, C., Salvi, L., Levy, J., and Notarangelo, L. D. (1995). Immunohistologic analysis of ineffective CD40-CD40 ligand interaction in lymphoid tissues from patients with X-linked immunodeficiency with hyper-IgM. Abortive germinal center cell reaction and severe depletion of follicular dendritic cells. *J. Immunol.* **154,** 6624–6633.

Falini, B., Pileri, S., Pizzolo, G., Durkop, H., Flenghi, L., Stirpe, F., Martelli, M. F., and Stein, H. (1995). CD30 (Ki-1) molecule: A new cytokine receptor of the tumor necrosis factor receptor superfamily as a tool for diagnosis and immunotherapy. *Blood* **85,** 1–14.

Fanslow, W. C., Anderson, D. M., Grabstein, K. H., Clark, E. A., Cosman, D., and Armitage, R. J. (1992). Soluble forms of CD40 inhibit biologic responses of human B cells. *J. Immunol.* **149,** 655–660.

Fanslow, W. C., Clifford, K. N., Seaman, M., Alderson, M. R., Spriggs, M. K., Armitage, R. J., and Ramsdell, F. (1994). Recombinant CD40 ligand exerts potent biologic effects on T cells. *J. Immunol.* **152,** 4262–4269.

Faris, M., Gaskin, F., Parsons, J. T., and Fu, S. M. (1994). CD40 signaling pathway: Anti-CD40 monoclonal antibody induces rapid dephosphorylation and phosphorylation of tyrosine-phosphorylated proteins including protein tyrosine kinase Lyn, Fyn, and Syk and the appearance of a 28-kD tyrosine phosphorylated protein. *J. Exp. Med.* **179,** 1923–1931.

Farrah, T., and Smith, C. A. (1992). Emerging cytokine family. *Nature* **358,** 26.

Farrington, M., Grosmaire, L. S., Nonoyama, S., Fischer, S. H., Hollenbaugh, D., Ledbetter, J. A., Noelle, R. J., Aruffo, A., and Ochs, H. D. (1994). CD40 ligand expression is defective in a subset of patients with common variable immunodeficiency. *Proc. Natl. Acad. Sci. USA* **91,** 1099–1103.

Fernandez, E., Vicente, A., Zapata, A., Brera, B., Lozano, J. J., Martinez, C., and Toribio, M. L. (1994). Establishment and characterization of cloned human thymic epithelial cell lines. Analysis of adhesion molecule expression and cytokine production. *Blood* **83,** 3245–3254.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–246.

Flores-Romo, L., Estoppey, D., and Bacon, K. B. (1993). Anti-CD40 antibody stimulates the VLA-4-dependent adhesion of normal and lymphocyte function-associated antigen-1-deficient B cells to endothelium. *Immunology* **79,** 445–451.

Fluckiger, A.-C., Garrone, P., Durand, I., Galizzi, J. P., and Banchereau, J. (1993). IL-10 upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J. Exp. Med.* **178,** 1473–1481.

Fluckiger, A. C., Rossi, J. F., Bussel, A., Bryon, P., Banchereau, J., and Defrance, T. (1992). Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B-cell tropic factors. *Blood* **80,** 3173–3181.

Fonatsch, C., Latza, U., Durkop, H., Rieder, H., and Stein, H. (1992). Assignment of the human CD30 (Ki-1) gene to 1p36. *Genomics* **14,** 825–826.

Foy, T. M., Laman, J. D., Ledbetter, J. A., Aruffo, A., Claassen, E., and Noelle, R. J. (1994). gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J. Exp. Med.* **180,** 157–163.

Foy, T. M., Shepherd, D. M., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J. Exp. Med.* **178,** 1567–1575.

Francis, D. A., Karras, J. G., Ke, X. Y., Sen, R., and Rothstein, T. L. (1995). Induction of the transcription factors NF-κB, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. *Int. Immunol.* **7,** 151–161.

Freudenthal, P. S., and Steinman, R. M. (1990). The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. USA* **87,** 7698–7702.

Fuchs, P., Strehl, S. D., M., Himmler, A., and Ambros, P. F. (1992). Structure of the TNF receptor 1 (p60) gene (TNFR1) and localization to chromosome 12p13. *Genomics* **13,** 219–224.

Fuleihan, R., Ramesh, N., Horner, A., Ahern, D., Belshaw, P. J., Alberg, D. G., Stamenkovic, I., Harmon, W., and Geha, R. S. (1994). Cyclosporin A inhibits CD40 ligand expression in T lymphocytes. *J. Clin. Invest.* **93,** 1315–1320.

Fuleihan, R., Ramesh, N., Loh, R., Jabara, H., Rosen, F. S., Chatila, T., Fu, S.-M., Stamenkovic, I., and Geha, R. S. (1993). Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc. Natl. Acad. Sci. USA* **90,** 2170–2173.

Funakoshi, S., Longo, D. L., Beckwith, M., Conley, D. K., Tsarfaty, G., Tsarfaty, I., Armitage, R. J., Fanslow, W. C., Spriggs, M. K., and Murphy, W. J. (1994). Inhibition of human B-cell lymphoma growth by CD40 stimulation. *Blood* **83,** 2787–2794.

Galibert, L., Durand, I., Rousset, F., and Banchereau, J. (1994). CD40 activated surface IgD positive lymphocytes constitute the long term IL-4 dependent proliferating B cell pool. *J. Immunol.* **152,** 22–29.

Galibert, L., Van Dooren, J., Durand, I., Rousset, F., Jefferis, R., Banchereau, J., and Lebecque, S. (1995). Anti-CD40 plus interleukin-4-activated human naive B cell lines express unmutated immunoglobulin genes with intraclonal heavy chain isotype variability. *Eur. J. Immunol.* **25,** 733–737.

Galibert, L., Burdin, N., de Saint-Vis, B., Garrone, P., van Kooten, C., Banchereau, J., and Rousset, F. (1996). CD40 and BCR dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. *J. Exp. Med.*, in press.

Galy, A. H., and Spits, H. (1992). CD40 is functionally expressed on human thymic epithelial cells. *J. Immunol.* **149,** 775–782.

Garrone, P., Neidhardt, E. M., Garcia, E., Galibert, L., van Kooten, C., and Banchereau, J. (1995). Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.*, in press.

Gauchat, J.-F., Aubry, J.-P., Mazzei, G., Life, P., Jomotte, T., Elson, G., and Bonnefoy, J.-Y. (1993a). Human CD40-ligand: Molecular cloning, cellular distribution and regulation of expression by factors controlling IgE production. *FEBS Lett.* **3,** 259–266.

Gauchat, J.-F., Henchoz, S., Fattah, D., Mazzei, G., Aubry, J.-P., Jomotte, T., Dash, L., Page, K., Solari, R., Aldebert, D., Capron, M., Dahinden, C., and Bonnefoy, J.-Y. (1995). CD40 ligand is functionally expressed on human eosinophils. *Eur. J. Immunol.* **25,** 863–865.

Gauchat, J.-F., Henchoz, S., Mazzei, G., Aubry, J.-P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J., Kishi, K., Butterfield, J., Dahinden, C., and Bonnefoy, J.-Y. (1993b). Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* **365,** 340–343.

Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Nayee, P., Owen, K., Patel, S., Thomas, W., Wells, G., Wood, L. M., and Woodley, K. (1994). Processing of tumour necrosis factor-α precursor by metalloproteinases. *Nature* **370,** 555–557.

Godfrey, W. R., Fagnoni, F. F., Harara, M. A., Buck, D., and Engleman, E. G. (1994). Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. *J. Exp. Med.* **180,** 757–762.

Gooding, L. R. (1992). Virus proteins that counteract host immune defenses. *Cell* **71,** 5–7.

Goodwin, R. G., Alderson, M. R., Smith, C. A., Armitage, R. J., Van den Bos, T., Jerzy, R., Tough, T. W., Schoenborn, M. A., Davis-Smith, T., Hennen, K., Falk, B., Cosman,

D., Baker, E., Sutherland, G. R., Grabstein, K. H., Farrah, T., Giri, J. G., and Beckmann, M. P. (1993a). Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to Tumor Necrosis Factor. *Cell* **73,** 447–456.

Goodwin, R. G., Anderson, D., Jerzy, R., Davis, T., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Smith, C. A. (1991). Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. *Mol. Cell. Biol.* **11,** 3020–3026.

Goodwin, R. G., Din, W. S., Davis-Smith, T., Anderson, D. M., Gimpel, S. D., Sato, T. A., Maliszewski, C. R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., Farrah, T., Armitage, R. J., Fanslow, W. C., and Smith, C. A. (1993b). Molecular cloning of a ligand for the inducible T cell gene 4-1BB: A member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur. J. Immunol.* **23,** 2631–2641.

Gordon, J., Millsum, M. J., Guy, G. R., and Ledbetter, J. A. (1987). Synergistic interaction between interleukin 4 and anti-Bp50 (CDw40) revealed in a novel B cell restimulation assay. *Eur. J. Immunol.* **17,** 1535–1538.

Gordon, J., Millsum, M. J., Guy, G. R., and Ledbetter, J. A. (1988). Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. A comparison with IL-4 mediated signaling. *J. Immunol.* **140,** 1425–1430.

Grabstein, K. H., Maliszewski, C. R., Shanebeck, K., Sato, T. A., Sprigg, M. K., Fanslow, W. C., and Armitage, R. J. (1993). The regulation of T cell-dependent antibody formation in vitro by CD40 ligand and IL-2. *J. Immunol.* **150,** 3141–3147.

Graf, D., Korthäuer, U., Mages, H. W., Senger, G., and Kroczek, R. A. (1992). Cloning of TRAP, a ligand for CD40 on human T cells. *Eur. J. Immunol.* **22,** 3191–3194.

Graf, D., Müller, S., van Kooten, C., Weise, C., and Kroczek, R. A. (1995). A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation. *Eur. J. Immunol.* **25,** 1749–1754.

Grammer, A. C., Bergman, M. C., Miura, Y., Fujita, K., Davis, L. S., and Lipsky, P. E. (1995). The CD40 ligand expressed by human B cells costimulates B cells responses. *J. Immunol.* **154,** 4996–5010.

Gravestein, L. A., Blom, B., Nolten, L. A., de Vries, E., van der Horst, G., Ossendorp, F., Borst, J., and Loenen, W. A. (1993). Cloning and expression of murine CD27: Comparison with 4-1BB, another lymphocyte-specific member of the nerve growth factor receptor family. *Eur. J. Immunol.* **23,** 943–950.

Gray, D., Dullforce, P., and Jainandunsing, S. (1994). Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. *J. Exp. Med.* **180,** 141–155.

Gray, D., Kosco, M., and Stockinger, B. (1991). Novel pathways of antigen presentation for the maintenance of memory. *Int. Immunol.* **3,** 141–148.

Gray, P. W., Aggarwal, B. B., Benton, C. V., Bringman, T. S., Henzel, W. J., Jarret, J. A., Leung, D. W., Moffat, B., Ng, P., Svedersky, L. P., Palladino, M. A., and Nedwin, G. E. (1984). Cloning and expression of cDNA for human lymphotoxin, lymphokine with tumor necrosis activity. *Nature* **312,** 721–724.

Gregory, C. D., Dive, C., Henderson, S., Smith, C. A., Williams, G. T., Gordon, J., and Rickinson, A. B. (1991). Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* **349,** 612–614.

Grimaldi, J. C., R., T., Kozak, C. A., Chang, R., Clark, E. A., Howard, M., and Cockayne, D. A. (1992). Genomic structure and chromosomal mapping of the murine CD40 gene. *J. Immunol.* **149,** 3921–3926.

Gruber, M. F., Bjorndahl, J. M., Nakamura, S., and Fu, S. M. (1989). Anti-CD45 inhibition of human B cell proliferation depends on the nature of activation signals and the state of B cell activation. *J. Immunol.* **142,** 4144–4152.

Gruss, H. J., Hirschstein, D., Wright, B., Ulrich, D., Caligiuri, M. A., Barcos, M., Strockbine, L., Armitage, R. J., and Dower, S. K. (1994). Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease. *Blood* **84,** 2305–2314.

Guinan, E. C., Bribben, J. G., Boussiotis, V. A., Freeman, G. J., and Nadler, L. M. (1994). Pivotal role of the B7: CD28 pathway in transplantation tolerance and tumor immunity. *Blood* **10,** 3261–3282.

Haluska, F. G., Brufsky, A. M., and Canellos, G. P. (1994). The cellular biology of the Reed-Sternberg cell. *Blood* **84,** 1005–1019.

Hamilton, M. S., Ball, J., Bromidge, E., and Franklin, I. M. (1991). Surface antigen expression of human neoplastic plasma cells includes molecules associated with lymphocyte recirculation and adhesion. *Br. J. Hematol.* **78,** 60–65.

Hasbold, J., and Klaus, G. G. (1994). B cells from CBA/N mice do not proliferate following ligation of CD40. *Eur. J. Immunol.* **24,** 152–157.

Heaney, M. L., and Golde, D. W. (1993). Soluble hormone receptors. *Blood* **82,** 1945–1948. [Editorial]

Heller, R. A., and Krönke, M. (1994). Tumor necrosis factor receptor-mediated signaling pathways. *J. Cell Biol.* **126,** 5–9.

Hermann, P., Blanchard, D., de Saint-Vis, B., Fossiez, F., Gaillard, C., Vanbervliet, B., Brière, F., Banchereau, J., and Galizzi, J.-P. (1993). Expression of a 32 kD ligand for the CD40 antigen on activated human T lymphocytes. *Eur. J. Immunol.* **23,** 961–964.

Hill, A., and Chapel, N. (1993). X-linked immunodeficiency—The fruits of cooperation. *Nature* **361,** 494.

Hintzen, R. Q., de Jong, R., Hack, C. E., Chamuleau, M., de Vries, E. F. R., Ten Berge, I. J. M., Borst, J., and van Lier, R. A. W. (1991). A soluble form of the human T cell differentiation antigen CD27 is released after triggering of the TCR/CD3 complex. *J. Immunol.* **147,** 29–35.

Hintzen, R. Q., de Jong, R., Lens, S. M., and van Lier, R. A. (1994). CD27: Marker and mediator of T-cell activation? *Immunol. Today* **15,** 307–311.

Holder, M. J., Wang, H., Milner, A. E., Casamayor, M., Armitage, R., Spriggs, M. K., Fanslow, W. C., MacLennan, I. C. M., Gregory, C. D., and Gordon, J. (1993). Suppression of apoptosis in normal and neoplastic human B lymphocytes by CD40 ligand is independent of Bcl-2 induction. *Eur. J. Immunol.* **23,** 2368–2371.

Hollenbaugh, D., Grosmaire, L. S., Kullas, C. D., Chalupny, N. J., Braesch-Andersen, S., Noelle, R. J., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992). The human T antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: Expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J.* **11,** 4313–4321.

Hollenbaugh, D., Mischel-Petty, N., Edwards, C., Simon, J. C., Denfeld, R. W., Kilner, P. A., and Aruffo, A. (1995). Expression of functional CD40 by vascular endothelial cells. *J. Exp. Med.* **182,** 33–40.

Hollenbaugh, D., Ochs, H. D., Noelle, R. J., Ledbetter, J. A., and Aruffo, A. (1994). The role of CD40 and its ligand in the regulation of the immune response. *Immunol. Rev.* **138,** 23–37.

Horner, A. A., Jabara, H., Ramesh, N., and Geha, R. S. (1995). Gamma/Delta T lymphocytes express CD40 ligand and induce isotype switching in B lymphocytes. *J. Exp. Med.* **181,** 1239–1244.

Howard, S. T., Chan, Y. S., and Smith, G. L. (1991). Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* **180,** 633–647.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell* **81,** 495–504.

Hu, F.-Q., and Pickup, D. J. (1991). Transcription of the terminal loop region of vaccinia virus DNA is initiated from telomere sequences directing DNA resolution. *Virology* **181,** 716–720.

Hu, H. M., O'Rourke, K., Boguski, M. S., and Dixit, V. M. (1994). A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J. Biol. Chem.* **269,** 30069–30072.

Huebner, K., Isobe, M., Chao, M., Bothwell, M., Ross, A. H., Finan, J., Hoxie, J. A., Sehgal, A., Buck, C. R., Lanahan, A., Nowell, P. C., Koprowski, H., and Croce, C. M. (1986). The nerve growth factor receptor gene is at human chromosome region 17q12-17q22, distal to the chromosome 17 breakpoint in acute leukemias. *Proc. Natl. Acad. Sci. USA* **83,** 1403–1407.

Ibanez, C. F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992). Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. *Cell* **69,** 329–341.

Inazawa, J., Itoh, N., Abe, T., and Nagata, S. (1992). Assignment of the human Fas antigen gene (Fas) to 10q24.1. *Genomics* **14,** 821–822.

Indzhiia, L. V., Yakovleva, L. A., Overbaugh, J., Licciardi, K. A., Chikobava, M. G., Klotz, I. N., Torres, R., Indzhiia, V. O., Lapin, B. A., Clark, E. A., and Valentine, M. A. (1992). Baboon T cell lymphomas expressing the B cell-associated surface proteins CD40 and Bgp95. *J. Clin. Immunol.* **12,** 225–236.

Inui, S., Kaisho, T., Kikutani, H., Stamenkovic, I., Seed, B., Clark, E. A., and Kishimoto, T. (1990). Identification of the intracytoplasmic region essential for signal transduction through a B cell activation molecule, CD40. *Eur. J. Immunol.* **20,** 1747–1753.

Islam, K. B., Nilsson, L., Sideras, P., Hammarström, L., and Smith, C. I. E. (1991). TGF-β1 induces germ-line transcripts of both IgA subclasses in human B lymphocytes. *Int. Immunol.* **3,** 1099–1106.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J. Biol. Chem.* **268,** 10932–10937.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S. I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66,** 233–243.

Jaattela, M. (1991). Biology of disease. Biologic activities and mechanisms of action of tumor necrosis factor-α/cachectin. *Lab. Invest.* **64,** 724–742.

Jabara, H. H., Fu, S. M., Geha, R. S., and Vercelli, D. (1990). CD40 and IgE: Synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* **172,** 1861–1864.

Jackson, N., Ling, N. R., Ball, J., Bromidge, E., Nathan, P. D., and Franklin, I. M. (1988). An analysis of myeloma plasma cell phenotype using antibodies defined at the IIIrd International Workshop on Human Leucocyte Differentiation Antigens. *Clin. Exp. Immunol.* **72,** 351–356.

Jacob, J., and Kelsoe, G. (1992). *In situ* studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. *J. Exp. Med.* **176,** 679–687.

Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986). Expression and structure of the human NGF receptor. *Cell* **47,** 545–554.

Johnson, P. W. M., Watt, S. M., Betts, D. R., Davies, D., Jordan, S., Norton, A. J., and Lister, T. A. (1993). Isolated follicular lymphoma cells are resistant to apoptosis and can be grown in vitro in the CD40/stromal cell system. *Blood* **82,** 1848–1857.

Jones, E. Y., Stuart, D. I., and Walker, N. P. C. (1989). Structure of tumor necrosis factor. *Nature* **338,** 225–228.

Jones, E. Y., Stuart, D. I., and Walker, N. P. C. (1992). Crystal structure of TNF. *In* "Tumor Necrosis Factors-Structure, Function and Mechanism of Action" (B. B. Aggarwal and J. Vilcek, Eds.). Marcel Dekker, New York.

Josimovic-Alasevic, O., Dürkop, H., Schwarting, R., Backé, E., Stein, H., and Diamantstein, T. (1989). Ki-1 (CD30) antigen is released by K1-1-positive tumor cells *in vitro* and *in vivo*. I. Partial characterization of soluble Ki-1 antigen and detection of the antigen in cell culture supernatants and in serum by an enzyme-linked immunosorbent assay. *Eur. J. Immunol.* **19,** 157–162.

Jumper, M. D., Splawski, J. B., Lipsky, P. E., and Meek, K. (1994). Ligation of CD40 induces sterile transcripts of multiple Ig heavy chain isotypes in human B cells. *J. Immunol.* **152,** 438–445.

Kansas, G. S., and Tedder, T. F. (1991). Transmembrane signals generated through MHC Class II, CD19, CD20, and CD40 antigens induce LFA-1-dependent and independent adhesion in human B cells through a tyrosine kinase-dependent pathway. *J. Immunol.* **147,** 4094–4102.

Kansas, G. S., Wood, G. S., and Tedder, T. F. (1991). Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes. *J. Immunol.* **146,** 2235–2244.

Karmann, K., Hughes, C. C. W., Schechner, J., Fanslow, W. C., and Pober, J. S. (1995). CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc. Natl. Acad. Sci. USA* **92,** 4342–4346.

Kato, Y., Salter, C. L., Flajnik, M. F., Kasahara, M., Namikawa, C., Sasaki, M., and Nonaka, M. (1994). Isolation of the Xenopus complement factor B complementary DNA and linkage of the gene to the frog MHC. *J. Immunol.* **153,** 4546–4554.

Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T., and Kikutani, H. (1994). The immune response in CD40-deficient mice: Impaired immunoglobulin class switching and germinal center formation. *Immunity* **1,** 167–178.

Kemper, O., Derre, J., Cherif, D., Engelmann, H., Wallach, D., and Berger, R. (1991). The gene for the type II (p75) tumor necrosis factor receptor (TNF-RII) is localized on band 1p36.2-p36.3. *Hum. Genet.* **87,** 623–624.

Kennedy, I. C., Hart, D. N., Colls, B. M., Nimmo, J. C., Willis, D. A., and Angus, H. B. (1989). Nodular sclerosing, mixed cellularity and lymphocyte-depleted variants of Hodgkin's disease are probable dendritic cell malignancies. *Clin. Exp. Immunol.* **76,** 324–331.

Kim, H.-S., Zhang, X., and Choi, Y. S. (1994). Activation and proliferation of follicular dendritic cell-like cells by activated T lymphocytes. *J. Immunol.* **153,** 2951–2961.

Klaus, G. G., Choi, M. S., and Holman, M. (1994a). Properties of mouse CD40. Ligation of CD40 activates B cells via a Ca(++)-dependent, FK506-sensitive pathway. *Eur. J. Immunol.* **24,** 3229–3232.

Klaus, S. J., Pinchuk, L. M., Ochs, H. D., Law, C. L., Fanslow, W. C., Armitage, R. J., and Clark, E. A. (1994b). Costimulation through CD28 enhances T cell-dependent B cell activation via CD40-CD40L interaction. *J. Immunol.* **152,** 5643–5652.

Kluin-Nelemans, H. C., Beverstock, G. C., Mollevanger, P., Wessels, H. W., Hoogendoorn, E., Willemze, R., and Falkenburg, J. H. (1994). Proliferation and cytogenetic analysis of hairy cell leukemia upon stimulation via the CD40 antigen. *Blood* **84,** 3134–3141.

Knipping, E., Debatin, K. M., Stricker, K., Heilig, B., Eder, A., and Krammer, P. H. (1995). Identification of soluble APO-1 in supernatants of human B- and T-cell lines and increased serum levels in B- and T-cell leukemias. *Blood* **85,** 1562–1569.

Knox, K. A., and Gordon, J. (1993). Protein tyrosine phosphorylation is mandatory for CD40-mediated rescue of germinal center B cells from apoptosis. *Eur. J. Immunol.* **23,** 2578–2584.

Koho, H., Paulie, S., Ben-Aissa, H., Jonsdottir, I., Hansson, Y., Lundblad, M. L., and Perlman, P. (1984). Monoclonal antibodies to antigens associated with transitional cell carcinoma of the human urinary bladder. I. Determination of the selectivity of six antibodies by cell ELISA and immunofluorescence. *Cancer Immunol. Immunother.* **17,** 165–172.

Kolesnick, R., and Golde, D. W. (1994). The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77,** 325–328.

Korthäuer, U., Graf, D., Mages, H. W., Brière, F., Padayachee, M., Malcolm, S., Ugazio, A. G., Notarangelo, L. D., Levinsky, R. J., and Kroczek, R. A. (1993). Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* **361,** 539–541.

Kosco, M. H., Burton, G. F., Kapasi, Z. F., Szakal, A. K., and Tew, J. G. (1989). Antibody-forming cell induction during an early phase of germinal centre development and its delay with ageing. *Immunology* **68,** 312–318.

Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S. D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* **53,** 45–53.

Kroczek, R. A., Graf, D., Brugnoni, D., Gilliani, S., Korthuer, U., Ugazio, A., Senger, G., Mages, H. W., Villa, A., and Notarangelo, L. D. (1994). Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)." *Immunol. Rev.* **138,** 39–59.

Kwekkeboom, J., de Rijk, D., Kasran, A., Barcy, S., de Groot, C., and de Boer, M. (1994). Helper effector function of human T cells stimulated by anti-CD3 mAb can be enhanced by co-stimulatory signals and is partially dependent on CD40-CD40 ligand interaction. *Eur. J. Immunol.* **24,** 508–517.

Kwon, B. S., Kozak, C. A., Kim, K. K., and Pickard, R. T. (1994). Genomic organization and chromosomal localization of the T-cell antigen 4-1BB. *J. Immunol.* **152,** 2256–2262.

Kwon, B. S., and Weissman, S. M. (1989). cDNA sequences of two inducible T-cell genes. *Proc. Natl. Acad. Sci. USA* **86,** 1963–1967.

Lafage-Pochitaloff, M., Hermann, P., Birg, F., Galizzi, J. P., Simonetti, J., Mannoni, P., and Banchereau, J. (1994). Localization of the human CD40 gene to chromosome 20, bands q12-q13.2. *Leukemia* **8,** 1172–1175.

Lagresle, C., Bella, C., Daniel, T., Krammer, P. H., and Defrance, T. (1995). Regulation of germinal center B cell differentiation. Role of the human APO-1/Fas (CD95) molecule. *J. Immunol.* **154,** 5746–5756.

Lagresle, C., Bella, C., and Defrance, T. (1993). Phenotypic and functional heterogeneity of the IgD^- B cell compartment: Identification of two major tonsillar B cell subsets. *Int. Immunol.* **5,** 1259–1268.

Lalmanach-Girard, A. C., Chiles, T. C., Parker, D. C., and Rothstein, T. L. (1993). T cell-dependent induction of NF-kappa B in B cells. *J. Exp. Med.* **177,** 1215–1219.

Lane, P., Burdet, C., Hubele, S., Schneidegger, D., Muller, U., McConnell, F., and Kosco-Vilbois, M. (1994). B cell function in mice transgenic for mCTLA4-H gamma 1: Lack of germinal centers correlated with poor affinity maturation and class switching despite normal priming of CD4+ T cells. *J. Exp. Med.* **179,** 819–830.

Lane, P., Burdet, C., McConnell, F., Lanzavecchia, A., and Padovan, E. (1995). CD40 ligand-independent B cell activation revealed by CD40 ligand-deficient T cell clones: Evidence for distinct activation requirements for antibody formation and B cell proliferation. *Eur. J. Immunol.* **25,** 1788–1793.

Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A., and Gray, D. (1992). Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur. J. Immunol.* **22,** 2573–2578.

Latza, U., Durkop, H., Schnittger, S., Ringeling, J., Eitelbach, F., Hummel, M., Fonatsch, C., and Stein, H. (1994). The human OX40 homolog: cDNA structure, expression and chromosomal assignment of the ACT35 antigen. *Eur. J. Immunol.* **24,** 677–683.

Law, C. L., Wörmann, B., and LeBien, T. W. (1990). Analysis of expression and function of CD40 on normal and leukemic human B cell precursors. *Leukemia* **4,** 732–738.

Lebman, D. A., Nomura, D. Y., Coffman, R. L., and Lee, F. D. (1990). Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching. *Proc. Natl. Acad. Sci. USA* **87,** 3962–3966.

Ledbetter, J. A., Clark, E. A., Norris, N. A., Shu, G., and Hellström, I. (1987a). Expression of a functional B-cell receptor CDW40 (Bp50) on carcinomas. *In* "Leukocyte Typing III. White Cell Differentiation Antigens" (A. J. McMichael *et al.*, Eds.), pp. 432–435. Oxford Univ. Press, Oxford.

Ledbetter, J. A., Shu, G., Gallagher, M., and Clark, E. A. (1987b). Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen BP50 (CDw40). *J. Immunol.* **138,** 788–794.

Lederman, S., Yellin, M. J., Cleary, A. M., Pernis, A., Inghirami, G., Cohn, L. E., Covey, L. R., Lee, J. J., Rothman, P., and Chess, L. (1994). T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death. *J. Immunol.* **152,** 2163–2171.

Lederman, S., Yellin, M. J., Inghirami, G., Lee, J. J., Knowles, D. M., and Chess, L. (1992a). Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J. Immunol.* **149,** 3817–3826.

Lederman, S., Yellin, M. J., Krichevsky, A., Belko, J., Lee, J. J., and Chess, L. (1992b). Identification of a novel surface protein on activated $CD4^+$ T cells that induces contact-dependent B cell differentiation (Help). *J. Exp. Med.* **175,** 1091–1101.

Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H. W., Chen, E. Y., and Goeddel, D. V. (1991). Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc. Natl. Acad. Sci. USA* **88,** 2830–2834.

Lichter, P., Walczak, H., Weitz, S., Behrmann, I., and Krammer, P. H. (1992). The human APO-1 (APT) antigen maps to 10q23, a region that is syntenic with mouse chromosome 19. *Genomics* **14,** 179–180.

Life, P., Gauchat, J.-F., Schnuriger, V., Estoppey, S., Mazzei, G., Durandy, A., Fischer, A., and Bonnefoy, J.-Y. (1994). T cell clones from an X-linked hyper-immunoglobulin (IgM) patient induce IgE synthesis in vitro despite expression of nonfunctional CD40 ligand. *J. Exp. Med.* **180,** 177–1784.

Lindhout, E., Lakeman, A., and de Groot, C. (1995). Follicular dendritic cells inhibit apoptosis in human B lymphocytes by a rapid and irreversible blockade of preexisting endonuclease. *J. Exp. Med.* **101,** 1985–1995.

Lindhout, E., Lakeman, A., Mevissen, M. L. C. M., and de Groot, C. (1994). Functionally active Esptein-Barr virus-transformed follicular dendritic cell-like cell lines. *J. Exp. Med.* **179,** 1173–1184.

Ling, N. R., MacLennan, I. C. M., and Mason, D. (1987). B-cell and plasma cell antigens: New and previously defined clusters. *In* "Leucocyte Typing III. White Cell Differentiation Antigens" (A. J. McMichael *et al.*, Eds.), pp. 302–335. Oxford University Press, Oxford.

Liu, Y.-J., Barthelemy, C., de Bouteiller, O., Arpin, C., Durand, I., and Banchereau, J. (1995). Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid upregulation of B7.1 and B7.2. *Immunity* **2,** 238–248.

Liu, Y. J., Joshua, D. E., Williams, G. T., Smith, C. A., Gordon, J., and MacLennan, I. C. M. (1989). Mechanisms of antigen-driven selection in germinal centers. *Nature* **342,** 929–931.

Loenen, W. A. M., de Vries, E., Gravestein, L. A., Hintzen, R. Q., van Lier, R. A. W., and Borst, J. (1992a). The CD27 membrane receptor, a lymphocyte-specific member of the nerve growth factor receptor family, gives rise to a soluble form by protein processing that does not involve receptor endocytosis. *Eur. J. Immunol.* **22,** 447–455.

Loenen, W. A. M., Gravestein, L. A., Beumer, S., Melief, C. J. M., Hagemeijer, A., and Borst, J. (1992b). Genomic organization and chromosomal localization of the human CD27 gene. *J. Immunol.* **149,** 3937–3943.

Loetscher, H., Pan, Y. C. E., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. (1990). Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell* **61,** 351–359.

Lynch, D. H., Watson, M. L., Alderson, M. R., Baum, P. R., Miller, R. E., Tough, T., Gibson, M., Davis-Smith, T., Smith, C. A., Hunter, K., Bhat, D., Din, W., Goodwin, R. G., and Seldin, M. F. (1994). The mouse Fas-ligand gene is mutated in gld mice and is part of a TNF family gene cluster. *Immunity* **1,** 131–136.

Macchia, D., Almerigogna, F., Parronchi, P., Ravina, A., Maggi, E., and Romagnani, S. (1993). Membrane tumor necrosis factor-α is involved in the polyclonal B-cell activation induced by HIV-infected human T cells. *Nature* **363,** 464–466.

MacLennan, I. C. M. (1994). Germinal centers. *Annu. Rev. Immunol.* **12,** 117–139.

Maliszewski, C. R., Grabstein, K., Fanslow, W. C., Armitage, R., Spriggs, M. K., and Sato, T. A. (1993). Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: Cooperative effects of cytokines. *Eur. J. Immunol.* **23,** 1044–1049.

Mallett, S., and Barclay, A. N. (1991). A new superfamily of cell surface proteins related to the nerve growth factor receptor. *Immunol. Today* **12,** 220–222.

Mallett, S., Fossum, S., and Barclay, A. N. (1990). Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule related to nerve growth factor receptor. *EMBO J.* **9,** 1063–1068.

Marshall, L. S., Shepherd, D. M., Ledbetter, J. A., Aruffo, A., and Noelle, R. J. (1994). Signaling events during helper T cell-dependent B cell activation. I. Analysis of the signal transduction pathways triggered by activated helper T cell in resting B cells. *J. Immunol.* **152,** 4816–4825.

Mayer, L., Kwan, S. P., Thompson, C., Ko, H. S., Chiorazzi, N., Waldmann, T., and Rosen, F. (1986). Evidence of a defect in switch T cells in patients with immunodeficiency and hyperimmunoglobulinemia M. *N. Engl. J. Med.* **314,** 409–413.

Mazzei, G. J., Edgerton, M. D., Losberger, C., Lecoanet-Henchoz, S., Graber, P., Durandy, A., Gauchat, J. F., Bernard, A., Allet, B., and Bonnefoy, J. Y. (1995). Recombinant soluble trimeric CD40 ligand is biologically active. *J. Biol. Chem.* **270,** 7025–7028.

McGeehan, G. M., Becherer, J. D., Bast, R. C., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghese, M., Warner, J., and Ways, J. P. (1994). Regulation of tumour necrosis factor-α processing by a metalloproteinase inhibitor. *Nature* **370,** 558–561.

McKenzie, A. N. J., Culpepper, J. A., de Waal Malefyt, R., Brière, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S., de Vries, J. E., Banchereau, J., and Zurawski, G. (1993). Interleukin-13, a novel T cell-derived cytokine that regulates human monocyte and B cell function. *Proc. Natl. Acad. Sci. USA* **90,** 3735–3739.

Merville, P., Dechanet, J., Desmoulière, A., Durand, I., de Bouteiller, O., Garrone, P., Banchereau, J., and Liu, Y.-J. (1995a). Bcl-2 positive tonsillar plasma cells are rescued from prompt apoptosis by bone marrow fibroblasts. *J. Exp. Med.*, in press.

Merville, P., Dechanet, J., Grouard, G., Durand, I., and Banchereau, J. (1995b). T cell-induced B cell blasts differentiate into plasma cells when cultured on bone marrow stroma with IL3 and IL-10. *Int. Immunol.* **7,** 635–643.

Minty, A., Chalon, P., Derocq, J.-M., Dumont, X., Guillemot, J.-C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P., and Caput, D. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* **362,** 248–250.

Miura, S., Ohtani, K., Numata, N., Niki, M., Ohbo, K., Ina, Y., Gojobori, T., Tanaka, Y., Tozawa, H., Nakamura, M., and Sugamura, K. (1991). Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40 *tax*. *Mol. Cell. Biol.* **11,** 1313–1325.

Mohan, C., Shi, Y., Laman, J. D., and Datta, S. K. (1995). Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J. Immunol.* **154,** 1470–1480.

Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994). Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* **370,** 218–220.

Möller, P., Henne, C., Leithäuser, F., Eichelmann, A., Schmidt, A., Brüderlein, S., Dhein, J., and Krammer, P. H. (1993). Coregulation of the APO-1 antigen with intercellular adhesion molecule-1 (CD54) in tonsillar B cells and coordinate expression in Follicular center B cells and in follicle center and mediastinal B-cell lymphomas. *Blood* **81,** 2067–2075.

Möller, P., and Mielke, B. (1989). Extensive analysis of tissue distribution of antigens defined by new clustered and unclustered B-cell antibodies. *In* "Leucocyte Typing IV. White Cell Differentiation Antigens" (W. Knapp *et al.*, Eds.), pp. 175–177. Oxford University Press, Oxford.

Moreau, I., Duvert, V., Banchereau, J., and Saeland, S. (1993). Culture of human fetal B-cell precursors on bone marrow stroma maintains highly proliferative CD20dim cells. *Blood* **81,** 1170–1178.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80,** 389–399.

Muller, U., Jongeneel, C. V., Nedospasov, S. A., Lindahl, K. F., and Steinmetz, M. (1987). Tumour necrosis factor and lymphotoxin genes map close to H-2D in the mouse major histocompatibility complex. *Nature* **325,** 265–267.

Nagata, S., and Golstein, P. (1995). The Fas death factor. *Science* **267,** 1449–1456.

Nishioka, Y., and Lipsky, P. E. (1994). The role of CD40-CD40 ligand interaction in human T cell-B cell collaboration. *J. Immunol.* **153,** 1027–1036.

Noelle, R. J., Ledbetter, J. A., and Aruffo, A. (1992a) CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. *Immunol. Today* **13,** 431–433.

Noelle, R. J., Roy, M., Shepherd, D. M., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992b). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA* **89,** 6550–6554.

Nonoyama, S., Penix, L. A., Edwards, C. P., Lewis, D. B., Ito, S., Aruffo, A., Wilson, C. B., and Ochs, H. D. (1995). Diminished expression of CD40 ligand by activated neonatal T cells. *J. Clin. Invest.* **95,** 66–75.

Notarangelo, L. D., Duse, M., and Ugazio, A. G. (1992). Immunodeficiency with hyper-IgM (HIM). *Immunodef. Rev.* **3,** 101–122.

Ochs, H. D., Davis, S. D., and Wedgwood, R. J. (1971). Immunologic responses to bacteriophage ø X174 in immunodeficiency diseases. *J. Clin. Invest.* **50,** 2559–2665.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., and Trauth, B. C. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor-nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.* **267,** 10709–10715.

O'Grady, J. T., Stewart, S., Lowrey, J., Howie, S. E., and Krajewski, A. S. (1994). CD40 expression in Hodgkin's disease. *Am. J. Pathol.* **144,** 21–26.

Padayachee, M., Feighery, C., Finn, A., McKeown, C., Levinsky, R. J., Kinnin, C., and Malcolm, S. (1992). Mapping the X-linked form of hyper-IgM syntrome (HIGMX1) to Xq26 by close linkage to HPRT. *Genomics* **14,** 551—553.

Parker, D. C. (1993). T cell-dependent B cell activation. *Annu. Rev. Immunol.* **11,** 331–360.

Parry, S. L., Hasbold, J., Holman, M., and Klaus, G. G. (1994). Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. *J. Immunol.* **152,** 2821–2829.

Pascual, V., Liu, Y.-J., Magalski, A., de Bouteiller, O., Banchereau, J., and Capra, J. D. (1994). Analysis of somatic mutation in five B cell subsets of human tonsil. *J. Exp. Med.* **180,** 329–339.

Paul, N. L., and Ruddle, N. H. (1988). Lymphotoxin. *Annu. Rev. Immunol.* **6,** 407–438.

Paulie, S., Ehlin-Henriksson, B., Mellstedt, H., Koho, H., Ben-Aissa, H., and Perlmann, P. (1985). A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. *Cancer Immunol. Immunother.* **20,** 23–28.

Paulie, S., Rosen, A., Ehlin-Henriksson, B., Braesch-Andersen, S., Jakobson, E., Koho, H., and Perlmann, P. (1989). The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction. *J. Immunol.* **142,** 590–595.

Peitsch, M. C., and Jongeneel, C. V. (1993). A 3-D model for the CD40 ligand predicts that is is a compact trimer similar to the tumor necrosis factors. *Int. Immunol.* **5,** 233–238.

Pellat-Deceunynck, C., Bataille, R., Robillard, N., Harousseau, J.-L., Rapp, M.-J., Juge-Morineau, N., Wijdenes, J., and Amiot, M. (1994). Expression of CD28 and CD40 in human myeloma cells: A comparative study with normal plasma cells. *Blood* **84,** 2597–2603.

Pène, J., Rousset, F., Brière, F., Chrétien, I., Bonnefoy, J. Y., Spits, H., Yokota, T., Arai, N., Arai, K. I., Banchereau, J., and De Vries, J. E. (1988a). IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc. Natl. Acad. Sci. USA* **85,** 6880–6884.

Pène, J., Rousset, F., Brière, F., Chrétien, I., Paliard, X., Banchereau, J., Spits, H., and De Vries, J. E. (1988b). IgE production by normal human B cells induced by alloreactive T cell clones is mediated by interleukin 4 and suppressed by interferon γ. *J. Immunol.* **141,** 1218–1224.

Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., Kohr, W. J., Aggarwal, B. B., and Goeddel, D. V. (1984). Human tumour necrosis factor: Precursor structure, expression and homology to lymphotoxin. *Nature* **312,** 724–729.

Pinchuk, L. M., Polacino, P. S., Agy, M. B., Klaus, S. J., and Clark, E. A. (1994). The role of CD40 and CD80 accessory cell molecules in dendritic cell-dependent HIV-1 infection. *Immunity* **1,** 317–325.

Porteu, F., and Nathan, C. (1990). Shedding of tumor necrosis factor receptors by activated human neutrophils. *J. Exp. Med.* **172,** 599–607.

Potocnik, A. J., Kinne, R., Menninger, H., Zacher, J., Emmrich, F., and Kroczek, R. A. (1990). Expression of activation antigens on T cells in rheumatoid arthritis patients. *Scand. J. Immunol.* **31,** 213–224.

Pulendran, B., Kannourakis, G., Nouri, S., Smith, K. G. C., and Nossal, G. J. V. (1995). Soluble antigen can cause enhanced apoptosis of germinal-centre B cells. *Nature* **375,** 331–334.

Punnonen, J., Aversa, G., and de Vries, J. E. (1993a). Human pre-B cells differentiate into Ig-secreting plasma cells in the presence of interleukin-4 and activated $CD4^+$ T cells or their membranes. *Blood* **82,** 2781–2789.

Punnonen, J., Aversa, G. G., Cocks, B. G., McKenzie, A. N. J., Menon, S., Zurawski, G., de Waal Malefyt, R., and de Vries, J. E. (1993b). Interleukin-13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acac. Sci. USA* **90,** 3730–3734.

Quilding-Järbrink, M., Lakew, M., Nordström, I., Banchereau, J., Butcher, E., Holmgren, J., and Czerkinsky, C. (1995). Human circulating specific antibody-forming cells after systemic and mucosal immunizations: Differential homing commitments and cell surface differentiation markers. *Eur. J. Immunol.* **25,** 322–327.

Raffioni, S., Bradshaw, R. A., and Buxser, S. E. (1993). The receptors for nerve growth factor and other neurotrophins. *Annu. Rev. Biochem.* **62,** 823–850.

Ramesh, N., Fuleihan, R., Ramesh, V., Lederman, S., Yellin, M. J., Sharma, S., Chess, L., Rosen, F. S., and Geha, R. S. (1993a). Deletions in the ligand for CD40 in X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1). *Int. Immunol.* **5,** 769–773.

Ramesh, N., Ramesh, V., Gusella, J. F., and Geha, R. (1993b). Chromosomal localization of the gene for human B-cell antigen CD40. *Somat. Cell Mol. Genet.* **19,** 295–298.

Ramsdell, F., Seaman, M. S., Clifford, K. N., and Fanslow, W. C. (1994a). CD40 ligand acts as a costimulatory signal for neonatal thymic gamma delta T cells. *J. Immunol.* **152,** 2190–2197.

Ramsdell, F., Seaman, M. S., Miller, R. E., Picha, K. S., Kennedy, M. K., and Lynch, D. H. (1994b). Differential ability of Th1 and Th2 T cells to express Fas ligand and to undergo activation-induced cell death. *Int. Immunol.* **6,** 1545–1553.

Ranheim, E. A., and Kipps, T. J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J. Exp. Med.* **177,** 925–935.

Ren, C. L., Morio, T., Fu, S. M., and Geha, R. S. (1994). Signal transduction via CD40 involves activation of lyn kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J. Exp. Med.* **179,** 673–680.

Renard, N., Duvert, V., Blanchard, D., Banchereau, J., and Saeland, S. (1994). Activated CD4+ T cells induce CD40 dependent proliferation of human B cell precursors. *J. Immunol.* **152,** 1693–1701.

Renshaw, B. R., Fanslow III, W. C., Armitage, R. J., Campbell, K. A., Liggitt, D., Wright, B., Davison, B. L., and Maliszewski, C. R. (1994). Humoral immune responses in CD40 ligand-deficient mice. *J. Exp. Med.* **180,** 1889–1900.

Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I. A. G., Debatin, K. M., Fischer, A., and de Villartay, J. P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* **268,** 1347–1349.

Roldan, E., and Brieva, J. A. (1991). Terminal differentiation of human bone marrow cells capable of spontaneous and high-rate immunoglobulin secretion: A role of bone marrow stromal cells and interleukin 6. *Eur. J. Immunol.* **21,** 2671–2677.

Romani, N., Gruner, S., Brang, D., Kämpgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. (1994). Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.

Romani, N., Lenz, A., Glassl, H., Stossel, H., Stanzl, U., Majdic, O., Fritsch, P., and Schuler, G. (1989). Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J. Invest. Dermatol.* **93,** 600–609.

Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78,** 681–692.

Rothstein, T. L., Wang, J. K. M., Panka, D. J., Foote, L. C., Wang, Z., Stanger, B., Cui, H., Ju, S.-T., and Marshak-Rothstein, A. (1995). Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature* **374,** 163–165.

Rousset, F., Garcia, E., and Banchereau, J. (1991). Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* **173,** 705–710.

Rousset, F., Garcia, E., Defrance, T., Péronne, C., Vezzio, N., Hsu, D. H., Kastelein, R., Moore, K. W., and Banchereau, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc. Natl. Acad. Sci. USA* **89,** 1890–1893.

Rousset, F., Peyrol, S., Garcia, E., Vezzio, N., Andujar, M., Grimaud, J.-A., and Banchereau, J. (1995). IL10 induces the differentiation of CD40-activated B lymphocytes into plasma cells. *Int. Immunol.*, **7,** 1243–1253.

Roy, M., Aruffo, A., Ledbetter, J., Linsley, P., Kehry, M., and Noelle, R. (1995). Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur. J. Immunol.* **25,** 596–603.

Roy, M., Waldschmidt, T., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). Regulation of the expression of gp^{39}, the CD40 ligand, on normal and cloned $CD4^+$ T cells. *J. Immunol.* **151,** 2497–2510.

Ruby, J., Bluethmann, H., Aguet, M., and Ramshaw, I. A. (1995). CD40 ligand has potent antiviral activity. *Nat. Med.* **1,** 437–441.

Saeland, S., Duvert, V., Caux, C., Pandrau, D., Favre, C., Vallé, A., Durand, I., Charbord, P., de Vries, J. E., and Banchereau, J. (1992). Distribution of surface-membrane molecules on bone marrow and cord blood $CD34^+$ hematopoietic cells. *Exp. Hematol.* **20,** 24–33.

Saeland, S., Duvert, V., Moreau, I., and Banchereau, J. (1993). Human B cell precursors proliferate and express CD23 after CD40 ligation. *J. Exp. Med.* **178,** 113–120.

Saiki, O., Tanaka, T., Wada, Y., Uda, H., Inoue, A., Katada, A., Izeki, M., Iwata, M., Nunoi, H., Matsuda, I., Kinoshita, N., and Kishimoto, T. (1995). Signaling through CD40 rescues IgE but not IgG or IgA secretion in X-linked immunodeficiency with hyper-IgM. *J. Clin. Invest.* **95,** 510–514.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.

Santos-Argumedo, L., Lund F. E., Heath, A. W., Solvason, N., Wu, W. W., Grimaldi, J. C., Parkhouse, R. M. E., and Howard, M. (1995). CD38 unresponsiveness of xid B cells implications Bruton's tyrosine kinase (btk) as a regulator of CD38 induced signal transduction. *Int. Immunol.* **7,** 163–170.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: A protein tyrosine phosphatase that associates with Fas. *Science* **268,** 411–415.

Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., *et al.* (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* **61,** 361–370.

Schattner, E., Elkon, K. B., Yoo, D. H., Tumang, J., Krammer, P., Crow, M. K., and Friedman, S. M. (1995). CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. *J. Exp. Med.*, in press.

Schriever, F., Freedman, A. S., Freeman, G., Messner, E., Lee, G., Daley, J., and Nadler, L. M. (1989). Isolated follicular dendritic cells display a unique antigenic phenotype. *J. Exp. Med.* **169,** 2043–2048.

Schwartz, R. H. (1992). Costimulation of T lymphocytes: The role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **71,** 1065–1068.

Schwarz, H., Tuckwell, J., and Lotz, M. (1993). A receptor induced by lymphocyte activation (ILA) is a new member of the human nerve growth factor/tumor necrosis factor receptor family. *Gene* **134,** 295–298.

Schwarz, H., Valbracht, J., Tuckwell, J., von Kempis, J., and Lotz, M. (1995). ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages. *Blood* **85,** 1043–1052.

Servet-Delprat, C., Bridon, J. M., Blanchard, D., Banchereau, J., and Brière, F. (1995). CD40-activated human naive surface IgD+ B cells produce IgG2 in response to activated T cell supernatant. *Immunology* **85,** 435–441.

Shchelkunov, S. N., Blinov, V. M., and Sandakhchiev, L. S. (1993). Genes of variola and vaccinia viruses necessary to overcome the host protective mechanisms. *FEBS Lett.* **319,** 80–83.

Shokat, K. M., and Goodnow, C. C. (1995). Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature* **375,** 334–338.

Shu, U., Kiniwa, M., Wu, C. Y., Maliszewski, C., Vezzio, N., Hakimi, J., Gately, M., and Delespesse, G. (1995). Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur. J. Immunol.* **25,** 1125–1128.

Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G., and Goodwin, R. G. (1991a). T2 open reading frame from the Shope Fibroma virsus encodes a soluble form of the TNF receptor. *Biochem. Biophys. Res. Commun.* **176,** 335–342.

Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). The TNF receptor superfamily of cellular and viral proteins: Activation, costimulation and death. *Cell* **76,** 959–962.

Smith, C. A., Gruss, H.-J., Davis, T., Anderson, D., Farrah, T., Baker, E., Sutherland, G. R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., Grabstein, K. H., Gliniak, B., McAlister, I. B., Fanslow, W., Alderson, M., Falk, B., Gimpel, S., Gillis, S., Din, W. S., Goodwin, R. G., and Armitage, R. J. (1993a). CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines a merging family of cytokines with homology to TNF. *Cell* **73,** 1349–1360.

Smith, C. I. E., Vorechovsky, I., Hammarstrom, L., Islam, K. B., Vetrie, D. and Sideras, P. (1993b). The molecular nature of immunoglobulin deficiencies—tyrosine kinase abnormalities and other mechanisms. *Immunologist* **1,** 81–88.

Smith, J. C., Ellenberger, H. H., Ballanyi, K., Richter, D. W., and Feldeman, J. L. (1991b). Pre-Botzinger complex: A brainstem region that may generate respiratory rhythm in mammals. *Science* **254,** 726–729.

Smith, K. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* **248,** 1019–1022.

Spies, T., Morton, C. C., Nedospasov, S. A., Fiers, W., Pious, D., and Strominger, J. L. (1986). Genes for the tumor necrosis factor alpha and beta are linked to the human major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* **83,** 8699–8702.

Spriggs, M. K., Armitage, R. J., Strockbine, L., Clifford, K. N., Macduff, B. M., Sato, T. A., Malisewski, C. R., and Fanslow, W. C. (1992). Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. *J. Exp. Med.* **176,** 1543–1550.

Stamenkovic, I., Clark, E. A., and Seed, B. (1989). A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J.* **8,** 1403–1410.

Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995). RIP: A novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* **81,** 513–523.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.

Stüber, E., Neurath, M., Calderhead, D., Fell, H. P., and Strober, W. (1995). Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* **2,** 507–521.

Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995). Expression of the Fas ligand in cells of T cell lineage. *J. Immunol.* **154,** 3806–3813.

Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the Tumor Necrosis Factor family. *Cell* **75,** 1169–1178.

Sumimoto, S., Heike, T., Kanazashi, S., Shintaku, N., Jung, E. Y., Hata, D., Katamura, K., and Mayumi, M. (1994). Involvement of LFA-1/intracellular adhesion molecule-1-dependent cell adhesion in CD40-mediated inhibition of human B lymphoma cell death induced by surface IgM crosslinking. *J. Immunol.* **153,** 2488–2496.

Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T., and Nagata, S. (1994a). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76,** 969–976.

Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T., and Nagata, S. (1994b). Human Fas ligand: Gene structure, chromosomal location and species specificity. *Int. Immunol.* **6,** 1567–1574.

Tamaru, J., Hummel, M., Zemlin, M., Kalvelage, B., and Stein, H. (1994). Hodgkin's disease with a B-cell phenotype often shows a VDJ rearrangement and somatic mutations in the VH genes. *Blood* **84,** 708–715.

Tanaka, T., Tanaka, K., Ogawa, S., Kurokawa, M., Mitani, K., Nishida, J., Shibata, Y., Yazaki, Y., and Hirai, H. (1995). An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. *EMBO J.* **14,** 341–350.

Taniguchi, T. (1995). Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* **268,** 251–255.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P. J., Duddy, L. P., Rajewsky, K., and Tybulewicz, V. L. J. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of *Vav*. *Nature* **374,** 467–470.

Tartaglia, L. A., and Goeddel, D. V. (1992). Two TNF receptors. *Immunol. Today* **13,** 151–153.

Tartaglia, L. A., Rothe, M., Hu, Y. F., and Goeddel, D. V. (1993). Tumor necrosis factors's cytotoxic activity is signaled by the p55 TNF receptor. *Cell* **73,** 213–216.

Tian, L., Noelle, R. J., and Lawrence, D. A. (1995). Activated T cells enhance nitric oxide production by murine splenic macrophages through gp39 and LFA-1. *Eur. J. Immunol.* **25,** 306–309.

Tong, A. W., Zhang, B. Q., Mues, G., Solano, M., Hanson, T., and Stone, M. J. (1994). Anti-CD40 antibody binding modulates human multiple myeloma clonogenicity in vitro. *Blood* **84,** 3026–3033.

Torres, R. M., and Clark, E. A. (1992). Differential increase of an alternatively polyadenylated mRNA species of murine CD40 upon B lymphocyte activation. *J. Immunol.* **148,** 620–626.

Tsubata, T., Wu, J., and Honjo, T. (1993). B cell apoptosis induced by antigen receptor cross-linking is blocked by T cell signal through CD40. *Nature* **364,** 645–648.

Uckun, F. M., Gajil-Peczalska, K., Myers, D. E., Jaszcz, W., Haissig, S., and Ledbetter, J. A. (1990). Temporal association of CD40 antigen expression with discrete stages of human B-cell ontogeny and the efficacy of anti-CD40 immunotoxins against clonogenic B-lineage acute lymphoblastic leukemia as well as B-lineage non-Hodgkin's lymphoma cells. *Blood* **76,** 2449–2456.

Uckun, F. M., Schieven, G. L., Dibirdik, I., Chandan-Langlie, M., Tuel-Ahlgren, L., and Ledbetter, J. A. (1991). Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified serine/threonine-specific protein kinases by the pan-B-cell receptor CD40/Bp50 at discrete development stages of human B-cell ontogeny. *J. Biol. Chem.* **266,** 17478–17485.

Unaneu, E. R., and Allen, P. M. (1986). Comment on the finding of Ia expression in nonlymphoid cells. *Lab. Invest.* **55,** 123–125. [Editorial]

Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* **184,** 370–382.

Urashima, M., Chauhan, D., Uchiyama, H., Freeman, G. J., and Anderson, K. C. (1995). CD40 ligand triggered interleukin-6 secretion in multiple myeloma. *Blood* **85,** 1903–1912.

Valent, P., Majdic, O., Maurer, D., Bodger, M., Muhm, M., and Bettelheim, P. (1990). Further characterization of surface membrane structures expressed on human basophils and mast cells. *Int. Arch. Allergy Appl. Immunol.* **91,** 198–203.

Valentine, M. A., and Licciardi, K. A. (1992). Rescue from anti-IgM-induced programmed cell death by the B cell surface proteins CD20 and CD40. *Eur. J. Immunol.* **22,** 3141–3148.

Vallé, A., Zuber, C. E., Defrance, T., Djossou, O., De Rie, M., and Banchereau, J. (1989). Activation of human B lymphocytes through CD40 and interleukin 4. *Eur. J. Immunol.* **19,** 1463–1467.

Van den Ackerveken, G. F., VanKan, J. A., Joosten, M. H., Muisers, J. M., Verbakel, H. M., and De Wit, P. J. (1993). Characterization of two putative pathogenicity genes of the fungal tomato pathogen. Cladosporium fulvum. *Mol. Plant. Microbe Interac.* **6,** 210–215.

Van den Eertwegh, A. J. M., Noelle, R. J., Roy, M., Shepherd, D. M., Aruffo, A., Ledbetter, J. A., Boersma, W. J. A., and Claassen, E. (1993). In vivo CD40-gp39 interactions are essential for thymus dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. *J. Exp. Med.* **178,** 1555–1565.

van Kooten, C., Gaillard, C., Galizzi, J.-P., Hermann, P., Fossiez, F., Banchereau, J., and Blanchard, D. (1994). B cells regulate expression of CD40-ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur. J. Immunol.* **24,** 787–792.

Vilcek, J., and Lee, T. H. (1991). Tumor Necrosis Factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **266,** 7313–7316.

Villa, A., Notarangelo, L. D., Di Santo, J. P., Macchi, P. P., Strina, D., Frattini, A., Lucchini, F., Patrosso, C. M., Giliani, S., Mantuano, E., Agosti, S., Nocera, G., Kroczek, R. A., Fischer, A., Ugazio, A. G., de Saint-Basile, G., and Vezzoni, P. (1994). Organization of the human CD40L gene: Implications for molecular defects in X chromosome-linked hyper-IgM syndrome and prenatal diagnosis. *Proc. Natl. Acad. Sci. USA* **91,** 2110–2114.

Wagner, D. J., Stout, R. D., and Suttles, J. (1994). Role of the CD40-CD40 ligand interaction in CD4+ T cell contact-dependent activation of monocyte interleukin-1 synthesis. *Eur. J. Immunol.* **24,** 3148–3154.

Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., van Arsdell, J. N., Yamamoto, R., and Mark, D. F. (1985). Molecular cloning, of the complementary DNA for human tumor necrosis factor. *Science* **228,** 149–154.

Wang, F., Gregory, C. D., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A. B., and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP-1)- and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP-1 cooperatively induce CD23. *J. Virol.* **64,** 2309–2318.

Ware. C. F., Crowe, P. D., Vanarsdale, T. L., Andrews, J. L., Grayson, M. H., Jerzy, R., Smith, C. A., and Goodwin, R. G. (1991). Tumor necrosis factor (TNF) receptor expression in T lymphocytes. Differential regulation of the type I TNF receptor during activation of resting and effector T cells. *J. Immunol.* **147,** 4229–4238.

Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992a). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356,** 314–317.

Watanabe-Fukunaga, R., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992b). The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J. Immunol.* **148,** 1274–1279.

Westendorf, J. J., Ahmann, G. J., Armitage, R. J., Spriggs, M. K., Lust, J. A., Greipp, P. R., Katzmann, J. A., and Jelinek, D. F. (1994). CD40 expression in malignant plasma cells. Role in stimulation of autocrine IL-6 secretion by a human myeloma cell line. *J. Immunol.* **152,** 117–128.

Worm, M., and Geha, R. S. (1994). CD40 ligation induces lymphotoxin α gene expression in human B cells. *Int. Immunol.* **6,** 1883–1890.

Xu, J., Foy, T. M., Laman, J. D., Elliott, E. A., Dunn, J. J., Waldschmidt, T. J., Elsemore, J., Noelle, R. J., and Flavell, R. A. (1994). Mice deficient for the CD40 ligand. *Immunity* **1,** 423–431.

Yellin, M. J., Sinning, J., Covey, L. R., Sherman, W., Lee, J. J., Glickman-Nir, E., Sippel, K. C., Rogers, J., Cleary, A. M., Parker, M., Chess, L., and Lederman, S. (1994). T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity. *J. Immunol.* **153,** 666–674.

Young, L. S., Dawson, C. W., Brown, K. W., and Rickinson, A. B. (1989). Identification of a human epithelial cell surface protein sharing an epitope with the C3d/Epstein-Barr virus receptor molecule of B lymphocytes. *Int. J. Cancer* **43,** 786–794.

Zhang, K., Clark, E. A., and Saxon, A. (1991). CD40 stimulation provides an IFN-γ-independent and IL-4 dependent differentiation signal directly to human B cells for IgE production. *J. Immunol.* **146,** 1836–1842.

Zhang, R., Alt, F. W., Davidson, L., Orkin, S. H., and Swat, W. (1995). Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the *vav* proto-oncogene. *Nature* **374,** 470–473.

Zhou, L.-J., and Tedder, T. F. (1995). Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol.* **154,** 3821–3835.

Zubler, R. H., Werner-Favre, C., Wen, L., Sekita, K.-I., and Straub, C. (1987). Theoretical and practical aspects of B-cell activation: Murine and human systems. *Immunol. Rev.* **99,** 281–299.

ADVANCES IN IMMUNOLOGY, VOL. 61

Antibody Class Switching

Janet Stavnezer

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122

I. Introduction: Overview and Functions of Different Classes of Antibodies

Class, or isotype, switching is the process whereby a B cell changes the heavy chain class of the antibody it synthesizes by changing the heavy chain constant (C_H) region expressed, but not the light chain or heavy chain variable (V_H) regions. The change in antibody class, except to IgD, is effected by a deletional DNA recombination event called switch recombination, which occurs between tandemly repeated sequences called switch regions. Switch recombination causes the recombined V(D)J gene segment, which is initially expressed with the $C\mu$ gene, to be subsequently expressed with one of six (mouse) or seven (human) downstream C_H genes (Shimizu *et al.*, 1982). IgD is an exception, as it is coexpressed with IgM by the mechanisms of alternative RNA processing and termination of transcription (Knapp *et al.*, 1982; Maki *et al.*, 1981; Moore *et al.*, 1981). Diagrams of the heavy chain gene loci in mouse and humans are shown in Fig. 1.

Because antibody specificity is determined by the variable regions of antibodies, class switching does not change the antigen-binding specificity. Because effector functions of antibodies are determined by the C_H regions, class switching does change the effector function of the antibody. Different Ig classes are found in most vertebrates examined, suggesting the importance of having different effector functions. There are five classes of immunoglobulins in mice, rats, and humans: IgM, IgD, IgG, IgE, and IgA. There are four subclasses of IgG (IgG1, IgG2b, IgG2a, and IgG3 in the mouse; IgG1, IgG2, IgG3, and IgG4 in human) and in human two subclasses of IgA: IgA1 and IgA2. The $C\gamma$ genes appear to have duplicated since divergence of the ancestors to mice and humans; thus, there is an incomplete correspondence between the subclasses. Antibodies of different heavy chain classes differ in size, *in vivo* half-life, ability to bind to cell surface Fc receptors, ability to activate complement, sensitivity to digestion by proteolytic enzymes, and the tendency to aggregate (reviewed in Snapper and Finkelman, 1993). Two of the classes, IgM and IgA, form pentamers/hexamers and dimers/trimers/tetramers, respectively, when secreted, thus increasing their avidity. IgG3 tends to aggregate after binding antigen, also increasing its avidity (Greenspan and Cooper, 1992).

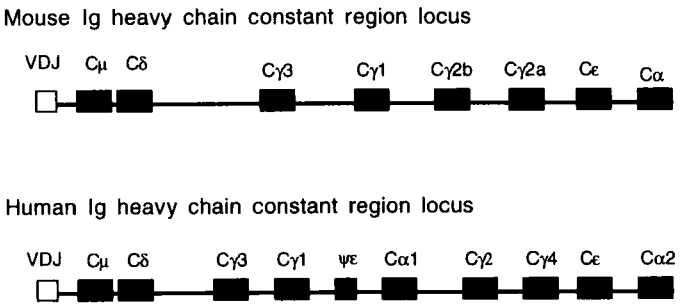


FIG. 1. (Top) Heavy chain gene locus of mouse. (Bottom) Heavy chain gene locus of human.

During a primary immune response, IgM is secreted first and predominates for a short time, but it has a short half-life (5 days). Because it is expressed prior to somatic mutation of the variable region it tends to be of low affinity, although its ability to form pentamers or hexamers greatly increases its effective affinity for antigens with multiple identical epitopes. IgM is highly effective in agglutination of particulate antigens, e.g., bacteria, and it can destroy or opsonize targets due to its efficient fixation of complement (Snapper and Finkelman, 1993). IgD exists as a monomer mainly on the surface of cells and participates in activation of B cells when cross-linked.

IgG is the predominant isotype in the blood and lymph, crosses the placenta, and is very stable [average half-life for human IgG is 23 days] (Carayannopoulos and Capra, 1993). The different subclasses of IgG have somewhat different effector functions, mainly due to their varying abilities to bind Fc receptors and to activate complement. Different subclasses tend to predominate during responses to different types of pathogens and to be well suited for resisting these pathogens, although it appears that the functions of the subclasses overlap (Snapper and Finkelman, 1993).

IgA predominates in respiratory, digestive, and urogenital secretions and is highly resistant to proteolytic enzymes. It cannot activate complement by the classical pathway, but can activate complement by the alternative pathway. IgA deficiency is quite common in humans and does not cause an obvious immune deficiency in most individuals, although in some there is a reduced ability to resist respiratory infections (Snapper and Finkelman, 1993).

IgE is involved in parasite defense but also mediates immediate hypersensitivity reactions. IgE binds to Fc receptors on basophil and mast cells; cross-linking IgE on these cells by antigen induces degranulation, resulting in symptoms of allergy.

In this review, I will discuss how isotype specificity is regulated, describe what is known about how switch recombination is induced and regulated, and discuss the mechanism of switching. Most of the data are derived from studies in mouse, although when the data are available I will compare the studies in humans.

A. Problems Found in Some Studies of Class Switching

Many studies assay class switching in cell culture by assaying secretion of antibodies of "switched" isotypes, and many of these assays fail to distinguish regulation of switch recombination from regulation of antibody synthesis and secretion, or even from B cell proliferation. This problem is avoided, for the most part, if mIg is measured using an antibody that does not bind to FcRs, or by measuring cytoplasmic Ig; however, this latter method is really only reliable in B cell lines. The intensity of staining of cytoplasmic Ig in mouse splenic B cells is extremely variable and generally does not provide reliable data. Most investigators still measure Ig secretion, which includes the effects of regulation of Ig production.

Another problem found is initiation of experiments with cells that have already undergone switching. This problem has been avoided in many experiments performed in the past few years by initiating cultures with purified B cells which are resting B cells (small or dense), B cells which are negative for the particular Ig class for which switching is being studied, or are $sIgM^+$ or $sIgD^+$ B cells. Another problem is that some experiments involving attempts to assay switching in human B cells have assayed secreted Ig more than two weeks after initiation of cultures. It seems likely that if it takes this long to obtain secretion of significant amounts of Ig from a downstream C_H gene, the investigators are not assaying switching, but rather they are assaying outgrowth of a very rare cell, or perhaps a combination of the two; the particular regulator being studied may contribute to either process. Data from these types of experiments will, for the most part, not be included. Still, antibody production in human systems is generally measured on Day 10, and in mouse spleen cell cultures on Day 6, although if one assays surface or membrane Ig (sIg) in mouse B cell cultures, switching is detected by Days 2 or 3. Thus, I will focus on results involving measurement of sIg, or actual switch recombination, if available. Furthermore, I will not discuss postswitch regulation, even if it might be

isotype specific, in order to concentrate on the regulation and mechanism of the class switch event itself.

II. Heavy Chain Isotype Expression during B Cell Differentiation

A. Antigen-Independent Differentiation: Mechanism of Expression and Function of IgD

Soon after emerging from the bone marrow, immature B cells which express IgM on their surface also start to express surface IgD, with the same V_H region as the IgM. This event occurs in virgin B cells in the absence of antigen stimulation. It is not known if a signal from a B cell surface receptor is required to induce IgD expression, although it is possible that a positive selection event involving some type of self-ligand initiates IgD expression. Cells that express IgM and IgD have a longer half-life than the newly emerged cells that express only IgM (Allman *et al.*, 1993). Coexpression of IgM and IgD is apparently effected by alternative RNA processing of the identical primary transcript because IgD expression is not accompanied by a change in sites of transcriptional termination (Yuan, 1986; Yuan and Witte, 1988). In resting mature B cells (IgM^+IgD^+) the mRNA for δ mRNA is two- to fourfold more stable than μ mRNA (Weiss *et al.*, 1989). There may also be an increase in the efficiency of translation of δ mRNA (Yuan, 1986). After antigen activation of B cells, IgD expression is reduced due to an increase in transcriptional termination 5′ to the δ exons. The expression of μ mRNA is simultaneously up-regulated due to an increase in transcription rate and an increase in μ mRNA stability (Yuan and Tucker, 1984).

The role of IgD is not understood, although experiments examining protein tyrosine kinase activity in stably transfected myeloma (J558L) cells expressing either IgM or IgD of the same antigen specificity suggest that signaling via sIgD may cause a more prolonged induction than signaling via sIgM (Kim and Reth, 1995). It will be very interesting to have results from similar experiments using B cell lines of earlier stages and also using splenic B cells from transgenic mice expressing either IgM or IgD with the same V_H region in order to compare signaling in normal sIg^+ B cells. Mice unable to express sIgD due to a knockout mutation do not have a pronounced phenotype, but they have a delayed response in the production of high-affinity antibodies to T cell-dependent antigens in the primary response (Nitschke *et al.*, 1993; Roes and Rajewsky, 1993). Thus, IgD may increase the efficiency of recruitment of B cells. Although both sIgM and IgD are associated with Ig-α and Ig-β chains, IgD has two additional polypeptides associated with it at the cell surface that are not associated

with sIgM, and vice versa (Kim *et al.*, 1994; Terashima *et al.*, 1994; Venkitaraman *et al.*, 1991).

B. Antibody Class Switching Occurs in Response to Encounters with Antigens or B Cell Mitogens

Unlike induction of IgD expression or the rearrangement of V, D, and J gene segments that occurs during pre-B cell development, switch recombination generally only occurs in mature B cells on exposure to antigen, thus allowing the heavy chain class to be influenced by the antigen. Antigen induces switching in conjunction with contact-dependent signals from T cells and signals from cytokines. T-independent responses also induce class switching, probably by signals from sIg cross-linking due to multivalent antigens, along with cytokines synthesized by cells other than T cells. Antibody class switching can also be induced in culture in mouse and human B cells by B cell mitogens (TI-1 antigens), e.g., by LPS for mouse B cells (Andersson *et al.*, 1978; Kearney *et al.*, 1976; Kearney and Lawton, 1975), by mimics of thymus-independent type 2 (TI-2) antigens, e.g., anti-Ig bound to Sepharose beads (Purkerson *et al.*, 1988) or anti-δ dextran (Snapper *et al.*, 1991, 1992), or by T-dependent antigens plus T cells (Lebman and Coffman, 1988), or by contact-dependent signals from activated T_H cells in the absence of antigen (Noelle *et al.*, 1992b; Snapper *et al.*, 1995a). One mouse $sIgM^+$ B lymphoma cell line (I.29μ) can be induced to switch by LPS (Stavnezer *et al.*, 1985), one $sIgM^+$ line can be induced to switch by IL-4 (CH12.LX) (Whitmore *et al.*, 1991), and a few pre-B lines transformed by Abelson leukemia virus (Burrows *et al.*, 1983) and a few mouse plasmacytoma lines switch spontaneously in culture (Spira *et al.*, 1994).

Mature virgin B cells which express surface IgM and IgD are capable of being induced to switch to any antibody class. This was first suggested by the finding that clones of activated splenic B cells can each express a few different heavy chain classes, and that the classes expressed appear to be determined by T cells (Gearhart *et al.*, 1975; Teale and Klinman, 1984). The most definitive data demonstrating the multipotentiality of $sIgM^+$ B cells have come from limiting dilution experiments in which it has been shown that up to 88% of splenic B cells stimulated with antigen, type 2 helper T (Th2) cells and IL-4 are precursors for IgG1-secreting cells, whereas in the absence of such stimuli a very low percentage of clones express IgG1 (Bergstedt-Lindqvist *et al.*, 1988; Layton *et al.*, 1984; Lebman and Coffman, 1988; Savelkoul *et al.*, 1988; reviewed in Coffman *et al.*, 1993). In bulk cultures it is often found that 20% or more of the LPS-activated splenic B cells can express IgG3 and IgG2b (McIntyre *et al.*, 1993). Thus, the numbers indicate that one B cell has the potential to

produce more than one isotype. The mechanisms of isotype regulation will be discussed below.

Antibody class switching begins within 4 days after activation by T cell-dependent antigens *in vivo*, initiating in B cell foci in the periarteriolar lymphoid sheaths and also in germinal centers prior to somatic mutation (Jacob *et al.*, 1991; Kraal *et al.*, 1982; McHeyzer-Williams *et al.*, 1993; Weinstein and Cebra, 1991). Thus, IgG, IgA, and IgE are made later than IgM during a primary response and account for most of the antibody made during a memory response. Although class switching and somatic mutation can occur independently, they occur simultaneously, with the result that these bivalent antibodies more than make up for the loss of avidity of pentameric IgM by increases in affinity of the variable regions (Fish *et al.*, 1989). T cell-independent antigens also induce class switching, but they are localized at different sites in the spleen.

III. Regulation of Isotype Specificity during Antigen-Dependent Class Switching and Induction of Class Switch Recombination

A. Cytokines Direct Switching by Regulation of Germline Transcripts

A large body of data indicate that cytokines direct and regulate isotype specificity of switching. The data have been obtained by analyzing class switching in culture and *in vivo* in normal mice and in a few mice with deficiencies of cytokine genes created by knockout experiments (Finkelman *et al.*, 1986, 1988; Kühn *et al.*, 1991). The mechanism of cytokine regulation of isotype specificity is generally by regulation of transcription of unrearranged C_H genes prior to class switching, yielding what are called germline transcripts (Jung *et al.*, 1993; Lutzker *et al.*, 1988; Stavnezer-Nordgren and Sirlin, 1986; Yancopolous *et al.*, 1986; Zhang *et al.*, 1993). Induction or suppression of germline transcription by particular cytokines have been directly correlated with subsequent switching to the same isotype after addition of a B cell activator (Berton *et al.*, 1989; Berton and Vitetta, 1992; Esser and Radbruch, 1989; Gaff *et al.*, 1992; Gauchat *et al.*, 1990; Goodman *et al.*, 1993; Islam *et al.*, 1991; Kitani and Strober, 1994; Lebman *et al.*, 1990a; Qiu *et al.*, 1990; Rothman *et al.*, 1988; Severinson *et al.*, 1990; Shockett and Stavnezer, 1991; Stavnezer *et al.*, 1988; Turaga *et al.*, 1993; Wakatsuki and Strober, 1993; Weinstein and Cebra, 1991; Xu and Rothman, 1994). Figure 2A shows a map and splicing diagram of germline α transcripts. Transcripts from all unrearranged C_H genes have an analogous structure.

The model originally posed was called the accessibility model, and it held that class switch recombination was directed to specific switch regions that had been induced to become accessible, as assayed by hypomethylation

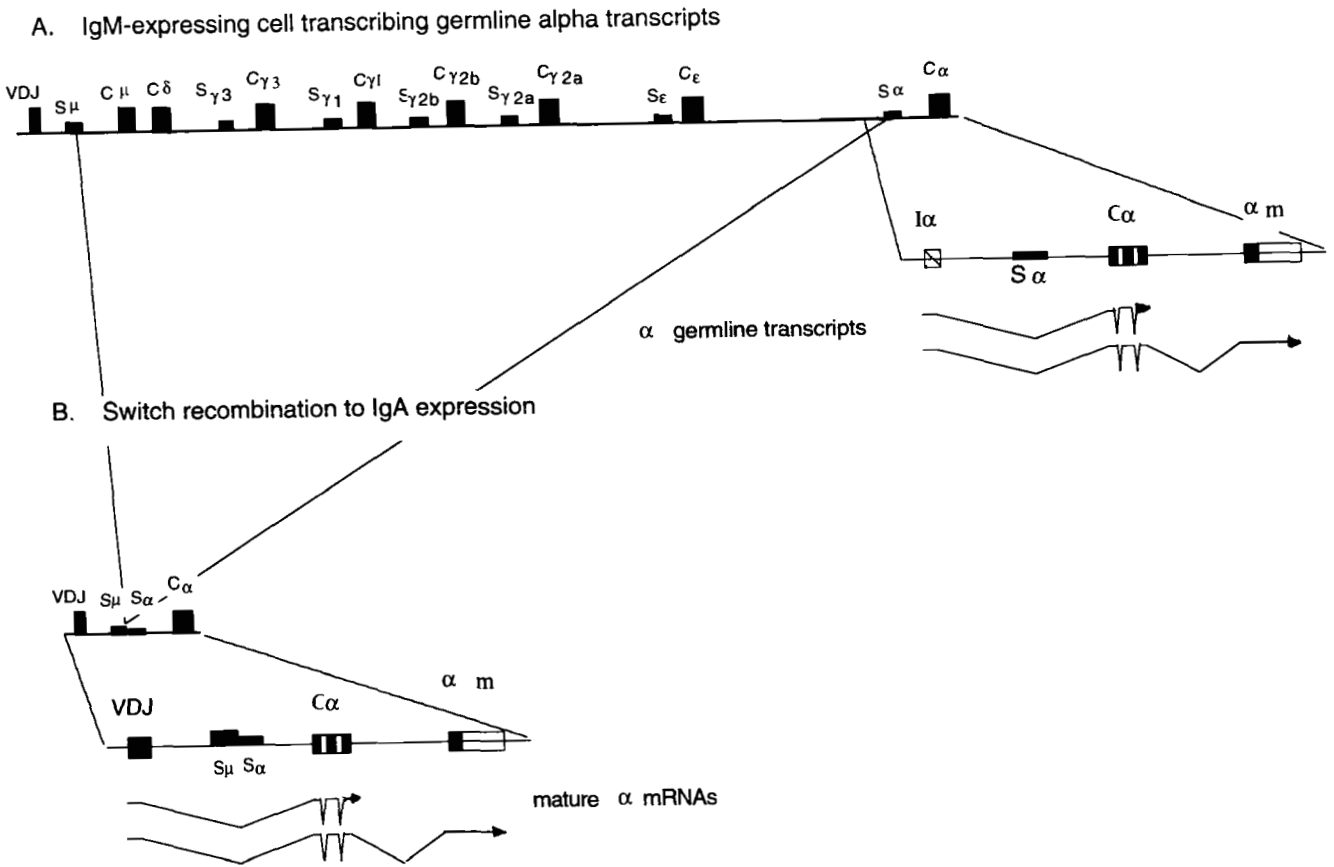


FIG. 2. (A) Diagram of heavy chain genes IgM-expressing mouse B cells. Map and splicing diagrams of germline α transcripts. (B) Diagram of switch recombination to IgA: genomic DNA residing between the Sμ and Sα regions is excised as a circle and deleted from the chromosome. Shown below the expanded map of the expressed alpha gene is a splicing diagram of mature α mRNA.

and germline transcripts (Stavnezer *et al.*, 1984; Stavnezer-Nordgren and Sirlin, 1986; Yancopolous *et al.*, 1986). This model is in agreement with the finding that switch recombination frequently occurs on both chromosomes and to the same isotype in both mice and humans, consistent with regulation by *trans*-acting factors (Hummel *et al.*, 1987; Irsch *et al.*, 1993; Kepron *et al.*, 1989; Winter *et al.*, 1987). This is a very general model, and recently several types of experiments (discussed below) have refined the model and further indicate the importance of the germline transcripts themselves in regulation of switching. Germline transcripts themselves do not induce class switch recombination, which requires addition of a B cell activator, e.g., mitogens like LPS or the human B cell activator *Staphylococcus aureus* Cowan I (SAC), or T_H cells or anti-δ dextran and IL-5. Inducers of switch recombination will be discussed below.

B. Cytokine Regulation of Switching Induced by LPS in Mouse B Cells, by SAC in Human B Cells, Or by T_H Membranes in Both Human and Mouse B Cells

The discovery that germline transcription precedes switch recombination to the same isotype has provided a powerful assay indicating whether a particular cytokine actually directs switching or affects postswitch events. Three cytokines have been shown to affect germline transcripts: IL-4, interferon-γ (IFN-γ), and transforming growth factor β1 (TGF-β). This section is organized according to cytokine, describing which isotypes each regulates when mouse B cells are activated *in vitro* with LPS. I will also mention available data from *in vivo* experiments and for human B cells. Because the nature of the B cell activator can sometimes alter the isotypes expressed in response to a cytokine, I will separately discuss experiments in which switching is induced by a mimic of TI-2 antigens or by CD40 signaling.

Treatment of mouse splenic B cells with LPS results in induction of germline γ3 and γ2b transcripts and subsequent switching to IgG3 and IgGb expression (Lutzker *et al.*, 1988; Rothman *et al.*, 1990b; Severinson *et al.*, 1990; Snapper and Paul, 1987).

1. IL-4

Addition of IL-4 to LPS-activated B cells decreases germline γ3 and γ2b transcripts and switching to IgG3 and IgG2b (Andersson *et al.*, 1978; Bergstedt-Lindqvist *et al.*, 1984; Coffman *et al.*, 1986; Isakson, 1982; Kearney *et al.*, 1976; Kearney and Lawton, 1975; Lutzker *et al.*, 1988; Rothman *et al.*, 1990b; Vitetta *et al.*, 1985). Unlike the results of experiments by Rothman *et al.* (1990b) in which T-depleted spleen cells were used, in experiments using whole spleen cells, two groups found that germline γ3

transcripts were not reduced by the addition of IL-4, relative to the level induced by LPS in the absence of IL-4 (Esser and Radbruch, 1989; Severinson *et al.*, 1990). Perhaps the inhibition of germline γ_3 transcripts by IL-4 is blocked by another cell type in these cultures.

IL-4 + LPS also induce germline $\gamma 1$ and ε transcripts in the mouse and subsequent switching to these isotypes (Bergstedt-Lindqvist *et al.*, 1984; Berton *et al.*, 1989; Coffman *et al.*, 1986; Stavnezer *et al.*, 1988; Vitetta *et al.*, 1985). IL-4 directs switching to IgG4 and IgE by human peripheral blood B cells stimulated with an activated helper T cell line (Lundgren *et al.*, 1989). Preliminary evidence indicates that IL-13 can substitute for IL-4, directing switching to IgG4 and IgE, when human B cells are activated to switch with membranes from an activated helper T cell line (Punnonen *et al.*, 1993).

IL-4 given in the absence of LPS induces lower levels of germline $\gamma 1$ transcripts and in most experiments cannot induce germline ε transcripts in mouse B cells. Treatment of high-density, $sIgG^-$ peripheral blood human B cells with IL-4 alone has been shown to induce germline $\gamma 4$ transcripts, and no other germline γ transcript, but germline ε transcripts were not examined in this study (Kitani and Strober, 1993). Treatment with SAC does not further increase the level of germline $\gamma 4$ transcripts, as measured by a reverse-transcriptase polymerase chain reaction (RT-PCR) (Kitani and Strober, 1993). If human splenic or peripheral blood B cells are purified by a method that does not remove activated cells, IL-4 alone induces low levels of germline ε transcripts (Gauchat *et al.*, 1990; Shapira *et al.*, 1992).

In conclusion, germline mouse $\gamma 1$ or human $\gamma 4$ transcripts can be induced by IL-4 in the absence of any other added B cell activator, perhaps to a lower extent than in activated cells, but germline ε transcripts are probably only induced by IL-4 in activated B cells. Consistent with this is the fact that germline ε transcripts can be induced in I.29μ B lymphoma cells, which represent activated B cells, by IL-4 alone (Stavnezer *et al.*, 1988).

Although IL-4 is well known for its ability to specifically direct switching to IgG1 in mouse, to IgG4 in human, and to IgE in both mouse and human, IL-4 also appears to increase switching to IgA in splenic B cells and in two B cell lines, CH12.LX and I.29μ (McIntyre *et al.*, 1995; Shockett and Stavnezer, 1991; Whitmore *et al.*, 1991). The mechanism of this effect is unknown, as it was shown in I.29μ cells that treatment with anti-IL-4 monoclonal antibody does not decrease the steady-state level of germline α transcripts nor inhibit DNA synthesis (Shockett and Stavnezer, 1991). In CH12.LX cells, IL-4 alone can induce switching to IgA, and also to other isotypes, and is required for DNA synthesis (Louie *et al.*, 1993).

2. *IFN-γ*

IL-4 and IFN-γ are mutually antagonistic. Addition of IFN-γ reduces germline γ1 and ε transcripts and switching to IgG1 and IgE in mouse B cells induced by LPS + IL-4 (Berton *et al.*, 1989; Coffman and Carty, 1986; Severinson *et al.*, 1990; Snapper and Paul, 1987; Xu and Rothman, 1994). The induction of germline γ4 transcripts in human B cells by IL-4 is inhibited by IFN-γ (Kitani and Strober, 1993). The IL-4-induced germline ε transcripts in purified human splenic B cells could not be inhibited, however, with IFN-γ or with IFN-α, although both of these IFNs inhibit switching to IgE by human B cells (Gauchat *et al.*, 1992).

Addition of IFN-γ to LPS-activated B cells induces germline γ2a transcripts and subsequent switching to IgG2a in mouse B cells (Collins and Dunnick, 1993; Severinson *et al.*, 1990; Snapper and Paul, 1987). A mouse lacking the receptor for IFN-γ has reduced antigen-specific IgG2a (fivefold) and IgG3 (twofold) responses to vaccinia virus (Huang *et al.*, 1993). However, the effects of IFN-γ on germline γ2a transcripts and on switching to IgG2a are not nearly as dramatic as the effects of IL-4 on switching to IgG1 or IgE. In limiting dilution experiments with LPS-activated splenic B cells (Bossie and Vitetta, 1991) found that IFNγ induced only a two- to fourfold increase in precursor frequency for IgG2a secreting cells, which they interpreted as due to an increase in IgG2a secretion rather than switching. These experiments are difficult to interpret, however, due to the very low frequency of switching in these cultures (at best: 1/1100 $sIgC^-$ cells).

IFN-γ also inhibits secretion of IgG3 by $sIgM^+$ B cells induced by LPS (Coffman and Carty, 1986; Severinson *et al.*, 1987; Snapper and Paul, 1987). However, it was found that IFN-γ induced germline γ3 transcripts somewhat on Day 2 in LPS-activated spleen cells (Severinson *et al.*, 1990). Again, as found in spleen cells treated with LPS + IL-4, there is a contradiction with expectation for IgG3, and again the discrepancy occurred in experiments in which germline γ3 transcripts were examined in whole spleen cell cultures. It is possible, for example, that IFN-γ may have induced a T_H cell or an adherent cell to secrete a cytokine which inhibits the function of IFN-γ. It is also possible, however, that switching to IgG3 is controlled by means other than, or in addition to, germline transcripts.

3. *TGF-β*

Addition of TGF-β increases germline α transcripts and subsequent switching to IgA in mouse and human B cells (Coffman *et al.*, 1989; Defrance *et al.*, 1992; Ehrhardt *et al.*, 1992; Islam *et al.*, 1991; Kim and Kagnoff, 1990; Kitani and Strober, 1994; Lebman *et al.*, 1990a; McIntyre *et al.*, 1995; Nilsson *et al.*, 1991; Shockett and Stavnezer, 1991; Sonoda *et al.*, 1989). TGF-β also increases germline γ2b transcripts and subsequent switching to IgG2b (Mc-

Intyre *et al.*, 1993; Sonoda *et al.*, 1992). It appears likely that TGF-β may also specifically inhibit switching to IgE, because it inhibits induction of germline ε transcripts by IL-4 and switching to IgE in mouse and human B cells (Shockett and Stavnezer, 1991; Gauchat *et al.*, 1992; Ichiki *et al.*, 1992).

C. Structure and Function of Germline Switch Transcripts

Germline C_H gene transcripts all have an analogous structure, initiating 5′ to the tandem repeats of the switch region, proceeding through the C_H gene, and terminating at either the normal poly(A) site for secreted or for membrane-bound Ig heavy chain mRNAs (Fig. 2A) (Gaff and Gerondakis, 1990; Gerondakis, 1990; Lebman *et al.*, 1990b; Lutzker and Alt, 1988; Radcliffe *et al.*, 1990; Rothman *et al.*, 1990a; Sideras *et al.*, 1989). The primary transcript is spliced to generate a germline RNA containing an upstream, or 5′ germline exon (I exon) spliced at the normal splice acceptor for mature heavy chain mRNA. After switch recombination, the segment of DNA 5′ to the switch region is deleted from the chromosome and thus, germline transcripts cannot be synthesized after class switching (Fig. 2B). Instead, the mature mRNA is transcribed.

Located 5′, or upstream, of the I exons are DNA sequences which have been shown to respond to cytokines by induction of DNase hypersensitive sites (Berton and Vitetta, 1990; Schmitz and Radbruch, 1989) and to function as promoters, regulating transcription of germline transcripts in reporter gene assays (Albrecht et al., 1994; Delphin and Stavnezer, 1995; Ichiki *et al.*, 1993; Lin and Stavnezer, 1992; Nilsson and Paschalis, 1993; Rothman *et al.*, 1991; Xu and Stavnezer, 1992).

The fact that all germline transcripts have the same overall structure suggests that the transcripts themselves might have a function. Consistent with this are results of experiments in which the powerful technique of targeted homologous recombination was used to create deletion mutants of various portions of the germline I exons and their promoters. Deletion of the promoter and I exons for ε, γ2b, and γ1 all result in no switching on that chromosome to that particular C_H gene, although the other allele and other C_H genes on that chromosome are unaffected (Jung *et al.*, 1993; Zhang *et al.*, 1993; Bottaro *et al.*, 1994). These data suggest that transcription of the unrearranged switch region and C_H genes are required for switching, but do not indicate the mechanism of their function. The results also do not indicate whether germline transcripts function *in cis* or *in trans*. For example, if the role of the RNA were to hybridize with the DNA segment upstream of the switch region, this segment of DNA has been deleted from the chromosome on the targeted allele.

D. Mechanism of Regulation of Transcription of Germline Transcripts

Nuclear run-on assays and RNA stability measurements indicate that the regulation of expression of germline transcripts may be solely at the

transcriptional level (Rothman *et al.*, 1991; Shockett and Stavnezer, 1991; Lorenz *et al.*, 1995). A contradictory experiment has been published, but appears flawed, as previously discussed (Lebman *et al.*, 1993; Stavnezer, 1995). Furthermore, transient or stable transfection of reporter genes driven by 5′ flanking segments of germline Iγ1, Iε, and Iα exons demonstrate that transcription driven by each of these promoters is inducible by the cytokine which directs switching to that antibody class (Albrecht *et al.*, 1994; Delphin and Stavnezer, 1995; Ichiki *et al.*, 1993; Lin and Stavnezer, 1992; Nilsson and Paschalis, 1993; Rothman *et al.*, 1991; Xu and Stavnezer, 1992).

Reporter gene assays have identified DNA elements involved in induction of transcription of germline RNAs by IL-4 or by TGF-β. The IL-4 responsive elements of the germline γ1 and ε promoters include binding sites for two transcription factors: (1) IL-4 Stat/Stat 6, which is a member of a family of transcription activators induced by Jak kinases and involved in transduction of signals from cytokine receptors; and (2) a binding site for members of the C/EBP family (Albrecht *et al.*, 1994; Berton and Linehan, 1995; Delphin and Stavnezer, 1995; Hou *et al.*, 1994; Ichiki *et al.*, 1993; Kohler and Rieber, 1993; Kotanides and Reich, 1993; Lundgren *et al.*, 1994; Xu and Stavnezer, 1992). Mutation of either of the adjacent IL-4 Stat or C/EBP binding sites eliminates IL-4 induction of the germline ε promoter (Delphin and Stavnezer, 1995). In addition, NF-κB family members are required for expression of the germline ε promoter because a mutation in a κB site in the promoter eliminates both constitutive and IL-4-induced transcription (Delphin and Stavnezer, 1995). A binding site for the B cell-specific transcriptional activator Pax-5 also appears to be necessary for LPS + IL-4-induced expression of the mouse germline ε promoter in some reporter constructs (Liao *et al.*, 1994; Rothman *et al.*, 1991).

Reporter genes driven by the 5′ flanking segment for germline α transcripts of both mouse and human can be induced by TGF-β, and a novel repeated element required for the induction of transcription by TGF-β has been identified (Lin and Stavnezer, 1992; Nilsson and Paschalis, 1993). Binding of an unidentified protein complex to this repeat element is competed by a DNA fragment containing the TGF-β-responsive region of another TGF-β-inducible gene, plasminogen activator inhibitor I (Keeton *et al.*, 1991; Sandler *et al.*, 1994; Shi and Stavnezer, submitted).

E. B Cell Activators also Determine Isotype Specificity

Cytokines which induce germline transcripts cannot by themselves induce B cells to switch. They must act together with a B cell activator that induces proliferation and differentiation, and switch recombination activity. Generally, B cell activators can induce switching to a variety of isotypes,

although in some cases the nature of the B cell activator influences the isotype specificity, depending on the cytokine present. TI-2 antigens, present in bacterial cell walls and exemplified by haptenated polysaccharides, induce IgM and IgG3 responses (Perlmutter *et al.*, 1978). Cytokines are synthesized by cells other than T cells, and so could also influence isotypes expressed during T cell-independent immune responses. The three cytokines which regulate isotype specificity and transcription of germline transcripts, IL-4, IFN-γ and TGF-β, are synthesized by T_H cells, and also by non-T cells. Mast cells and their precursors synthesize IL-4; NK cells and macrophages synthesize IFN-γ; and TGF-β is secreted by numerous cell types, including B cells and macrophages (Assoian *et al.*, 1987; Bancroft *et al.*, 1987; Ben-Sasson *et al.*, 1990; Fultz *et al.*, 1993; Kehrl *et al.*, 1986a; Plaut *et al.*, 1989).

In the presence of certain cytokines, class switching can be induced in culture by anti-δ-dextran, anti-μ-dextran, or by anti-Ig coupled to Sepharose beads, each of which cross-link sIg, thereby mimicking TI-2 antigens (Purkerson and Isakson, 1992; Snapper *et al.*, 1992; Snapper and Mond, 1993). Interestingly, the response to cytokines differs somewhat from responses when B cells are activated by LPS or T_H cells. IL-5 appears to be required to obtain switch recombination, whereas the role of IL-5 in responses to LPS is at the postswitch level, to increase expression of mRNAs for mature Igs (Mandler *et al.*, 1993a). In the presence of anti-δ dextran, IFN-γ induces germline γ3 transcripts and addition of IL-5 induces class switch recombination to IgG3, whereas in the presence of LPS, IFN-γ inhibits switching to IgG3 (Snapper *et al.*, 1992; Snapper and Paul, 1987). Furthermore, small resting B cells do not express germline ε transcripts nor secrete IgE when activated by anti-δ dextran plus IL-4 in the presence or absence of IL-5 (Purkerson and Isakson, 1992; Snapper *et al.*, 1991; Zelazowski *et al.*, 1995). The isotypes induced by anti-δ dextran are not completely discrepant from those induced by LPS, however, since anti-δ dextran induces secretion of IgG2a in the presence of supernatant from T_{H1} cells (which contains IFN-γ) and induces secretion of IgA in the presence of TGF-β and a T_{H2} supernatant (which contains IL-4 and IL-5) (Snapper *et al.*, 1991).

Unlike LPS, anti-δ dextran induces germline γ1 transcripts in purified small resting B cells in the absence of IL-4, but IL-4 can synergize to greatly increase the level of germline γ1 transcripts and, if IL-5 is added, switching to IgG1 (Mandler *et al.*, 1993a). The fact that IL-5 induces switching in this system has been demonstrated by detection of mIgG1$^+$ B cells and by digestion circularization-PCR (DC-PCR), which directly measures switch recombination in genomic DNA (Chu *et al.*, 1992). Anti-Ig Sepharose + IL-4 also induces germline γ1 transcripts and, if IL-5 is added, switching to IgG1 (Purkerson and Isakson, 1992).

From these data, Mandler *et al.* (1993) propose a three-component model for induction of switching in which the requirements are (1) an inducer of proliferation, e.g., anti-δ dextran; (2) an inducer of some component of the switch recombination machinery, which is the role of IL-5 in these experiments; and (3) activation of germline C_H gene transcription. Apparently, when LPS is used to induce switching it performs both of the first two functions, and it performs all three functions in induction of switching to IgG3. This group also tested the effect of anti-δ bound to Sepharose beads and found that, unlike soluble anti-δ dextran, IgE secretion is induced in the presence of IL-4, suggesting that this form of anti-δ delivers a different signal (Snapper *et al.*, 1991).

The finding that anti-δ dextran can induce switching to IgG1 in the absence of IL-4 fits with observations by other investigators, including the findings that switching to IgG1 occurs in IL-4 knockout mice and that the promoter for germline γ1 transcripts can be induced by phorbol ester or by anti-IgM (although IL-4 synergizes to further induce the promoter) (Kühn *et al.*, 1991; Xu and Stavnezer, 1992). By contrast, switching to IgE is completely dependent on IL-4, and the promoter for germline ε RNA is not inducible by phorbol ester nor by anti-IgM (Kühn *et al.*, 1991) (S. Delphin and J. Stavnezer, unpublished results). Although the promoters for germline γ1 and ε transcripts both have adjacent binding sites for IL-4 Stat and C/EBP, the orientation of these two sites relative to each other differs (Delphin and Stavnezer, 1995; Lundgren *et al.*, 1994). It is unknown whether this difference accounts for the difference in regulation of these two promoters.

F. CD40 Signaling Can Induce Switch Recombination and Some Germline Transcripts

Contact-dependent signals from T_H cells have been shown to induce class switching (Lebman and Coffman, 1988; Vercelli *et al.*, 1989). The most important component of contact-dependent T cell help for B cells appears to be the CD40 ligand (CD40-L or gp39), a 33-kDa glycoprotein which is transiently expressed on activated T_H cells and provides a mitogenic signal to B cells (Fanslow *et al.*, 1992; Lane *et al.*, 1992, 1993; Noelle *et al.*, 1992a; reviewed in Banchereau *et al.*, 1994). Soluble CD40-L or antibody to CD40 synergizes with IL-4 to induce proliferation of purified mouse and human B cells. CD40 signaling also induces switching to most isotypes.

Humans lacking CD40-L have hyper-IgM syndrome due to the fact that they express large amounts of serum IgM and do not express other Ig classes, except small amounts of IgG3 (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993; Fuleihan *et al.*, 1993; Korthauer *et al.*, 1993). In addition to lack of switching to isotypes dependent on T cells, their

antibodies do not undergo affinity maturation, and they do not develop B cell memory. Mice lacking CD40-L or CD40 have a deficit similar to the human syndrome (Castigli *et al.*, 1994; Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994). In both types of knockout mice, some T-dependent antigens induce a normal IgM response, perhaps due to an ability to deliver a signal through sIg, but no other isotypes. CD40-L or CD40 knockout mice respond perfectly well to TI-2 antigens, e.g., DNP–Ficoll, making abundant antigen-specific IgM and IgG3 and normal amounts of IgG1, IgG2b, and IgA. Sera of nonimmunized 3-month-old CD40-L knockout mice contain normal levels of IgM, 2- to 3-fold reduced levels of IgG2b and IgG3, 10- to 100-fold reduced levels of IgG1 and IgG2a, and no IgE (Xu *et al.*, 1994). IgE appears to be completely dependent on CD40-L.

$CD4^+$ T cells from CD40-L knockout mice can be activated normally *in vivo* or *in vitro* with anti-CD3 antibody and antigen-presenting cells. If effector T cells generated by such treatment are used to activate resting splenic B cells from wild-type mice *in vitro* in the presence of IL-4 and IL-5, the B cells will secrete normal amounts of IgM, but no IgG1, whereas control T cells induce secretion of both isotypes (Xu *et al.*, 1994). Other isotypes were not examined. Altogether the data support the hypothesis that CD40 signaling is required for induction of class switching by activated T cells and suggest that secretion of IgM does not require T cell help (other than IL-4 and IL-5), or that it requires a different molecule on the T cells.

Signaling via CD40 has also been shown to induce B cells to undergo class switching *in vitro,* although it has not been determined if it can induce switching to all isotypes. The amount of switching to some isotypes *in vitro* is increased by simultaneously cross-linking sIg. *In vivo* it is likely that antigen up-regulates costimulatory signals on B cells, thereby stimulating T_H cells, but antigen also appears to activate B cells directly by cross-linking sIg, providing a signal to the B cell in addition to the CD40 signal (Cooke *et al.*, 1994; Goroff *et al.*, 1991; Thornton *et al.*,1994). The use of antibody to CD40 has allowed the first relatively efficient means for induction of switching in human B cell cultures. Most studies using CD40 signaling to induce switching in culture have used human systems, possibly because more human antibodies are available and due to the lack of other good methods of inducing class switching in human B cells. I will discuss the research in human systems first.

1. IgE

Anti-CD40 monoclonal antibody in the presence of IL-4 can induce purified $sIgM^+$ or $sIgE^-$ human B cells to secrete IgE after 10–14 days

culture (Gascan *et al.*, 1991; Jabara *et al.*, 1990; Splawski *et al.*, 1993). Antibody to CD40 cannot induce germline ε transcripts, although it synergizes with IL-4 to induce large amounts of germline ε transcripts (Gauchat *et al.*, 1992; Shapira *et al.*, 1992).

2. *IgG*

One group has shown that treatment of purified $sIgD^+$ cells from tonsils with antibody to CD40 in the presence of IL-4 induces secretion of all IgG isotypes except IgG2 (Fujieda *et al.*, 1995). The finding that CD40 antibody in the presence of IL-4 actually induces switching, rather than simply secretion of IgG from previously switched cells, was supported by detection of excised circles of DNA containing Sγ–Sμ sequences (except Sγ2) (Fujieda *et al.*, 1995). Excised switch circles do not appear to replicate and thus are diluted out as cells divide subsequent to switching, so the presence of circles indicates that switching actually occurred in the cultures. Evidence that IL-4 directs switching in these cultures was provided by the finding that IL-4 alone induces germline γ1, γ3, and γ4 transcripts, but not γ2 transcripts, and that antibody to CD40 does not induce germline transcripts for any of the four subclasses, nor does it synergize with IL-4 to further induce Cγ transcripts (Fujieda *et al.*, 1995). These results differ somewhat from the results discussed previously, in which it was shown that IL-4 alone induces germline γ4 transcripts in dense $sIgG^-$ peripheral blood B cells, but no other γ transcripts (Kitani and Strober, 1993). Yet another group, Jumper *et al.* (1994), found that treatment of total peripheral blood B cells with antibody to CD40 for 7 days induces germline γ1, γ3, and γ2 transcripts but not γ4 transcripts, and that addition of IL-4 results in induction of γ4 transcripts. One potentially important difference between these experiments is that all three groups used different types of B cells: Fujieda *et al.* initiated their studies with $sIgD^+$ tonsil B cells, Kitani and Strober used high-density peripheral blood $sIgG^-$ B cells, and Jumper *et al.* used total peripheral blood B cells. Thus, the B cells used by Fujeida *et al.* and by Jumper *et al.* include activated B cells, and in the case of Jumper *et al.* would include previously switched cells. Consistent with this hypothesis, Kitani and Strober (1993) have shown that germline Cγ1 and Cγ3 transcripts are constitutively expressed in both high-density and low-density peripheral blood B cells, but not in high-density IgG^- B cells.

Kitani and Strober (1993) also found that treatment of high-density $sIgG^-$ B cells with IFN-γ alone induces germline γ2 transcripts, but no other germline transcripts. Induction of germline γ1 and γ3 transcripts requires B cell activation (by SAC in this report).

3. *IgA*

Defrance *et al.* (1992) reported that in limiting dilution cultures initiated with purified $sIgD^+$ tonsillar B cells, precursors for IgA-secreting

cells can be induced with antibody to CD40 presented on FcγRII-bearing fibroblasts (L cells) + TGF-β + IL-10 + SAC. In other experiments they demonstrated that optimal IgA secretion (16 μg/ml) was induced by 10 days of treatment with the CD40 antibody-presenting L cells + anti-IgM + TGF-β + IL-10. They obtained about threefold less IgA in the absence of anti-IgM, but the levels were still high. It is possible that the fibroblast line to which the mAb for CD40 is bound is providing an additional signal(s).

These data were extended by Kitani and Strober (1994) who examined the effect of stimulating dense $sIgA^-$ peripheral blood B cells by CD40 antibody presented on L cells, assaying induction of germline α transcripts and mature α mRNA by RT-PCR. Kitani and Strober found that TGF-β alone, CD40 antibody, or SAC alone induce germline α1 and α2 transcripts in these B cells. They demonstrated that the lack of requirement for exogenous TGF-β is because CD40 activation, like other forms of B-cell activation, induces TGF-β synthesis (Kehrl *et al.*, 1986b). Addition of antibody to TGF-β eliminated the germline α transcripts. Expression of mature α1 mRNA was induced by treatment for 3 days with CD40 antibody + TGF-β + IL-10, or by SAC + TGF-β + IL-10, but only the former induction regimen induced α2 mRNA. Addition of IL-4 increased the amount of mature α2 mRNA detected.

IL-10 is required to obtain expression of mature α mRNA is this system, although the mechanism of this effect is unknown. IL-10 has not been shown to induce germline transcripts, and it induces secretion of several human antibody classes, suggesting that it may have a function at the postswitch level (Briere *et al.*, 1994).

Vasoactive intestinal peptide (VIP), given along with antibody to CD40, has been shown to induce secretion of IgA1 and IgA2 by $sIgA^-$ dense tonsillar B cells (Kimata and Fujimoto, 1994). Antibody to TGF-β does not inhibit the VIP-induced IgA secretion. Although the amounts of IgA secreted were very low (100 ng/ml), the induction was about 20-fold and no other isotype was induced. Kimata and Fujimoto point out that VIP levels in intestinal mucosa are high, and thus VIP could be immunomodulator for IgA. It is unknown if VIP itself is directing switching, if it is inducing cytokine production by other cells, or if it has some other indirect effect. Consistent with the latter possibility, they measure IgA production quite late, after 14 days of culture.

4. IgG1 and IgE in Mouse

In the mouse system there are very few reports about the effect of CD40 ligation on class switching, although many published reports document its effect on B cell proliferation and antibody secretion. It has been shown that a soluble CD40-L–CD8α fusion protein in the presence of IL-4 induces

switching to IgG1 by purified resting splenic B cells, by the criterion of mIg expression (Lane *et al.*, 1993; Snapper *et al.*, 1995a). Addition of IL-5 does not increase the percentage of $IgG1^+$ cells. Snapper *et al.* (1995) also found that addition of anti-δ or anti-μ dextran slightly increases the percentage of $sIgG1^+$ cells at optimal doses of CD40-L, but at low doses of CD40-L the effect is synergistic. They also found that anti-δ dextran strongly synergizes with optimal doses of CD40-L to induce proliferation of B cells and secretion of IgG1 and IgE in these cultures. Altogether these data demonstrate that soluble CD40-L in the presence of IL-4 induces switching to IgG1, and also to IgE (by the criterion of Ig secretion), and that the effects of anti-Ig cross-linking are mostly to increase Ig secretion and B cell proliferation.

Activated helper T cells or membranes from activated mouse helper T cells can induce germline γ1 transcripts even in the absence of IL-4 (membranes from T cells from IL-4 knockout mice work), although addition of IL-4 further increases the level of germline transcripts (Schultz *et al.*, 1992). In these experiments Schultz *et al.* (1992) also examined germline γ3,γ2b, ε, and α transcripts and found that membranes from activated T_H cells do not induce these transcripts. Because it has been shown that the most important ligand for B-cell activation present on membranes from activated T cells is CD40 ligand, it is reasonable to expect that cross-linking CD40 on mouse B cells will induce germline γ1 transcripts. Consistent with this hypothesis, it has recently been found that cross-linking CD40 induces germline γ1 transcripts on splenic B cells (L. Strom, A. Miskinien and E. Severinson, unpublished). Furthermore, we have recently found that treatment of the M12.4.1 B cell lymphoma with CD40-L induces the promoter for germline γ1 transcripts in a transient reporter gene assay (Lin and Stavnezer, submitted). The finding that membranes from activated T_H cells in the absence of IL-4 can induce germline γ1 transcripts provides another explanation for why mice bearing a homozygous targeted disruption of their IL-4 can still express IgG1, although they cannot express IgE (Kühn *et al.*, 1991). Germline γ1 transcripts and the promoter can also be induced in the absence of IL-4 by anti-IgM, by anti-δ dextran, or by phorbol ester (Mandler *et al.*, 1993a; Xu and Stavnezer, 1992; Zelazowski *et al.*, 1995).

5. *IgA in Mouse*

The only other isotype which has been tested for the ability to be induced by CD40 signaling in mouse is IgA. It was found that $sIgA^+$ cells are induced by soluble CD40-L + TGF-β, but only a low percentage are induced unless anti-δ dextran + IL-4 + IL-5 are also added (McIntyre *et al.*, 1995). Interestingly, McIntyre *et al.* also found that anti-δ dextran greatly increases the percentages of $sIgA^+$ cells induced by LPS + TGF-β + IL-4 + IL-5. Thus, unlike other isotypes induced by LPS or by

CD40 signaling, induction of switching to IgA in both human and mouse appears to be greatly increased (~10-fold in mouse) by cross-linking of sIg.

In conclusion, although the data are incomplete at this time, signaling via CD40 induces resting B cells to undergo class switching to some isotypes, but there appears to be some isotype specificity to this induction. This isotype specificity is manifest by differential effects of CD40 signaling on germline transcripts; for example, CD40 alone appears to be able to induce germline $\gamma 1$ transcripts in the mouse, but requires synergy with IL-4 to induce germline ε transcripts. Consistent with these results, soluble CD40-L alone induces the germline $\gamma 1$ promoter better than the germline ε promoter in a transiently transfected mouse B cell line, but synergizes better with IL-4 to induce the ε promoter to a higher final level than the $\gamma 1$ promoter (Lin and Stavnezer, submitted; L. Iciek and J. Stavnezer, unpublished data).

As described above, other B cell activators besides CD40 also demonstrate isotype specificity in their induction of germline transcripts. In the case of LPS, it appears that this specificity can be overcome by addition of the appropriate cytokine. For example, LPS induces germline $\gamma 3$ transcripts and this induction can be inhibited by addition of IL-4. It is possible that with addition of the appropriate cytokine CD40, like LPS, will be able to induce switching to any isotype. It appears, however, that the TI-2 mimic, anti-δ dextran, cannot induce switching to all isotypes as it cannot induce switching to IgE (Snapper *et al.*, 1991).

6. CD58 (LFA-3)

Preliminary data have been presented which suggest that a few other receptors/ligands may be able to induce switching. An especially interesting example is CD58, an adhesion molecule on B cells that is up-regulated during activation, for example, on intestinal lamina propria B cells, and whose ligand is CD2, which is present on T and NK cells. CD2 levels are especially high on CD45RO T cells in the intestinal lamina propria. Diaz-Sanchez *et al.* (1994) have found that treatment of purified tonsillar B cells with IL-4 + soluble antibody to CD58 induces secretion of IgE detectably at 7 days, but not maximally until 22 days (>30-fold). A CD40–Fc fusion protein did not block the induction, distinguishing the response from a CD40 signal. Although the response was late, they found that at Day 10 they could detect mature ε RNA by RT-PCR in the cultures stimulated with IL-4 and CD58 antibody, but not in the presence of IL-4 or CD58 antibody alone. The requirement for IL-4 suggests, but does not prove, that they were not simply stimulating a postswitch event. CD58 does not, however, induce germline ε transcripts, nor synergize with IL-4 to induce them. They reported that CD58 + IL-4 does not induce IgG4.

7. *cAMP*

Prostaglandin E_2 (PGE), which induces cAMP production and which is secreted constitutively at high levels by monocytes from atopic (allergic) individuals, synergizes with IL-4 to induce germline ε transcripts in purified mouse splenic B cells (Roper *et al.*, 1995). Treatment with PGE + IL-4 does not induce switching, however, because this requires the subsequent addition of LPS + IL-4. Pretreatment with PGE for 15 hr results in a 10-fold increase in levels of germline ε RNA and mature ε mRNA relative to LPS + IL-4 alone and an increase in secretion of IgE. Another inducer of cAMP, cholera toxin, has been previously shown to synergize with IL-4 to increase germline $\gamma 1$ transcripts and switching to IgG1 in the presence of LPS + IL-4 in experiments performed with total mouse spleen cells (Lycke *et al.*, 1990). It is not known if inducers of cAMP increase switching to all isotypes, although the available data suggest they may also increase switching to IgG2a and IgA (Lycke *et al.*, 1990; Stein and Phipps, 1991).

G. Cytokines Can Regulate Class Switching without Regulating Germline Transcripts: IFN, IL-4, IL-5, and IL-10

In the preveious sections, a few examples were described in which cytokines influenced switching to specific isotypes without regulating levels of the corresponding germline transcripts. IFN-γ treatment of LPS-activated spleen cells reduces switching to IgG3, but does not reduce germline $\gamma 3$ transcripts (Severinson *et al.*, 1990). IFN-γ or -α treatment of human splenic B cells activated with T cells and IL-4 does not reduce levels of germline ε transcripts, but does reduce switching to IgE (Gauchat *et al.*, 1992). It is possible, however, that a kinetics experiment would demonstrate that IFN-γ decreases expression of germline transcripts in these cultures. Shockett and Stavnezer (1991) found that treatment of LPS-induced I.29μ cells greatly inhibits germline α transcripts on Days 1 and 2, but by Day 3 the levels are as high as in the absence of IFN-γ. Switching to IgA is inhibited by 60% by addition of IFN-γ to LFS-activated I.29μ cells.

Treatment of I.29μ cells with antibody to IL-4 inhibits switching to IgA induced with LPS + TGF-β by 80% but has no effect on levels of germline α transcripts, nor on DNA synthesis (Shockett and Stavnezer, 1991). IL-5 is required for switch recombination by splenic B cells induced to switch to IgG1 with anti-δ dextran, although it does not regulate germline $\gamma 1$ transcripts (Mandler *et al.*, 1993a; Purkerson and Isakson, 1992). The levels at which these cytokines regulate switching to these isotypes are unknown.

Recently, IL-10 has been added to this list. IL-10 increases switching by LPS-activated mouse splenic B cells to IgG3 by three- to fivefold, as assayed by sIg expression and by DC-PCR, and yet has no effect on steady-

state levels of germline γ3 transcripts (Shparago *et al.*, submitted). Furthermore, IL-10 decreases switching by approximately fivefold to IgA in splenic B cells induced with LPS + anti-δ dextran + IL-4 + IL-5 + TGF-β, but has no effect on germline α transcripts in these cells. In contrast, if CD40-L is substituted for LPS in the latter experiment, IL-10 has no effect on switching to IgA. Shparago *et al.* also found that IL-10 has no effect on membrane expression of any other isotype. IL-10 inhibits DNA synthesis in both of the LPS-induced cultures, although it increases switching to IgG3 and decreases switching to IgA. It does not inhibit DNA synthesis in the CD40-L-induced culture. IL-10 has also been reported to increase expression of certain isotypes in human B cells cultures, including IgM, IgG1, IgG3, and IgA, but not IgG2 or IgG4. The effects on IgG1 and IgG3 appear to be to increase switching, but germline transcripts were not examined (Briere *et al.*, 1994; Defrance *et al.*, 1992; Kim and Kagnoff, 1990). Shparago *et al.* speculate that because $CD5^+$ B cells synthesize IL-10, this cytokine may be involved in negatively regulating IgA production in T-independent responses in gut-associated lymphoid tissue where $CD5^+$ B cells are localized.

IV. Mechanism of Switch Recombination

A. Switch Recombination Occurs by Intrachromosomal Deletion of Intervening DNA

Several classical studies were published in 1980 in which Southern blotting, electron microscopy, and DNA sequencing were used to analyze the structure of Ig heavy chain genes in plasmacytomas and hybridoma cells lines. The data indicated that switching is effected by recombination between DNA sequences located in the intergenic regions upstream of C_H genes, resulting in deletion of the chromosomal DNA residing between the recombining switch regions from the cell (Coleclough *et al.*, 1980; Cory and Adams, 1980; Davis *et al.*, 1980a; Hurwitz *et al.*, 1980; Rabbits *et al.*, 1980; Sakano *et al.*, 1980; Yaoita and Honjo, 1980). Data were later provided suggesting this deletion event also occurs in culture when mouse splenic B cells are induced with LPS to undergo class switching (Hurwitz and Cebra, 1982; Radbruch and Sablitsky, 1983). These experiments also allowed a preliminary ordering of the C_H genes in mice, which was confirmed when Shimizu *et al.* (1982) cloned the entire Ig C_H locus in overlapping λ phage clones.

Class switching generally results in switching from expression of 5′ C_H genes to expression of more 3′ C_H genes, although early analyses of the products of switch recombination in plasmacytomas suggested that switch recombination occasionally occurs from C_H genes located 3′ on the chromosome to C_H genes located more 5′ (Obata *et al.*, 1981; Radbruch *et al.*, 1980). This

led to the hypothesis that switch recombination occurs by sister-chromatid exchange, although recombination between homologous chromosomes could also explain the data. However, the bulk of the Southern blotting data obtained at this time indicated that deletions of C_H genes occur on both chromosomes, which is more consistent with switch recombination occurring by an intrachomosomal deletion event, rather than by sister-chromatid exchange. Subsequently, very strong evidence supporting the intrachromatid deletion hypothesis, or "looping out" model, was provided by Wabl *et al.* (1985) in an extensive analysis of the products of switch recombination in an Abelson leukemia virus transformed pre-B line, 18-81, in which switching from μ to γ2b heavy chain expression occurs spontaneously (Burrows *et al.*, 1983; Wabl *et al.*, 1985). Wabl *et al.* searched for cells containing three Cμ genes, which would be predicted by a sister-chromatid exchange mechanism or by recombination between homologous chromosomes. They found no such cells. The subsequent finding of excised circles of DNA containing the segment originally residing between the recombined switch regions in cells undergoing switch recombination has further proven that switching occurs by an intrachromosomal deletion (Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; von-Schwedler *et al.*, 1990).

B. Transchromosomal Switching Also Occurs

Although intrachromosomal deletion has been established as the predominant mechanism, it is clear that transchromosomal switching can also occur. Due to the fact that rabbit V_H genes and C_H genes each have allotype markers, it has been possible to ask whether the C_H genes expressed before and after switching are encoded by the same chromosome. Using serological assays, it has been shown that 3–8% of serum IgA in an F1 rabbit expresses the Cα gene of a different haplotype from the V_H gene (Knight *et al.*, 1974). Knight *et al.* (1995) determined the nucleotide sequence of several cDNAs derived from two different chromosomes in F1 rabbits. Because the expressed J_{H4} gene segments from the two haplotypes analyzed have an allelic difference, they were able to demonstrate that transchromosomal recombination occurs during class switching and not during V(D)J recombination. They further argue that the recombination is unlikely to be occurring by RNA splicing because if it were they would expect to find both *cis* and *trans* α chains in one B cell, but they never have. They also effectively rule out the possibility that gene conversion between chromosomes explains the data because the difference in the sequences of the variable regions of the two haplotypes is spread over the entire variable region, thus requiring the hypothetical gene conversion event to extend over a larger distance than gene conversion events observed previously. Thus, these data indicate that switch recombination occurs transchromosomally at a surprisingly high frequency.

These data further suggest that switch recombination may occur at specific location(s) in the nucleus. This possibility is further supported by the finding that an expressed V(D)J-Cμ transgene in mice is able to undergo switch recombination with endogenous heavy chain C_H genes (Gerstein *et al.*, 1990). Gerstein *et al.* found that a transgene VDJ segment could recombine with endogenous Sγ segments. This recombination occurred in all five of the lines of transgenic miced examined and involved both the Cγ1 and the Cγ2a genes. It is possible, however, that transchromosomal class switching in these transgenic mice does not involve normal switch recombination activities since the sites of recombination are localized slightly 5′ to the tandem repeats where switch recombination normally occurs.

C. Does Switching to Isotypes Other Than IgD Occur by RNA Splicing?

As described previously, virgin mature B cells express both IgM and IgD with the same antigenic specificity on their surfaces until activated by antigen and costimulatory signals. A long-running controversy has existed as to whether other heavy chain isotypes can also be expressed by a RNA processing mechanism because investigators have often observed cells which simultaneously stain with antibodies for two different isotypes. No one, however, has ever convincingly purified normal cells expressing two isotypes, nor provided any compelling evidence that normal cells expressing a second isotype other than IgD do so by a nondeletional mechanism. Thus, these double-expressing cells may be a transient population that has recently undergone switch recombination. Further supporting the conclusion that switching always occurs by DNA recombination, Chu *et al.* (1993), using a DC-PCR which quantitatively assays recombination between chromosomal Sμ and Sγ1 regions, demonstrated an excellent correlation between the frequency of switch recombination and the numbers of sIgG$^+$ cells. Their data indicate that the majority of B cells induced with LPS and IL-4 switch to IgG1 by recombination between Sμ and Sγ1 regions on both chromosomes. The recombination events were detected 2.5 days after addition of LPS + IL-4, which is the earliest time at which sIgG1$^+$ cells can be detected. Their data suggest that in this system all switching to IgG1 expression occurs by DNA recombination. These data do not disprove the hypothesis that switching is induced by other mechanisms; for example, a hypothetical mechanism that does not simultaneously induce differentiation to secretion might use a RNA processing mechanism. It might be expected that if cells are being induced to produce lots of antibody, as in LPS induction, recombination would occur in order to make the process more efficient.

Attempts to demonstrate that switching can occur by RNA processing rely on demonstrating that cell populations which express two isotypes have Cμ

genes joined to VDJ segments, as do IgM^+ cells (Perlmutter and Gilbert, 1984; Shimizu *et al.*, 1991). However, in all these systems except one, the double-expressing cells were not demonstrated to be clonal, and thus the cells that contained the $C\mu$ genes may not be the ones expressing the switched isotype. Cells expressing the switched isotype are in the minority, and thus would be difficult to detect by Southern blotting. The one exception is a subclone of the BCL_1 B lymphoma which expresses sIgM and produces variants that also express low levels of sIgG1 in the absence of switch recombination (Chen *et al.*, 1986a, 1986b). Nolan-Willard *et al.* (1992) have presented evidence suggesting that in the double-expressing clones of BCL_1 there may be discontinuous transcription of μ pre-mRNA and a $C\gamma1$ RNA which is initiated 5′ to $C\gamma1$, followed by trans-splicing or ligation. Whether this mechanism occurs in normal cells is completely unknown, but it is clear that if it does occur, it occurs infrequently.

D. Sites of Switch Recombination

Antibody class switching induced by exposure to antigens or certain B cell mitogens *in vivo* or *in vitro* occurs by recombination between or near to switch (S) regions located 5′ to each C_H gene, except $C\delta$. Unlike V-D-J recombination, switch recombination can occur at many different sites within S regions, and there is no precise sequence specificity for the site of recombination within the S regions. S regions consist of G-rich, simple tandem repetitive sequences 1-10 kb in length (Table 1) (reviewed in Gritzmacher, 1989). Mouse $S\mu$, $S\varepsilon$, and $S\alpha$ sequences are composed of variations of pentamers, e.g., GGGGT, GAGCT, and GGGCT. The unit repeat length of $S\mu$ tends to be 20 bp, that of $S\alpha$, 80 bp, and that of $S\varepsilon$, 40 bp (Arakawa *et al.*, 1993; Davis *et al.*, 1980b; Nikaido *et al.*, 1981, 1982; Obata *et al.*, 1981). $S\gamma$ regions also contain these elements, but are organized in 49- or 52-bp repeats (Dunnick *et al.*, 1980; Gritzmacher, 1989; Kataoka *et al.*, 1981; Mowatt and Dunnick, 1986; Nikaido *et al.*, 1982; Sakano *et al.*, 1980; Stanton and Marcu, 1982; Szurek *et al.*, 1985). $S\mu$, $S\alpha$, and $S\varepsilon$ are closely related but the four $S\gamma$ regions are less related to $S\mu$, the homology decreasing with increasing distance from $S\mu$ in the mouse locus. Mouse $S\gamma$ sequences, being at least 70% identical with each other are more closely related to each other than to the other switch regions (Stanton and Marcu, 1982). On Southern blots $S\mu$ and $S\gamma3$ sequences can cross-hybridize, indicating an overall homology of $\geq$70%. Recombination in human B cells also occurs in S regions with similar sequences, although they have a more irregular pattern of repetition (Mills *et al.*, 1990). Table 1 shows sequences of the consensus tandem repeats from the mouse switch regions. The mouse $S\gamma1$ region, which is 10 kb in length, has a complicated pattern of higher order longer direct repeats within it (Mowatt and Dunnick, 1986). The mouse $S\gamma2b$ region also has a higher order of longer direct repeats

TABLE I

STRUCTURAL CHARACTERISTICS OF MURINE SWITCH REGIONS[a]

Region	Consensus Sequence[b]	Length of Repeat Unit (bp)	Approximate Total Length (kb)	Reference
Sμ	GAGCTGAGCTGGGGTGAGCT	10–40	3.2[c]	Nikaido *et al.* (1981)
Sε	GGGCTGGGCTGAGCTGRGCTGAGCTGRGCTGAGCTRARNT	40–50	1.0	Nikaido *et al.* (1982)
Sα	ATGAGCTGGGATGRRCTGAGCTAGGCTGGAATAGGCTGGG CTGGGCTGGTGTGAGCTGGGTTAGGCTGAGCTGAGCTGGA	80	4.2	Arakawa *et al.* (1993)
Sγ3	GGGGACCAGGCTGGGCAGCTCTNGGGGAGCTGGGGTAGGTTGGGAGTGT	49	2.5[c]	Szurek *et al.* (1985)
Sγ1	GGTGACCCAGGCAGAGCAGCTCCAGGGGAGCCAGGACAGGTGGAAGTGT	49	10[c]	Mowatt and Dunnick (1986)
Sγ2b	AGGGACCAGWCCTAGCAGCTRTGGGGGAGCTGGGGAWGGTGGGAATGTG	49	5.0	Kataoka *et al.* (1981)
Sγ2a	GGGACCAGGCAGTACAGCTCTGGGTRGGGRNCAGGCAGTACAGCTCTGNGTG	52	2.5	Nikaido *et al.* (1982)

[a] Much of the information in this table is from Gritzmacher (1989).

[b] Nucleotides: A, adenine; G, guanine; C, cytidine; T, thymidine; R, purine; Y, pyrimidine; W, A or T; N, any nucleotide.

[c] Lengths vary between different strains of mice.

(Wu *et al.*, 1984). The segments surrounding the regions of tandem repeats tend to have the same elements but interspersed with other sequences.

Class switch recombination occurs within the tandem repeats of switch regions in a few B lineage lines in culture: the pre-B line 18-81, the I.29μ B lymphoma line, and in the B cell hybridoma PC1.4 (DePinho *et al.*, 1984; Dunnick *et al.*, 1989; Spira *et al.*, 1994; Stavnezer *et al.*, 1982, 1985). The frequency of switching in these lines is low relative to that of normal B cells. In other B cell hybridomas and in plasmacytomas which undergo class switching at an even lower frequency, recombination occurs exclusively outside the tandem repeats of switch regions, appearing to be mediated by homologous recombination and by recombination between TC repeats (Katzenberg *et al.*, 1989; Kipps and Herzenberg, 1986; Sablitsky *et al.*, 1982). Generally, plasmacytomas and hybridomas appear to "switch" by sister-chromatid exchange or by recombination between homologues, rather than by intrachromatid deletion (Tilley and Birshtein, 1985).

Although the Cδ gene does not have an associated S region consisting of tandem repeats, switching to IgD expression by DNA deletion events does infrequently occur. It might occur at a slightly higher frequency in humans than in mice because higher levels of serum IgD are observed in humans. By examination of genomic DNA in myelomas, it has been determined that switching to IgD occurs by recombination between specific sequences located upstream of the Cμ and Cδ genes having varying lengths and degrees of homology, in both human and mouse (Owens *et al.*, 1991; White *et al.*, 1990; Yasui *et al.*, 1989).

In the most thorough analysis thus far of switch recombination junctions, Dunnick *et al.* (1993) compared more than 150 known sites of switch recombination and made several observations that inform us about the mechanism of switch recombination, some of which had also been noted previously. Consistent with conclusions made by others, they found that the sites of switch recombination in genomic DNA of splenic B cells, hybridomas, myelomas, and lymphomas occur almost exclusively within the tandem repeats of switch regions, with the exception of Sμ. About 40% of recombinations with Sμ occur outside the region of tandem repeats and most of these occur 5′ to the tandem repeats. The sites of recombination in the excised circles are almost exclusively found to be within the tandem repeats of switch regions, even for Sμ, so it has been speculated that the recombinations occurring 5′ to Sμ are secondary events (Dunnick *et al.*, 1993). The sites of recombination occur throughout the regions of tandem repeats. These conclusions differ from those of Iwasoto *et al.* (1992) obtained by examining clones of deleted circles; Iwasoto *et al.* found the recombinations to be localized at the 5′ end of Sμ and the 3′ end of Sα. Dunnick *et al.* suggest this apparent bias in the deleted circles may

be due to the fact that the λ bacteriophage vector has quite stringent size requirements, thus restricting the possible recombination junctions which might be able to be cloned. Dunnick *et al.* also concluded that secondary recombinations occur only very rarely after cell transformation, within myeloma and hybridoma cell lines, because sites of recombination in a particular cell line determined by disparate labs at different times are in complete agreement.

Dunnick *et al.* (1993) questioned whether there are homologies between the donor and acceptor switch regions at sites of recombination. They found that if they compared the actual frequency of recombination at short bits (≥2 bp) of identity between the two switch regions to the frequency predicted if the sequences were random there was some preference for recombination at these bits of identity. However, this analysis was by comparison to random sequences, whereas switch regions are not random because they have numerous homologies, so they tentatively concluded there may be no preference for recombination at homologies. It would be interesting for a similar analysis to be performed in comparison to sequence elements present in switch regions.

E. Sequential Switching Can Occur

In addition to switch recombination between Sμ and other S regions, abundant evidence demonstrates that switching frequently occurs between downstream switch regions, in the 5′ to 3′ direction. Numerous examples of sequenced switch region junctions from chromosomal DNA indicate that recombination occurs between a variety of switch regions (Dunnick *et al.*, 1993; Mills *et al.*, 1992; Petrini *et al.*, 1987a; Siebenkotten *et al.*, 1992). Although junctions in excised switch circles also often contain sequences suggesting sequential switching, e.g., Sμ–Sγ1–Sε junctions, these data do not provide as strong evidence for sequential switching because theoretically they could be derived from recombination within the switch circle subsequent to excision.

It has been proposed that switching to IgE in mouse B cells usually occurs via an intermediate switch to IgG1 for several reasons: switching to IgE is a much rarer event than switching to IgG1, switching to IgE in LPS-activated cultures requires addition of much more IL-4, it occurs with slower kinetics than switching to IgG1, and it can be inhibited by addition of anti-IgG1 to the cultures (Mandler *et al.*, 1993b; Siebenkotten *et al.*, 1992). Furthermore, it was found that if sIgG1$^+$ cells were purified by sorting from these cultures, they could be induced to secrete IgE upon reculture (Mandler *et al.*, 1993b). Restriction enzyme mapping data also suggest that splenic B cells which switch to IgE have switched to IgG1 on the nonexpressed chromosome more frequently than to any other isotype.

However, it has been shown that an IgG1 switch is not an obligate intermediate because mice with a homozygous deletion of the Iγ1 exon and promoter, whose B cells cannot switch to IgG1, express just as much IgE as normal mice (Jung *et al.*, 1994). Furthermore, when B cells from these mice are cultured with LPS + IL-4, IgE switching occurs even more frequently than in normal mice, suggesting that there may be competition between IgG1 and IgE for switch recombination with Sμ.

Altogether the data suggest that switching to IgG1 may occur more frequently than to IgE, and that $IgG1^+$ cells may appear more rapidly than IgE^+ cells perhaps because germline γ1 transcripts can be more readily induced than ε transcripts. Unlike germline ε transcripts, γ1 transcripts can be induced by anti-Ig or by CD40 ligation and require much less IL-4 for induction. The data also indicate, however, that IgG1-expressing cells can be induced to switch to IgE.

Sequential switching has also been demonstrated to have occurred in IgE^+ human B cells by finding rearranged ε genes having recombined Sγ–Sε sequences (Mills *et al.*, 1992). The sequences of these recombined switch regions indicate that switching can occur from any of the four subclasses to IgE (Mills *et al.*, 1995). In a different study, nucleotide sequences were determined of several examples of recombined switch regions in IgE^+ cells from atopic patients and from B cells from these patients induced with EL-4 T cells + PMA to switch to IgE (van der Stoep *et al.*, 1994). No evidence of sequential switching was found, as all switch regions had Sμ–Sε junctions. Thus, these data further support the conclusion that switching is autonomously determined, and there is no programed switching from one specific switch region to another. This conclusion is consistent with the variety of switch regions found to be joined together in the analysis of excised switch circles and genomic switch junctions (Matsuoka *et al.*, 1990; Dunnick *et al.*, 1993).

1. Germline Iμ Transcripts

In addition to germline transcripts for the downstream C_H regions, germline μ transcripts that initiate heterogeneously within the μ intron enhancer have been identified and characterized (Lennon and Perry, 1985; Nelson *et al.*, 1983). Furthermore, these transcripts continue to be expressed after switch recombination. After switching, transcription initiating at the Iμ exon continues through the recombinant switch junction and through the newly recombined C_H gene (Li *et al.*, 1994). These hybrid transcripts appear to be constitutively expressed after switching, and Li *et al.* proposed that they may participate in sequential switching events. This same group has shown that measurement of the levels of these recombinant

Iμ transcripts by S1 assay provides a sensitive assay for switch recombination.

F. Do the Sites of Switch Recombination Have Additional Fine Sequence Specificity?

Several investigators have noted that sites of recombination occur preferentially near particular short motifs within switch regions, including YAGGTTG (Marcu *et al.*, 1982), TGAG, AGCT, TGGG, and GGGG (Nikaido *et al.*, 1982; Petrini *et al.*, 1987b), CCAG (Chou and Morrison, 1993), or G/AA/G GCT (Dunnick *et al.*, 1993). Although the data are intriguing, the correlation is never 100%, frequently being 80% at best, even allowing the motif to be on either side of the junction and at a variable close distance from it. However, some of these sequences are present more frequently than expected by chance, even after taking into account the fact that these particular sequences are present at higher than random frequency simply because they contain portions of the consensus repeat sequences. One of the most convincing examples is the sequence CCAG or its complement CTGG, which is present within seven nucleotides on either side of 77% of the 70 switch recombination sites analyzed, which differs from chance ($p = 0.00048$) (Chou and Morrison, 1993). This sequence is also found more frequently near sites of nonhomologous recombination in B cells (92% of the 14 analyzed) than in non-B cells (28% of 35 sequences). Because the CCAG sequence is not found at 23% of switch recombination sites, it may not be required, or alternatively it may be involved in an early step such as endonucleolytic digestion and then removed during subsequent exonuclease or DNA synthesis events. See below for a discussion of the mechanism of switch recombination. CTGG is also found at sites of recombination which effect a switch to IgD (Chou and Morrison, 1993). Interestingly, this sequence is a portion of the *Chi* sequence, 5′ GCTGGTGG 3′, which is important for directing recombination in prokaryotes. *Chi* is present at a higher frequency within switch regions than in the rest of the genome (Kenter and Birstein, 1981). CTGG is within the Sμ consensus repeat (Table 1) and within the binding site for a nuclear protein (NF-Sμ or SNUP) that binds to the Sμ repeat (Wuerffel *et al.*, 1990).

Data have been provided indicating that 80% of 45 switch recombination events between Sμ and $S_{\gamma 3}$, $S_{\gamma 1}$, or $S_{\gamma 2b}$ regions occur within the 5′ 60% of the Sγ tandem repeat when the repeat unit is as shown in Table 1 (Kenter *et al.*, 1993; Wuerffel *et al.*, 1992). This frequency is greater than expected by chance ($p = 0.01$). Kenter and colleagues hypothesize that this preference is due to the localization of particular protein-binding sites

in the consensus repeats. See below for a discussion of proteins and factors known to bind switch regions.

G. Switch Recombination Sites Are Often Associated with Mutations

By sequencing five different products of a single switch recombination event which occurred in I.29μ B lymphoma cells, it was found that point mutations, insertions, and deletions accompany switch recombination (Dunnick and Stavnezer, 1990; Dunnick *et al.*, 1989). Among the five independent λ phage clones containing the shared recombination site, the DNA sequences fell into two sets, one of which appeared to have mutations in the Sμ region. Subsequent analyses of other switch recombination sites also indicate that mutations appear to occur only on one side of the junction (Dunnick *et al.*, 1993). The mutations extend at least 200 bp from the site of recombination. These data suggest that an error-prone DNA synthesis event is involved in switch recombination.

H. An Illegitmate Priming Model for Antibody Class Switching

From these data, Dunnick *et al.* (1993) proposed the illegitimate priming model (also called the copy-choice model) for switch recombination shown in Fig. 3 (Dunnick *et al.*, 1993; Dunnick and Stavnezer, 1990). Recombination is initiated by a double-strand break, followed by exonuclease digestion, creating a single-strand end (donor sequence), which anneals by short segments of homology to the acceptor switch region and primes DNA synthesis using the acceptor switch sequence as a template (Figs. 3A and 3B). A reciprocal event involving a priming event using the downstream switch region sequence as a primer on upstream switch region sequence also occurs. These DNA synthesis events actually effect the recombination, and due to the frequent occurrence of mutations they appear to be error prone. If the two priming events are not opposite each other a gap may result, which will be filled using either or both strands as templates (Fig. 3C). The existence of this gap may explain the finding that sometimes sequences at sites of switch recombination do not appear to derive from either parental switch region. After switch recombination, the recombined chromosome replicates by normal mechanisms and the chromatids segregate, with the result that one daughter cell has a newly synthesized mutated donor switch region and the parental acceptor switch sequence (Fig. 3D). The other daughter cell receives the mutated acceptor switch region and the parental donor switch region.

This model can be reconciled with the existence of circles of excised switch regions. One possibility would be that prior to creation of the double-strand breaks which initiate recombination, the two switch regions

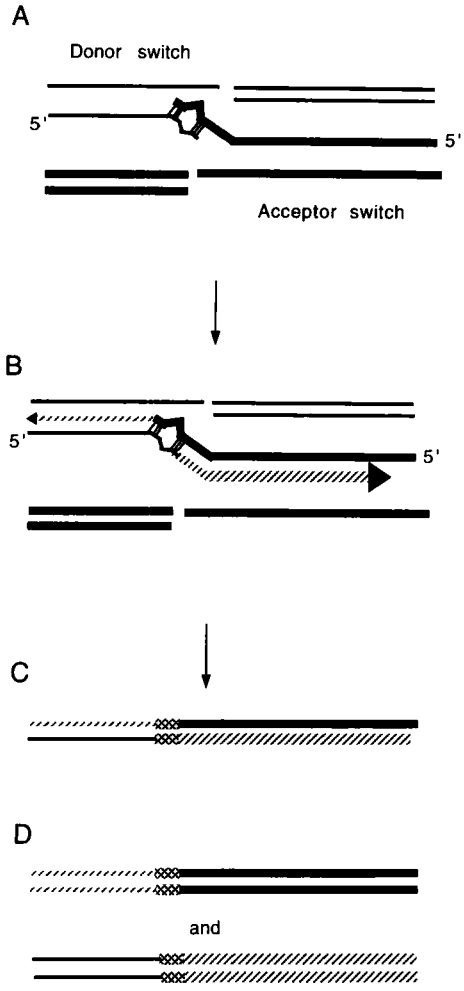


FIG. 3. Diagram of illegitimate priming model for switch recombination (Dunnick *et al.*, 1993). Parental donor and acceptor switch regions are indicated by the solid lines and are thin for donor switch and thick for acceptor switch regions, respectively. Newly synthesized strands are hatched and thin for donor switch switch region and hatched and thick for acceptor region. See text for further description.

which will be recombined are brought into juxtaposition. Thus, when the breaks are made the ends of the excised DNA segment simply ligate to create the circle. This model is similar to the finding for V-D-J recombination, in which the actions of exonuclease and terminal transferase are restricted to the coding sequences and do not occur on the excised circles

containing signal joints. This model, however, does not fit with the finding of mutations in the excised switch circles (Dunnick *et al.*, 1993). Thus, it appears more likely that after the double-strand breaks (which must occur in a concerted fashion in order to create circles), exonuclease activity and illegitimate priming of error-prone DNA synthesis also occur on the excised segment to effect circle formation.

The illegitimate priming model requires that the short segments of homology between different switch regions be sufficient for priming DNA synthesis, whereas it is believed that DNA synthesis generally involves a perfect match between primer and template. Thus, this model predicts that proofreading mechanisms should be inhibited during switch recombination. This prediction is consistent with the finding that inhibition of a ubiquitous nuclear enzyme, poly (ADP-ribose) polymerase (PARP), which is involved in mismatch repair, increases the frequency of switch recombination by fivefold in I.29μ cells and by a lesser, variable extent, in spleen B cells (Shockett and Stavnezer, 1993). Inhibition of PARP may stimulate switching by preventing DNA repair which would remove the single-strand ends and double-strand breaks, which presumably are intermediates in switch recombination, and also by preventing destruction of the primer–template hybrid by mismatch repair. Inhibition of PARP has previously been shown to stimulate homologous recombination between two nearby thymidine kinase genes (Waldman and Waldman, 1991).

1. Homeologous Recombination

The finding that inhibition of PARP stimulates switching suggests that switch recombination may be similar to homeologous recombination. Homeologous recombination is the term applied to recombination that occurs in eukaryotes between similar but not identical sequences (Mezard *et al.*, 1992). The reported examples of homeologous recombination appear to use mechanisms similar to those used in homologous recombination because the frequency of recombination is higher among more closely related sequences, the products of recombination indicate that the recombining segments align during the recombination process to match their overall homology, and identities of 2–20 nucleotides are present at the sites of recombination. Furthermore, homeologous recombination is stimulated by mutations in genes encoding mismatch repair enzymes (Bailis and Rothstein, 1990).

Switch recombination, however, differs somewhat from the typical model for homeologous recombination because genes whose sequences are 70% homologous recombine correctly by this mechanism, i.e., recombination results in preservation of their overall structure, whereas sequences which are 52% homologous misalign when they recombine. Sμ and some

Sγ regions differ by more than 70% overall, and the entire switch regions do not appear to align, because recombination does not occur at comparable sites within a tandem repeat between the donor and acceptor sequences (Dunnick *et al.*, 1993).

The two models for homologous (and homeologous) recombination that are most favored are the single-strand annealing (SSA) model (Lin *et al.*, 1984) and the double-strand break repair (DSBR) model (Szotak *et al.*, 1983). Both of these models have similarities to the illegitimate priming model. In the SSA model two single-strand ends (one from the donor and one from the acceptor) anneal with each other and then recombine by mechanisms involving DNA synthesis, repair, and ligation. In the DSBR model, a single-strand end invades an intact DNA duplex, followed by strand displacement involving DNA synthesis, repair, and ligation. These types of mechanisms could occur in switch recombination, but switch recombination also differs somewhat from both these models in that double-strand breaks occur on both the donor and the acceptor sequences because excised circles are produced. Because there are two sets of breaks in switch recombination, the ends must be held together and the recombination must be concerted in order to prevent loss of the chromosome. The model for switching described previously will be expanded after discussion of possible functions of germline transcripts.

I. Possible Roles for Germline Transcripts

Extremely interesting results using gene knockout technology to analyze the function of germline transcripts have been published by Alt's and Radbruch's groups. Both groups demonstrated that if the promoter-Iε or promoter-Iγ1 segments are replaced by heterologous strong promoters (an Ig μ intron enhancer/V_H promoter cassette or a metallothionein promoter, respectively), each of which were shown to drive high levels of transcription, switching occurs only very infrequently on that allele (Bottaro *et al.*, 1994; Lorenz *et al.*, 1995) (See Figs. 4D and 4E). These data indicate that transcription *per se* is not sufficient to direct switching to the switch region. Lorenz *et al.* (1995) further showed that if a 114-bp DNA segment containing the splice donor for the Iγ1 exon is restored back to its original location and immediately 3′ to the metallothionein promoter, switching is recovered (Fig. 4F). LPS alone induces switching to IgG1 on this allele. Addition of the splice donor segment greatly increases (approximately 20-fold) the transcription rate driven by the metallothionein promoter as measured in nuclear run-on assays, and also greatly increases the stability of the RNA produced as assayed by a Northern blot. The increased transcription rate and steady-state levels of RNA do not appear to account for the increased ability to switch, however, because in the experiments of

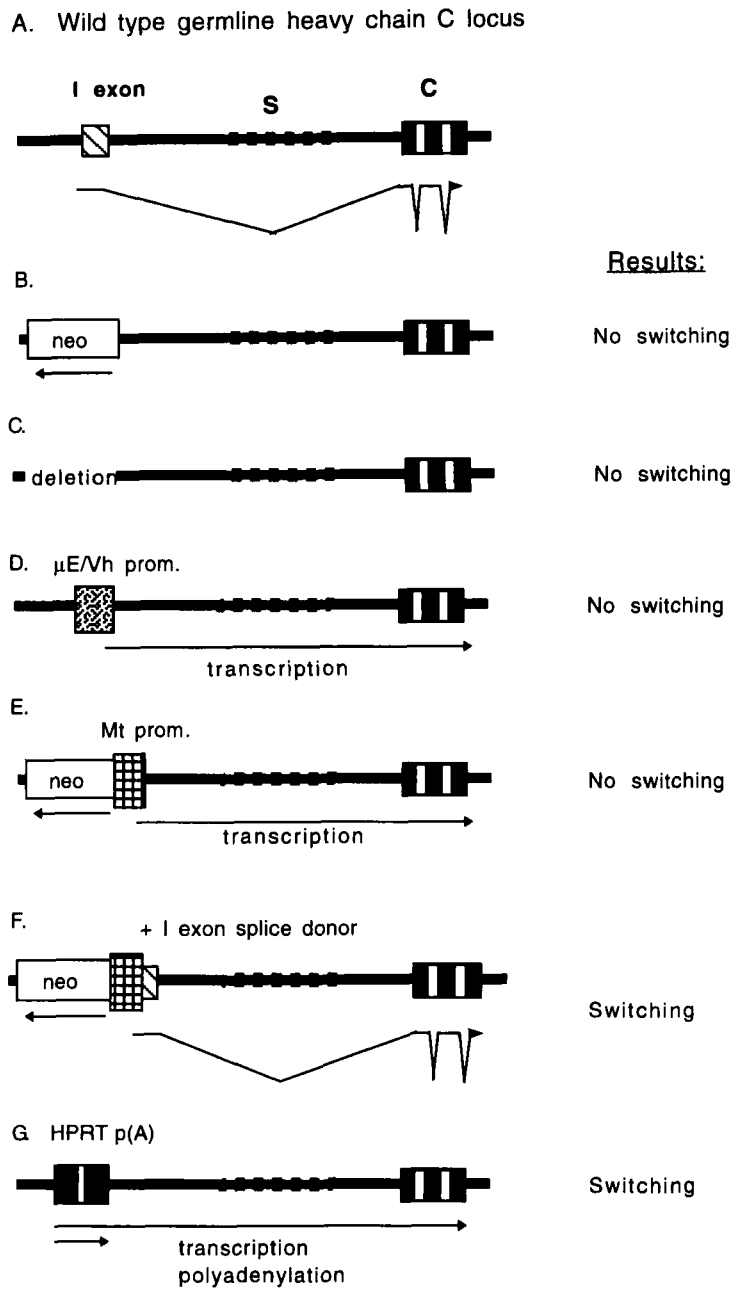


FIG. 4. Diagrammatic representations of germline I exon knockout mutations and results. See text for further description of mutations and results. A, diagram of wild-type allele; B, Zhang *et al.* (1993); C, Jung *et al.* (1993); D, Bottaro *et al.* (1994); E and F, Lorenz *et al.* (1995); G, Harriman *et al.* (1996).

Bottaro *et al.* (1994) (Fig. 4D) abundant amounts of large, probably unspliced, transcripts are obtained from the allele driven by the Ig μ enhancer/V_H promoter, as detected on Northern blots, and yet switching does not occur. Lorenz *et al.* suggest that the 114-bp DNA segment is probably not functioning by providing a binding site on the chromosome for factors necessary for switch recombination because the sequence of the segment containing the splice donor is not conserved between human Iγ4 and mouse Iγ1. In conclusion, the results of both Alt's and Radbruch's groups suggest that a splice donor in the transcript may be necessary for switching.

Because abundant amounts of transcripts from the mutated germline allele are detectable in the experiments of Bottaro *et al.* (1994), in which significant switching does not occur, it appears likely that the presence of a stable transcript theoretically capable of hybridizing with the allele from which it is transcribed is not sufficient for directing switch recombination. These data suggest that germline RNA does not function *in trans*, although a possible caveat is that the transcripts in these cells are unspliced. Consistent with the lack of a *trans* effect, previous attempts to inhibit switching with antisense oligonucleotides or transfected plasmids transcribing antisense RNA for germline α transcripts have been equivocal at best (Wakatsuki and Strober, 1993), or negative (G. Qiu and J. Stavnezer, unpublished data). The remarkable mitogenic effect on B cells of an antisense oligonucleotide complementary to the Iγ2b exon, reported by Tanaka *et al.* (1992), was probably due to the fact that the oligonucleotide contained an unmethylated CpG sequence, which has been demonstrated to be mitogenic for B cells (Krieg *et al.*, 1995).

The data from the knockout mice described previously are most consistent with the hypothesis that the splice donor is necessary, although there is no evidence in any system that splice donors contribute to recombination. One possibility is that the splice donor attracts a nuclear factor involved in switch recombination. Because RNA splicing appears to occur in domains in cell nuclei containing localized concentrations of splicing factors, it is possible that the binding of a splicing factor localizes both of the switch regions to be recombined to specific domains in the nuclei (Xing *et al.*, 1993). Another possibility is that the splice donor could be attracting enzymatic activities involved in DNA ligation or DNA repair. Both of these hypotheses are unprecedented.

Recent results obtained from another interesting knockout mouse in which the germline Iα exon was replaced by a mini hypoxanthyl ribosyl transferase (HPRT) gene in the sense direction suggest another explanation for the requirement of a splice donor (Harriman *et al.*, 1996) (Fig. 4G). The replacement of the Iα exon with the HPRT gene does not reduce switching to IgA, as these mice have normal levels of serum IgA, and B

cells from these mice are induced by LPS to switch to IgA. TGF-β does not further increase switching. The HPRT gene is followed by a poly(A) site and gives rise to a stable readily detectable RNA species. Harriman *et al.* have also demonstrated that transcription continues through the intron and Sα region and into the Cα gene, although no spliced RNA containing both HPRT and Cα sequences is detectable, even by a PCR assay. Thus, it appears that transcripts initiated at the HPRT gene are endonucleolytically cut and polyadenylated, but that transcription continues through the Sα region–Cα segment. Because the HPRT gene has an intron, and thus presumably undergoes splicing, the intron may provide the function of the normal I exon splice donor. It is difficult to imagine, however, how splicing of a transcript in which both the donor and the acceptor are on the same side of the switch region would help to localize proteins or enzyme activities to switch regions.

It appears more likely that the presence of a splice donor or a poly(A) site upstream of the switch region causes an endonuclease cleavage of the primary transcript, thus allowing the intron to be excised from the exons, which form the germline RNA and leave the DNA template. This might allow the intron RNA to remain associated in a complex with the duplex switch region DNA segment. This hypothesis is supported by data described below.

1. RNA–Duplex Switch Region DNA Complex

Two provocative papers have demonstrated that if a plasmid containing a 2.3-kb segment of the Sα region is transcribed *in vitro* with T7 RNA polymerase in the direction it is normally transcribed *in vivo*, a purine-rich portion of the RNA product remains in a complex with the double-stranded DNA template, but not if it is transcribed in the antisense direction with T3 polymerase (Reaban and Griffin, 1990; Reaban *et al.*, 1994). Previously, it was reported that Sα sequences can form triple-stranded DNA structures, but results presented by Reaban *et al.* (1994) suggest that a triple-strand DNA structure is not involved in the RNA–DNA complex. Generally, newly synthesized RNA leaves the template as it is transcribed, but it has been demonstrated in other systems, i.e., yeast and *Escherichia coli*, that RNA, which is highly G rich, as are transcripts from switch regions, can stably complex with the duplex DNA template (Xu and Clayton, 1995). For example, the beginning of the RNA transcript which initiates at an origin of replication in yeast mitochondrial DNA has a 17-nt sequence termed CSBII, GGGGGAGGGGGTGGGTG, followed by a more normal type of sequence. Transcripts that are 228 nt in length, beginning with this G-rich sequence, remain associated with the DNA template, as assayed by RNase A resistance and RNase H sensitivity (the

latter enzyme being specific for RNA in a RNA–DNA hybrids). If the CSBII sequence is inverted causing the product RNA to have a C-rich sequence instead, it does not remain associated with the DNA template. The G-rich sequence normally begins 4 nt from the site of initiation, and if this distance is increased, formation of this complex with yeast mitochondrial DNA is greatly decreased. If ≥90 nt precedes the G-rich sequence no complex is formed. Because switch regions are at least 1 kb 3′ to the splice donor of the I exons, it is not obvious that such a complex could form with switch region transcripts, although one could speculate, in the absence of data, that formation of such a complex with switch region sequences might only be possible if splicing of the germline RNA helped to stabilize it by removing the I exon sequences 5′ to the switch region. Switch regions consist of much longer stretches of G-rich sequences than present in mitochondrial DNA, which might help stabilize the complex. Another consideration is that not all RNA polymerases are equally capable of generating such complexes (Xu and Clayton, 1995).

The significance of such putative RNA–duplex DNA complexes is unknown, although it is known that triple-strand DNA structures are intermediates in homologous recombination and that RecA binds to, aligns, and stabilizes triple-strand structures (Hsieh *et al.*, 1990). Furthermore, a recent paper suggests that RNA may promote homologous recombination mediated by RecA. The standard assay for RecA-mediated recombination involves assaying creation of joint molecules formed between a single-strand circle and a duplex DNA homologous to a portion of the single-strand circle. Using such an assay, it has been shown that transcription of a 224-bp segment of the Sγ1 region in the duplex DNA increases the frequency of RecA-mediated recombination between a duplex DNA and a single-strand circle containing the $S_{\gamma 1}$ segment (Kotani and Kmiec, 1994). Transcription reduces the length of homology required from greater than 50 bp to less than 15 bp. In these experiments the RNA was found to remain in a complex of both strands of the duplex DNA and the single-strand circle, although the nature of the complex was not determined. In order to promote recombination, the RNA transcript has to be complementary to the single-strand circle, but it can consist of either the G-rich top strand or the C-rich bottom strand of the Sγ1 insert (E. Kmiec, personal communication). The fact that the C-rich RNA also promotes recombination in this assay may be surprising, but this RNA still remains in the complex in their experiments.

Thus, it is possible that RNA transcribed from the switch regions may remain in a complex with the duplex genomic switch region and be involved in stimulating the invasion of one switch region by the other. It is possible that the RNA may distort the duplex to aid in strand invasion or alignment

of a single-strand end of one switch region with another switch region. RNA may stabilize the recombination complex by participating in a three-stranded complex which may be recognized by a RecA-like activity.

A different model for the role of such a RNA–DNA complex is that the RNA serves to target the endonuclease activity required to initiate switch recombination, perhaps by distorting the DNA duplex. RNA transcription has been shown to initiate within 4 hr after treatment of splenic B cells with IL-4 and LPS prior to detection of nicks in the switch region (see below) and before switch recombination, which is first detected at 2.5 days by the DC-PCR assay (Berton, *et al.*, 1989; Chu *et al.*, 1993; Kenter and Wuerffel, 1995).

Yet another possible role of germline transcripts is to attract DNA repair machinery, as it is known that an actively transcribed template DNA strand is repaired five times more rapidly than the rest of the genome (Bohr *et al.*, 1985; Mellon *et al.*, 1987). Some components of the nucleotide excision repair machinery necessary for repair of mismatches in DNA have affinity for RNA polymerase II bound to DNA and appear to have activity in both transcription and in DNA repair (reviewed by Drapkin *et al.*, 1994). In particular, one factor which is deficient in one type of Cockayne's syndrome is attracted to stalled RNA polymerase II and appears necessary for coupling repair to transcription. If germline transcription were to play this role, it should be independent of RNA splicing or stable germline transcripts, and it should only depend on transcription. Thus, attraction of DNA repair machinery cannot be the entire function of germline transcripts.

J. Control of Switch Recombination during the Cell Cycle

It has also been shown that switching occurs in cells undergoing proliferation and that inhibition of DNA synthesis prevents switch recombination (Kenter and Watson, 1987; Lundgren *et al.*, 1995; Severinson-Gronowicz *et al.*, 1979). Furthermore, analysis of elutriated cells suggests that germline $\gamma 1$ transcripts are present in LPS + IL-4 − activated mouse splenic B cells in G1 and S phases, are slightly reduced in G2 and M phases, and are absent from Go cells in these cultures (Lundgren *et al.*, 1995). Lundgren *et al.* suggest that cell cycle regulation of germline $\gamma 1$ transcripts occurs at the transcriptional level because proteins which bind near the start sites of germline $\gamma 1$ transcripts show cell cycle regulation being most abundant in G1 and S phases, much reduced in G2 and M phases, and absent in Go cells. In addition, the data suggest that during G2 and M phases the complexes leave the DNA and reappear during the next cell cycle.

It is expected that switch recombination would be completed by the end of S phase in order to repair any double-stranded breaks which would otherwise prevent proper chromosome duplication. Thus, the data of Lundgren *et al.* (1995) support the hypothesis that germline transcripts are

present at the time switch recombination appears most likely to occur, during late G1 and early S phase.

K. Protein-Binding Sites within Consensus Tandem Repeats of Switch Regions

Several protein complexes have been demonstrated to bind near or at switch regions. Some have only a few binding sites, whereas others bind to the tandem repeat sequences. Although very litte is known about most of these proteins and even less about their role in switching, the information available is very interesting and indicates that further study will be fruitful.

1. NF-κB/p50 and E47/E12

As mentioned previously, Kenter and colleagues (Kenter *et al.*, 1993; Wuerffel *et al.*, 1992) have analyzed Sγ sequences for localization of sites of recombination with Sμ and found that switch recombination occurs more frequently in the first 30 bp of the 49-bp repeats of the $S_{\gamma 3}$, $S_{\gamma 1}$, and $S_{\gamma 2b}$ regions than expected by chance. They find a 14-bp motif within this region that has a somewhat higher concentration of junctions (two times more frequent than if random) ($p = 0.003$) and that binds nuclear protein complexes they term SNAP in electrophoretic mobility shift assays. They have provided evidence indicating that the complexes contain proteins identical to or similar to the helix–loop–helix E2 box-binding proteins E47 and E12 (Kenter and Hu, submitted). Furthermore, their data suggest that the complexes bind cooperatively and form tetramers, and that the binding is inducible by LPS + dextran sulfate (Wuerffel *et al.*, 1992). E47 and E12 have previously been shown to bind to the Ig μ intron enhancer (Ephrussi *et al.*, 1985; Murre and Baltimore, 1992).

Just 5′ to the A site, at the first 10 bp of the consensus repeat of the Sγ3 sequence, is a binding site for a complex they term SNIP, which they have demonstrated to be an NF-κB/p50 homodimer (Kenter *et al.*, 1993; Wuerffel *et al.*, 1992). Although the sequences of p50 binding sites are not always recognizeable in every consensus repeat, even sites that appear quite diverged from a consensus κB site have been shown to bind p50 (Kenter *et al.*, 1993). Furthermore, due to cooperative binding by NF-κB, it appears likely that most of the repeats do indeed bind p50. Kenter *et al.* hypothesize that both the NF-κB/p50 and the SNAP complexes are important for specifying the sites of switch recombination.

2. Localization of Induced Double-Stranded Breaks in Sγ3 Regions

Kenter and colleagues have detected, by linker-mediated PCR, double-stranded (ds) breaks at a specific nucleotide sequence within the tandem repeats of the Sγ3 region induced 1 day after LPS treatment of splenic B

cells (Kenter and Wuerffel, 1995). The ds breaks occur at the GGGG/A sequence present in both the p50 and the SNAP sites. The breaks are detected within 18 hr (the earliest time point examined) after treatment with LPS, but they are not detected in uninduced B cells (A. Kenter and R. Wuerffel, manuscript in preparation). However, the cell type specificity is unknown. Due to their specificity in sequence and occurrence after mitogen activation, these double-stranded breaks appear to be strong candidates for the initiating events in switch recombination.

3. *NF-κB/p50 Knockout Mice*

It is clear, however, that NF-κB/p50 is not required for switch recombination, although it is important for normal levels of switching. Deletion of the gene encoding the precursor for NF-κB/p50 by gene targeting produces mice with an impaired ability to express some classes of serum antibody and also certain classes of antigen-specific antibodies secreted in response to a T-dependent antigen (Sha *et al.*, 1995). However, not all four IgG subclasses are reduced to the same extent, although it appears that at least three consensus Sγ tandem repeats bind NF-κB/p50 (Kenter *et al.*, 1993). It is unknown if Sε and Sα regions also bind NF-κB/p50.

In p50 knockout mice, there is an incomplete correspondence between the IgG subclasses reduced in serum antibody and those reduced in an antigen-specific response. For example, IgG1 is reduced 40- and 10-fold, respectively, in the antibody specific for a T-dependent antigen and in natural serum antibody relative to wild-type littermates, and IgG2b and IgG3 are reduced 40-fold in a T-dependent response, but less than 2-fold in natural serum antibody. Since Sha *et al.* (1995) measured Ig production rather than sIg or actual switch recombination, the effect of p50 could be partially due to an effect on regulation of synthesis of antibody, and/or proliferation of cells after switching. B cells from these mice do not proliferate in response to LPS.

It has been demonstrated that a NF-κB/p50-binding site in the promoter for germline ε transcripts is necessary for transcription of a reporter gene driven by the promoter for germline ε RNA in mouse B cell lines (Delphin and Stavnezer, 1995). In agreement with this, the level of serum IgE in the p50 knockout mice is reduced 40-fold. Furthermore, splenic B cells from these mice induced to switch in culture with CD40-L + IL-4 + IL-5 do not transcribe germline ε RNA, nor do they switch to IgE (Snapper *et al.*, 1996). In addition, there are three NF-κB sites in the promoter for mouse germline γ1 transcripts which are required for induction of transcription by CD40-L, consistent with the 40-fold reduction of IgG1 in a T-dependent antibody response found in the p50 knockout mice (Lin and Stavnezer, submitted). Although there is a NF-κB/p50 site in the

germline α promoter, mutation of this site does not reduce activity of the promoter in transient transfection assays in two B cell lines induced with TGF-β and/or PMA (Shi and Stavnezer, submitted). This result may be consistent with the small reduction (4-fold) in IgA levels in natural serum antibody found in the p50 knockout mice, although it is inconsistent with the finding of a large reduction in the ability of splenic B cells to be induced to switch to IgA with CD40-L + anti-δ dextran + IL-4 + IL-5 reported by Snapper *et al.* (1996). This latter result suggests that p50 may be required for another process besides germline transcription in the switch to IgA.

In conclusion, NF-κB/p50 appears to have several different activities that are known to be, or are likely to be, important for class switching because it contributes to regulation of some germline promoters, binds to the tandem repeats of Sγ, and is also necessary for proliferation in response to the B cell mitogen LPS.

4. *Sμbp-2*

Another protein, Sμbp-2, which binds a segment of the Sμ repeat and is induced by LPS in splenic B cells, has been described. The cDNA has been cloned from both human and mouse cells by Honjo and co-workers (Fukita *et al.*, 1993; Mizuta *et al.*, 1993). This protein binds phosphorylated single-stranded oligonucleotides containing 10 nucleotides of the Sμ repeat, with the requirement that the oligonucleotide has a 5′ G and at least one stretch of 3 G's. They speculate that Sμbp-2 is a helicase, as it has seven conserved motifs found in helicases. The activity of this protein is probably not specific for switch recombination because it is expressed in every tissue examined. Because it binds single-stranded DNA, Fukita *et al.* suggest that it may be involved in recognition and unwinding of G-rich DNA.

5. *NF-Sμ*

Other protein-binding sites have been defined in the tandem consensus repeats, but the proteins which bind have not been defined. These sites include a complex called NF-Sμ (and renamed SNUP) which binds a double-stranded oligonucleotide containing 15 bp of the Sμ tandem repeat GAGCTGGGGTGAGCT and is induced within 20 hr by LPS + dextran sulfate treatment of spleen cells (Wuerffel *et al.*, 1990). It is unknown whether NF-Sμ is B cell specific. Variations of the Sμ consensus sequence do not bind as well, nor do double-stranded oligonucleotides containing only 10 nucleotides of the repeat unit; thus, this protein has different binding specificities from Sμbp-2.

L. Proteins That Bind to a Single Site or a Few Sites in Switch Regions

Two DNA-binding proteins have been identified in mouse B cells which bind to sequence motifs present in or near switch regions that are not elements of the consensus repeats: Pax-5/BSAP/Sα-BP/NF-HB and LR1. Pax-5 and LR1 are each present in pre-B and B cell lines, but not in terminally differentiated plasma cells, T cells, fibroblasts, or any other lineage tested; Pax-5 is also expressed in specific regions of the developing central nervous system. Each has been shown to act as a transcriptional activator in B cells when its binding site is present in a promoter, but Pax-5 is also a repressor when bound to the 3′ Ig α enhancer. LR1 is induced by LPS in splenic B cells, but Pax-5 is constitutive although it can be further induced. These proteins may have additional functions in isotype regulation besides regulation of transcription because each binds to a few sites 5′ to or within some switch regions and the functions of these sites have not been determined.

1. LR1

LR1 was first defined as a LPS-inducible protein that binds to a site in the Sγ1 region and which may also bind to sites in Sγ3 and the Ig μ intron enhancer (Williams *et al.*, 1993; Williams and Maizels, 1991). The authors speculate from sequence analysis that there exist multiple sites for LR1 in these switch regions, but they have not addressed this possibility experimentally and the consensus sequence required for binding has not been determined. Binding to the Sγ1 site is induced within 2 hr of treatment of mouse splenocytes with LPS, but is constitutive in B lymphoma lines from human and mouse (Brys and Maizels, 1994; Williams and Maizels, 1991). Binding is inhibited by treatment of nuclear extracts with acid phosphatase. A binding site for LR1 has been defined in the c-*myc* promoter and shown to be important for transcriptional activity of the promoter in reporter gene assays (Brys and Maizels, 1994). LR1 has been partially purified, but its gene has not been cloned (Williams *et al.*, 1993).

2. Pax-5/BSAP

Much more is known about Pax-5. Pax-5 is a member of a family of regulatory genes encoding the paired domain box proteins, which are conserved during evolution and are important regulators of development (Adams *et al.*, 1991; Gruss and Walther, 1992; Strachan and Read, 1994). Pax-5 is expressed equally in pro-B, pre-B, and mature sIg^+ B cells where it can be further induced by LPS. It is not expressed in plasmacytoma cells (Barberis *et al.*, 1990; Waters *et al.*, 1989; Watsuki *et al.*, 1994). By creation of a knockout mutation of the Pax-5 gene, Urbanek *et al.* (1994)

have demonstrated that Pax-5 is essential for differentiation of B lineage cells because homozygous knockout mice have no B lineage cells more mature than large $B220^+$ $CD43^+$ cells present in the bone marrow. These mice have no serum Ig. Pax-5 is known to bind to the promoters of a few genes expressed very early in the B lineage. Pax-5 has been shown to bind to a site in the promoter for the B cell surface protein CD19, which is a coreceptor with sIg, and to activate transcription when this site is placed upstream of a minimal β-globin promoter (Kozmik *et al.*, 1992). Binding sites for Pax-5 are also found in the promoters for $\lambda 5$, VpreB1, and *blk* (Okabe *et al.*, 1992; Zwollo and Desiderio, 1994).

Antisense oligonucleotides or antisense transcripts of Pax-5 have been shown to greatly reduce proliferation of splenic B cells in response to LPS and to also reduce the constitutive proliferation of the sIg^+ B cell lymphoma CH12.LX (Wakatsuki *et al.*, 1994). Pax-5 antisense oligonucleotides do not inhibit proliferation of plasmacytoma or T cell lines, consistent with the lack of expression of Pax-5 in these cells. The location of binding sites for Pax-5 which might regulate expression of genes required for proliferation is unknown.

Binding sites for Pax-5 are located 5′ to several switch regions: Sμ, Sγ1, Sγ2a, Sε, and Sα and also within, but at the 5′ end, of Sα, but the roles of most of these sites have not been defined (Liao *et al.*, 1992; Waters *et al.*, 1989; Xu *et al.*, 1992). Evidence suggesting that Pax-5 may be involved in class switching comes from the finding that it binds to the promoter for germline ε transcripts and that mutation of this site greatly decreases expression of this promoter in transient transfection experiments (Liao *et al.*, 1994).

3. *Pax-5 and the 3′ Cα Enhancer*

Two sites for Pax-5 are located within the 3′ Cα enhancer, which is located 16 kb 3′ to Cα in the mouse and 25 kb 3′ to Cα in the rat (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991; Neurath *et al.*, 1994; Pettersson *et al.*, 1990; Singh and Birshtein, 1993). These sites have been shown to function as negative elements in transient transfection reporter gene assays of the function of the 3′ Cα enhancer in sIg^+ B cells, but to have no function in plasmacytoma cells, as expected from the lack of Pax-5 in plasmacytoma cells. Neurath *et al.* (1995) examined the mechanism of this inhibition and found that the binding of Pax-5 to one of the two sites in the 3′ α enhancer prevents binding of an Ets family member to the enhancer which functions as a transcriptional activator in the B cell lymphoma CH12.LX. They further demonstrated that prevention of binding of Pax-5 to this site by the addition of oligonucleotides capable of forming a triple-stranded structure with the Pax-5-binding site increases binding of

the Ets family protein, enhancer activity, and levels of mature α, γ2b, or γ3 mRNA, depending on the particular CH12.LX cell line used. The level of Ig μ mRNA was not increased. Thus, these data suggest that one function of the 3′ α enhancer is to increase transcription from the heavy chain locus in mature plasmacytoma cells after class switching and after Pax-5 is no longer expressed. Inhibition of binding of Pax-5 to the 3′ Cα enhancer has no affect on proliferation (W. Strober, personal communication).

In conclusion, it appears that Pax-5 is required for development of the B cell lineage and for proliferation of B cells, and it also stimulates transcription of germline ε transcripts prior to class switching; however, Pax-5 also inhibits transcription of mature mRNAs for γ2b, γ3, and α heavy chains. The functions of the binding sites for Pax-5, which are located 3′ to the I exons and within several S regions, are still unknown, however.

M. The 3′ Cα Enhancer Regulates Expression of Germline Switch Transcripts and Class Switching

Although the mechanism of the effect is unknown, the 3′ α enhancer is very important in regulating class switching because mice with a homozygous knockout of this enhancer show pronounced defects in switching to most isotypes (Cogne *et al.*, 1994). Reminiscent of the NF-κB/p50 knockout phenotype, not all isotypes are reduced. The phenotype differs, however, from the p50 knockout because B cells from these mice proliferate as well as wild-type B cells in response to LPS and the isotype specificity differs somewhat. Switching induced by LPS or by LPS + IL-4, as assayed by expression of sIg and also by secretion, is greatly reduced to all isotypes except to IgG1. IgA was not examined in the LPS cultures. The effect of the loss of the 3′ Cα enhancer on switching appears to be due to its effects on germline transcripts, because Cogne *et al.* showed that in the knockout mice LPS does not induce germline γ transcripts (of any subclass), nor does treatment with LPS + IL-4 induce germline ε transcripts.

Natural serum antibody is not as reduced as is Ig secreted by the LPS-activated B cells in culture as only IgG3 and IgG2a are significantly reduced (10- to 100-fold). Much of the natural serum antibody is believed to be secreted by CD5$^+$ B cells, which are believed to be induced by cross-linking sIg (Wortis *et al.*, 1995). LPS induction of B cells does not utilize the same signaling pathways as does signaling by sIg. The 3′ Cα enhancer may be important for induction of germline transcripts that are induced by LPS, but not for those induced by Ig cross-linking. Consistent with this hypothesis, germline γ1 and γ2b transcripts can be induced by anti-δ dextran, and the 3′ Cα enhancer knockout mice have normal levels of these serum antibody classes (Cogne *et al.*, 1994; Zelazowski *et al.*, 1995). Also consistent with this hypothesis, these mice have serum IgA, and it

has been shown that phorbol ester induces the germline α promoter (Nilsson and Paschalis, 1993; Shi and Stavnezer, submitted). Regulation of germline $\gamma 3$ transcripts is inconsistent, as has been found before. Germline $\gamma 3$ transcripts are inducible by anti-δ dextran, although serum IgG3 is greatly reduced in these mice. The effect of the 3′ Cα enhancer on T-dependent switching has not been reported.

In conclusion, the data suggest that the 3′ Cα enhancer regulates transcription of several germline RNAs during B cell activation by LPS plus cytokines. At this stage in differentiation, Pax-5 is probably bound to the 3′ Cα enhancer because it is present in mature sIg^+ B cells and is further up-regulated by LPS. This is consistent with data indicating that Pax-5 binding is required for optimal expression of germline ε transcripts by LPS + IL-4 induction. After switching, expression of Pax-5 is lost, allowing the binding of an Ets family transcription factor to the 3′ Cα enhancer, which contributes to activation of transcription of mature Ig heavy chain mRNAs. How the 3′ Cα enhancer functions in these roles is unknown, although Madisen and Groudine (1994) have proposed that it serves as a locus control region because it has copy number-independent enhancer activity.

N. Switch Recombination Substrates

It has been a long-standing goal of several groups to create transfectable substrates that are suitable for assaying switch recombination. The development of such assays for V-D-J recombination allowed a detailed characterization of the specificity requirements of this recombination and was crucial to the identification and cloning of *rag-1* and *rag-2* genes, two lymphoid-specific genes required for V-D-J recombination. It has also allowed identification of several DNA repair genes required for this recombination. Several groups have reported different approaches to creation of plasmids or retroviral vectors containing switch regions that could be used to assay switch recombination in cell transfection assays. None of the switch substrate plasmids that have been developed so far are completely satisfactory because none of them show inducible recombination which occurs specifically within switch regions, although some have properties suggesting that some switch recombination activities are being assayed.

1. Retroviral Switch Recombination Vector

The first successful attempts to develop switch vectors were published by Ott and Marcu, in which they used a retroviral vector containing a 1.8-kb segment of Sμ tandem repeats, followed by a *Herpes simplex* virus thymidine kinase (TK) gene, followed by a 1.4-kb segment having the 3′ end of the Sγ2b region, followed by a gene encoding resistance to G418 (Neo^R gene) (Ott *et al.*, 1987; Ott and Marcu, 1989). This vector was used

to infect several TK-negative cell lines: pre-B lines, a B cell hybridoma, and a fibroblast line. Function of the TK gene was lost more frequently in pre-B cells ($\sim 10^{-4}$) than in a B cell hybridoma (10^{-5}) or fibroblast line ($\sim 10^{-7}$), as assayed by bromodeoxyuridine (BUdR) resistance. One of the pre-B lines used (18-81) has been shown to undergo switch recombination of its endogenous genes (Burrows *et al.*, 1983; DePinho *et al.*, 1984). The hybridoma used undergoes infrequent class switching of its endogenous genes, but presumably not by recombination within switch regions (Sablitsky *et al.*, 1982). Because retroviral vectors have LTRs, which contain strong promoter and enhancer activities, this vector is probably actively transcribed in all cell types studied, and thus its cell type specificity is presumably not due to lack of accessibility. Ott and Marcu demonstrated that BUdR-resistant pre-B cell clones had deleted the TK gene by recombination between the two switch region segments of the vectors. In contrast, the BUdR-resistant hybridoma and fibroblast clones retained the TK gene, and therefore must have inactivated it by some other mechanism. Upon mapping and sequencing of the recombination sites in the pre-B cells, they found that the sites are localized within or near the tandem repeats which are present in only a subregion of each of the switch fragments used in the vector. They also demonstrated that replacing the Sγ2b fragment with a segment of the c-*myc* gene which is involved in chromosomal translocation reduces recombination 100-fold, further indicating the specificity for switch regions. The switch substrate construct of Ott and Marcu is attractive because the recombination shows cell type specificity, as far as has been examined, and the recombination is localized near tandem repeats within the switch region segments of the vector. This latter result contrasts with results obtained from plasmids that replicate as episomes, which are described below.

2. *Transient Assays Using Episomal Vectors*

Another approach that has been taken by three other labs is to create a plasmid that replicates as an episome in order to be able to recover replicated plasmids after transient transfection. By inserting a 3.6-kb segment containing the origin of replication and initiation sites for both early and late transcription from the mouse DNA virus Polyoma, transfected plasmids will replicate and can be recovered and subsequently used to transform *E. coli* cells. This is the same type of vector which has been successfully used to assay V-D-J recombination. Using this approach, Leung and Maizels (1992, 1994) created a plasmid containing in addition to the Polyoma segment : in the 5′ to 3′ direction, a cassette containing the Ig μ intron enhancer and a V_H promoter, followed by a 2.2-kb Sμ segment, followed by a λ phage promoter (λP_L, which interferes with plasmid replica-

tion except in *E. coli* containing particular lysogenic phage), followed by a 2.5-kb Sγ3 segment. If recombination results in deletion of the λ phage promoter, the plasmid recovered from transiently transfected cells will be able to transform an *E. coli* strain lacking a λ lysogen. The protocol is to transfect spleen cells after activation with LPS for 3 days, continue to culture with LPS for 2 additional days, and then to recover the plasmid. Plasmids are treated with the restriction enzyme *DpnI*, which digests plasmids that have not undergone replication (due to the presence of a bacterial pattern of methylation), and recombination is assayed by the ability to transform nonlysogenic *E. coli*. Leung and Maizels find that 25% of the replicated plasmids delete the λ P_L promoter, although most of the recombinations involve sequences outside the switch region segments: 78% are 5′ to Sμ and 86% 3′ to Sγ3. The frequency of recombination in a T cell line was 7%, only threefold reduced compared to LPS-induced spleen cells.

A cytomegalovirus (CMV) promoter can substitute for the Ig enhancer/promoter, but if no enhancer or promoter are present, recombination is reduced by 10-fold. Leung and Maizels also demonstrated that the Ig μ intron enhancer alone, in either orientation, promotes an identical frequency of recombination, although it is a much weaker promoter in the absence of the V_H promoter segment (Leung and Maizels, 1994). Further evidence that transcription across switch regions is not necessary for recombination in this plasmid is their finding that if the Ig enhancer/promoter cassette is placed 3′ to the Sμ segment, recombination is only reduced 2-fold. They find that the position of the enhancer/promoter or CMV promoter appears to serve as a target for the site of recombination, i.e., when these activator elements are located 3′ to Sμ, fewer of the recombination junctions occur 5′ to Sμ and more occur within Sμ. Because of this result, Leung and Maizels speculate that the activator elements in the Polyoma origin fragment may also serve as targets for recombination events, which may help to explain the high frequency of recombinations outside of switch regions reported in all three types of switch recombination vectors containing the Polyoma origin fragment (see below). In conclusion, although these plasmids have a high frequency of recombination that appears to depend on an enhancer element, there is very little evidence that switch recombination activities are being assayed in these experiments because most of the recombinations occur outside the switch regions and recombination is only 3-fold lower in the one non-B cell type examined.

Daniels and Lieber (1995) have taken a similar approach, but have solved some of the problems in the Leung and Maizels experiments. Their plasmids also have the 3.6-kb fragment containing the Polyoma origin and T antigen gene, and switch recombination is also assayed in transient

transfection experiments. Their plasmids differ from those of Leung and Maizels by having viral promoters upstream of each switch region and by having a *supF* tRNA gene positioned between the Sμ and Sγ3 segments, which when expressed, produces blue bacterial colonies and when deleted, produces white colonies. Although the high percentage of bacterial colonies which are white in plasmids recovered 20 hr after transfection demonstrates high levels of recombination in a variety of B cells and non-B cells, Daniels and Leiber discovered that only in pre-B and sIg^+B cell lines do the recombinants continue to increase 20 hr after transfection. They suggest that immediately after transfection the plasmids may be nicked and repaired in all cell types, but only in these B-lineage lines does recombination continue. The percentage of colonies which are white increases linearly between 20 and 48 hr after transfection, which they report as percentage recombination per hour. It is approximately 1% per hour in several B cell lines and in two pre-B lines, and 3–50 times lower in T cells and nonlymphoid cells.

To determine whether recombination is localized to switch regions, Daniels and Lieber have placed a kanamycin-resistance gene immediately upstream of the viral promoter located 5′ to Sμ and also an origin of replication of the plasmid immediately 3′ to Sγ3. They found that 50% of the recombinations delete the kanamycin gene, and of the ones that do not, 50% still recombine 5′ to Sμ, i.e., within the viral promoter segment. Thus, only about 25% of the sites of recombination occur within the Sμ region, whereas about 60% of endogenous switches occur within Sμ. Any recombination occurring 3′ to Sγ3 would not result in a recoverable plasmid. Sequences required for localization of endogenous switch recombination sites may simply be missing from the plasmid, and/or a portion of the recombination events assayed may be due to the activities of enzymes not involved in switch recombination. The fact that many of the recombination events are not B cell specific would support, but not prove, the latter possibility.

Furthermore, if one replaces each switch segment with the same eukaryotic gene segment (nonswitch sequences), which could presumably undergo homolgous recombination, the frequency of recombination is only reduced 2- to 4-fold, again suggesting that recombination activities other than switch recombination may be contributing to the events observed. Like Leung and Maizels, Daniels and Lieber find that deletion of one of the two promoters has no affect on frequency of recombination, and deletion of the other promoter only reduces the frequency by 2-fold. Most interestingly, they find that if the switch segments are inserted in reverse, but maintaining their polarity relative to each other, so that they are transcribed backwards, recombination frequency is reduced 65-fold. In contrast, re-

versing the direction of the two identical nonswitch DNA segments has no affect on their recombination frequency. These data strongly suggest that the mechanism of recombining the switch segments differs from homologous recombination.

In conclusion, the experiments of Daniels and Lieber suggest that transiently transfected switch plasmids containing a Polyoma origin of replication may assay switch recombination activities due to their finding of B cell specificity for continuation of recombination after the initial repair events and their intriguing finding that reversed switch regions do not recombine. However, the lack of localization of the recombination events to switch regions suggests that many of the events detected may be due to nonswitch recombination activities. This conclusion is consistent with the finding of high levels of recombination events at the 20-hr time point in T cells and fibroblasts (M. Lieber, personal communication).

3. *Recombination of Episomal Switch Plasmids in Stably Transfected Cells*

A third, but related, approach has been taken by Lepse *et al.* (1994) who have also used episomal plasmids containing the identical Polyoma origin fragment, but they select for stably transfected cells that have undergone recombination by the ability to express a neomycin-resistance gene (neo^R). Their constructs have (in the 5′ to 3′ direction) a herpes virus TK promoter, expressed in all cell types tested, followed by a Sγ2b or Sγ2a segment, a transcription terminator from the bovine growth hormone gene, a Sμ segment, and a neo^R gene. Thus, the plasmids contain the switch segments in reversed positions relative to the endogenous genes, although they have normal polarity relative to transcription. If recombination between switch region segments occurs, the transcriptional terminator is deleted and the neo^R gene is expressed. The plasmids are transfected, subjected to treatment with G418 2 days later, and after a few weeks neo^R-resistant clones are counted and analyzed by Southern blotting. Although the frequency of recombination cannot be determined because the authors cannot score cells that have not undergone recombination, they do find that the appearance of wells containing G418-resistant cells shows that there is excellent B cell specificity. Thirty-three of 120 wells of the pre-B line 18-81 contained G418-resistant cells, compared to 0 of 40 wells of a myeloma or a T cell line and 1 of 40 wells of a fibroblast line had G418-resistant cells. A positive control plasmid which expresses neomycin resistance constitutively yielded 37 of 60 G418-resistant lines, suggesting the recombination frequency in 18-81 cells may be quite high. By preliminary Southern blotting of a few lines, Lepse *et al.* (1994) determined that most of the recombinations occurred within switch regions. These results

do not necessarily support a role for switch recombination activities, however, because recombinations outside the switch regions would prevent G418 resistance, either by deleting the TK promoter or the neo^R gene itself. The strongest argument for their conclusion that this plasmid assays switch recombination is the excellent B cell specificity.

4. Summary of Results Using Switch Recombination Vectors

A compilation of all results from episomal switch plasmids containing the Polyoma origin indicates that there is evidence for B cell-specific recombination in these plasmids if one excludes the early events which may be due to repair of plasmids nicked during transfection. These non-B cell-specific activities may be the source of the large amount of recombination events which occur outside the switch region segments of these plasmids. Although one can exclude some of these events by selectable markers placed on either side of the switch region fragments, some nonspecific events may also occur within switch regions. The results of Lepse *et al.* (1994) suggest that if one excludes the events that occur outside of switch regions, the recombinations in the switch regions are B cell specific, suggesting that they may be mostly due to switch recombination activities.

Unlike endogenous recombination, transcription may not be necessary to obtain recombination in these plasmids, although it may help target the recombination (Daniels and Lieber, 1995; Leung and Maizels, 1994). However, placement of the switch regions in the reverse orientation relative to transcription very much reduces the frequency of recombination, whereas homologous recombination is unaffected by reversing the orientation of the fragments. This result suggests that accessibility, or open chromatin, is not sufficient for directing recombination, and that recombination of switch segments in these plasmids may utilize activities specific for switch recombination.

The results using retroviral vectors appear to show much more specificity than those using the transiently transfected plasmids in that recombination appears to occur generally within the switch regions and to occur in pre-B cells, but not in a hybridoma or fibroblast line. The drawback to this vector is the overall low frequency of recombination, which is much lower than observed for the transiently transfected vectors.

All three approaches previously described show signs of specificity for switch recombination, and yet none are completely satisfactory for utilization in experiments aimed at understanding the mechanism and sequence specificity of switch recombination, nor for an assay for enzymes and other factors involved in switch recombination. An important criterion of specificity has yet to be demonstrated: that recombination within switch

plasmids is induced under conditions in which endogenous switching is induced.

O. Revisit Models for Switch Recombination

After discussion of all the data described previously, it is useful to reconsider the models for switch recombination. The initial event in switch recombination is likely to be a double-stranded break, due to the finding of excised switch circles. The finding of nicks and double-strand breaks induced at specific sites in tandem repeats of Sγ3 regions by LPS treatment is consistent with this expectation. It is unknown, however, whether endonuclease acts specifically on switch regions that will undergo switching or on all switch regions, nor if the breaks occur only in B cells.

Germline transcripts could be involved in formation of a RNA–double-strand DNA complex which directs the initiating endonuclease cuts. If the induced double-strand breaks are only detected in transcribed switch regions, this model will be supported.

Another possibility is that all switch regions are subject to double-strand breaks, but most breaks are simply repaired before the next cell division by an active DNA repair mechanism. The endonuclease that creates the breaks could be an ubiquitous enzyme with specificity for stretches of G nucleotides (Cote *et al.*, 1989). In this case some of the proteins which bind to switch regions may help to more specifically target the endonuclease activity because NF-κB/p50 and some members of the helix–loop family are ubiquitous. Switch regions that will undergo switch recombination would be treated differently, presumably due to the presence of germline RNA, which may initiate strand invasion/alignment of switch regions by the formation of the RNA–DNA complex.

The requirement for the splice donor or poly(A) site may be to enable the formation of such a RNA–duplex DNA complex by cutting away the exons. The apparent lack of a requirement for a splice donor or poly(A) site upstream of the switch segments in the switch plasmids may be because transcription from these plasmids initiates relatively near the switch segments. The effect of adding a splice donor to these plasmids has not yet been tested.

DNA repair mechanisms might be inhibited specifically at sites of double-strand breaks that subsequently undergo switch recombination. This is possible for several reasons. Because switching appears to have some degree of localization in the nucleus due to the observation of trans-chromosomal recombination, it may be possible to exclude DNA repair enzymes from this site. It has been found that PARP, for example, is concentrated in domains in the nucleus. It is possible that the splice donor of the germline transcript is important because one or more components

of the splicing machinery contribute to bring the switch regions to a specific domain in the nucleus. Polyadenylation may also occur in the same nuclear domains, which is consistent with the suggestion that a poly(A) site may substitute for the splice site. According to this hypothesis for the requirement for a splice donor, splice donors might not be required for recombination within the artifical switch constructs because these constructs contain switch sequences very closely positioned with each other, and thus may not require a mechanism for colocalization.

ACKNOWLEDGMENTS

I am grateful to Amy Kenter, Eva Severinson, Wes Dunnick, Penny Shockett, Kenan Murphy, Martin Gellert, Ken Marcu, and Selina Chen-Kiang for discussions leading to a greater understanding and insights into class switching. I also thank Amy Kenter, Michael Lieber, Gregory Harriman, Cliff Snapper, and Eric Kmiec for sharing unpublished data. I thank Wes Dunnick and Amy Kenter for suggestions for this manuscript. I acknowledge support from the National Institutes of Health, Grant AI23287. Its contents are solely the responsibility of the author and do not necessarily represent the official views of the National Institutes of Health.

REFERENCES

Adams, D. H., Hathaway, M., Shaw, J., Burnett, D., Elias, E., and Strain, A. J. (1991). TGF-β induces human T lymphocyte migration in vitro. *J. Immunol.* **147,** 609–612.

Albrecht, B., Peiritsch, S., and Woisetschlager, M. (1994). A bifunctional control element in the human IgE germline promoter involved in repression and IL-4 activation. *Int. Immunol.* **6,** 1143–1151.

Allen, R. C., Armitage, R. J., Conley, M. E., Rosenblatt, H., Jenkins, N. A., Copeland, N. G., Bedell, M. A., Edelhoff, S., Disteche, C. M., Simoneaux, D. K., Fanslow, W. C., Belmont, J., and Spriggs, M. K. (1993). CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* **259,** 990–993.

Allman, D. M., Ferguson, S. E., Lentz, V. M., and Cancro, M. P. (1993). Peripheral B cell maturation. II. Heat stable antigen^{hi} splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* **151,** 4431–4444.

Andersson, J., Coutinho, A., and Melchers, F. (1978). The switch from IgM to IgG secretion in single mitogen-stimulated B-cell clones. *J. Exp. Med.* **147,** 1744–1754.

Arakawa, H., Iwasato, T., Hayashida, H., Shimizu, A., Honjo, T., and Yamagishi, H. (1993). The complete murine immunoglobulin class switch region of the α heavy chain gene-hierarchic repetitive structure and recombination breakpoints. *J. Biol. Chem.* **268,** 4651–4655.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L., Stenkamp, R., Neubauer, M., Roberts, R., Noelle, R., Ledbetter, J., Franke, U., and Ochs, H. (1993). The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* **72,** 291–300.

Assoian, R. K., Fleurdelys, B. E., Stevenson, H. C., Miller, P. J., Madtes, D. K., Raines, E. W., Ross, R., and Sporn, M. B. (1987). Expression and secretion of type β TGF by activated human macrophages. *Proc. Natl. Acad. Sci. USA* **84,** 6020–6024.

Bailis, A. M., and Rothstein, R. ((1990). A defect in mismatch repair in Saccharomyces cerevisiae stimulates ectopic recombination between homeologous genes by an excision repair dependent process. *Genetics* **126,** 535–547.

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C. V., Liu, Y. J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annu. Rev. Immunol.* **12,** 881–922.

Bancroft, G. J., Schreiber, R. D., Bosma, G. C., Bosma, M. J., and Unanue, E. R. (1987). A T-cell independent mechanism of macrophage activation by interferon-γ. *J. Immunol.* **139,** 1104–1110.

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1990). A novel B-cell lineage-specific transcription factor present at early not late stages of differentiation. *Genes Dev.* **4,** 849–859.

Ben-Sasson, S., LeGros, G., Canrad, D., Finkelman, F., and Paul, W. (1990). Cross-linking Fc receptors stimulate splenic non-B, non-T cells to secrete interleukin 4 and other lymphokines. *Proc. Natl. Acad. Sci. USA* **87,** 1421–1425.

Bergstedt-Lindqvist, S., Moon, H.-B., Persson, U., Moller, G., Heusser, C., and Severinson, E. (1988). Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. *Eur. J. Immunol.* **18,** 1073–1077.

Bergstedt-Lindqvist, S., Sideras, P., Macdonald, H. R., and Severinson, E. (1984). Regulation of Ig class secretion by soluble products of certain T-cell lines. *Immunol. Rev.* **78,** 25–50.

Berton, M. T., Uhr, J. W., and Vitetta, E. (1989). Synthesis of germline γ1 immunoglobulin heavy-chain transcripts in resting B cells: Induction by interleukin 4 and inhibition by interferon γ. *Proc. Natl. Acad. Sci. USA* **86,** 2829–2833.

Berton, M. T., and Linehan, L. A. (1995). IL-4 activates a latent DNA-binding factor that binds a shared IFN-γ and IL-4 response element present in the germ-line γ1 promoter. *J. Immunol.* **154,** 4513–4525.

Berton, M. T., and Vitetta, E. S. (1990). Interleukin 4 induces changes in the chromatin structure of the γ1 switch region in resting B cells before switch recombination. *J. Exp. Med.* **172,** 375–378.

Berton, M. T., and Vitetta, E. S. (1992). IL-4-induced expression of germline γ1 transcripts in B cells following cognate interactions with T helper cells. *Int. Immunol.* **4,** 387–396.

Bohr, V. A., Smith, C. A., Okumota, D. S., and Hanawalt, P. C. (1985). DNA repair in an active gene: Removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. *Cell* **40,** 359–369.

Bossie, A., and Vitetta, E. S. (1991). IFN-γ enhances secretion of IgG_{2a} from IgG_{2a}-committed LPS-stimulated murine B cells: Implications for the role of IFN-γ in class switching. *Cell. Immunol.* **135,** 95–104.

Bottaro, A., Lansford, R., Xu, L., Zhang, J., Rothman, P., and Alt, F. (1994). I region transcription (*per se*) promotes basal IgE class switch recombination but additional factors regulate the efficiency of the process. *EMBO J.* **13,** 665–674.

Briere, F., Servet-Delprat, C., Bridon, J.-M., Saint-Remy, J.-M., and Banchereau, J. (1994). Human IL-10 induces naive surface IgD^+ B cells to secrete IgG1 and IgG3. *J. Exp. Med.* **179,** 757–762.

Brys, A., and Maizels, N. (1994). LR1 regulates c-myc transcription in B-cell lymphomas. *Proc. Natl. Acad. Sci. USA* **91,** 4915–4919.

Burrows, P. D., Beck-Engeser, G. B., and Wabl, M. R. (1983). Immunoglobulin heavy-chain class switching in a pre-B cell line is accompanied by DNA rearrangement. *Nature* **306,** 243–246.

Carayannopoulos, L, and Capra, J. D. (1993). Immunoglobulin: Structure and function. *In* "Fundamental Immunology," Raven Press, pp. 283–314. New York.

Castigli, E., Alt, F. W., Davidson, L., Bottaro, A., Mizoguchi, E., Bhan, A. K., and Geha, R. S. (1994). CD40-deficient mice generated by recombination-activating gene-2-deficient blastocyst complementation. *Proc. Natl. Acad. Sci. USA* **91,** 12135–12139.

Chen, Y.-W., Word, C. J., Dev, V., Uhr, J. W., Vitetta, E. S., and Tucker, P. W. (1986a). *J. Exp. Med.* **164,** 562–579.

Chen, Y.-W., Word, C. J., Jones, S., Uhr, J. W., Tucker, P. W., and Vitetta, E. S. (1986b). *J. Exp. Med.* **164,** 548–561.

Chou, C. L., and Morrison, S. L. (1993). A common sequence motif near nonhomologous recombination breakpoints involving Ig sequences. *J. Immunol.* **150,** 5350–5360.

Chu, C. C., Max, E. E., and Paul, W. E. (1993). DNA rearrangement can account for in vitro switching to IgG1. *J. Exp. Med.* **178,** 1381–1390.

Chu, C. C., Paul, W. E., and Max, E. E. (1992). Quantitation of immunoglobulinμ-γ heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA* **89,** 6978–6982.

Coffman, R., and Carty, J. (1986). A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J. Immunol.* **136,** 949–956.

Coffman, R. L., Lebman, D. A., and Rothman, P. B. (1993). Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* **54,** 229–270.

Coffman, R. L., Lebman, D. A., and Shrader, B. (1989). Transforming growth factor-β specifically enhances IgA production by lipopolysaccharide-stimualated murine B lymphocytes. *J. Exp. Med.* **170,** 1039–1044.

Coffman, R. L., Ohara, J., Bond, M. W., Cary, J., Zlotnik, A., and Paul, W. E. (1986). B cell stimulatory factor-1 enhances the IgE responses of lipopolyasaccharide-activated B cells. *J. Immunol.* **136,** 4538–4545.

Cogne, M., Lansford, R., Bottaro, A., Zhang, J., Gorman, J., and Alt, F. W. (1994). A class switch control region at the 3′ end of the immunoglobulin heavy chain locus. *Cell* **77,** 737–747.

Coleclough, C., Cooper, D., and Perry, R. P. (1980). Rearrangement of Ig H chain genes during B lymphocyte development as revealed by studies of mouse plasmacytoma cells. *Proc. Natl. Acad. Sci. USA* **77,** 1422–1426.

Collins, J. T., and Dunnick, W. A. (1993). Germline transcripts of the murine immunoglobulin γ_{2a} gene: Structure and induction by IFN-γ. *Int. Immunol.* **5,** 885–891.

Cooke, M. P., Heath, A. W., Shokat, K. M., Zheng, Y., Finkelman, F. D., Linsley, P. S., Howard, M., and Goodnow, C. C. (1994). Immunoglobulin signal transduction guides the specificity of B cell–T cell interactions and is blocked in tolerant self-reactive B cells. *J. Exp. Med.* **179,** 425–434.

Cory, S., and Adams, J. (1980). Deletions are associated with somatic rearrangement of immunoglobulin heavy chain genes. *Cell* **19,** 37–51.

Cote, J., Renaud, J., and Ruiz-Carrillo, A. (1989). Recognition of (dG)n.dC)n sequences by endonuclease G. *J. Biol. Chem.* **264,** 3301–3310.

Daniels, G. A., and Lieber, M. R. (1995). Strand specificity in the transcriptional targeting of recombination at immunoglobulin switch sequences. *Proc. Natl. Acad. Sci. USA* **92,** 5625–5629.

Dariavach, P., Williams, G. T., Campbell, K., Petterson, S., and Neuberger, M. S. (1991). The mouse IgH 3′ enhancer. *Eur. J. Immunol.* **21,** 1499–1504.

Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R., Weissman, I. L., and Hood, L. (1980a). An immunoglobulin heavy-chain gene is formed by at least two recombination events. *Nature* **283,** 733–739.

Davis, M. M., Kim, S. K., and Hood, L. E. (1980b). *Science* **209,** 1360–1365.

Defrance, T., Vanbervliet, B., Brière, F., Durand, I., Rousset, F., and Banchereau, J. (1992). Interleukin 10 and transforming growth factor β cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J. Exp. Med.* **175,** 671–682.

Delphin, S. A., and Stavnezer, J. (1995). Characterization of an IL-4 responsive region in the immunoglobulin heavy chain ε promoter: Regulation by NF-IL4, a C/EBP family member and NF-κB/p50. *J. Exp. Med.* **181,** 181–192.

DePinho, R., Kruger, K., Andrews, N., Lutzker, S., Baltimore, D., and Alt, F. W. (1984). Molecular basis of heavy-chain class switching and switch region deletion in an Abelson Virsus-transformed cell line. *Mol. Cell. Biol.* **4,** 2905–2910.

Diaz-Sanchez, D., Chegini, S., Zhang, K., and Saxon, A. (1994). CD58 (LFA-3) stimulation provides a signal for human isotype switching and IgE production distinct from CD40. *J. Immunol.* **153,** 10–20.

DiSanto, J. P., Bonnefoy, J. Y., Gauchat, J. F., Fischer, A., and de Saint Basile, G. (1993). CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* **361,** 541–543.

Drapkin, R., Sancar, A., and Reinberg, D. (1994). Where transcription meets repair. *Cell* **77,** 9–12.

Dunnick, W., Hertz, G. Z., Scappino, L., and Gritzmacher, C. (1993). DNA sequences at immunogloubulin switch region recombination sites. *Nucleic Acids Res.* **21,** 365–372.

Dunnick, W., Wilson, M., and Stavnezer, J. (1989). Mutations, duplication, and deletion of recombined switch regions suggest a role for DNA replication in the immunoglobulin heavy-chain switch. *Mol. Cell. Biol.* **9,** 1850–1856.

Dunnick, W., Rabbits, T. H., and Milstein, C. (1980). *Nature* **286,** 669–675.

Dunnick, W., and Stavnezer, J. (1990). Copy choice mechanism of immunoglobulin heavy chain switch recombination. *Mol. Cell. Biol.* **10,** 397–400.

Ehrhardt, R. O., Strober, W., and Harriman, G. R. (1992). Effect of TGFβ1 on IgA isotype expression: TGFβ1 induces a small increase in $sIgA^+B$ cells regardless of the method of B cell activation. *J. Immunol.* **148,** 3830–3836.

Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985). B-lineage specific interactions of an immunoglobulin enhancer with cellular factors in vivo. *Science* **227,** 134–140.

Esser, C., and Radbruch, A. (1989). Rapid induction of transcription of unrearranged Sγ1 switch regions in activated murine B cells by interleukin 4. *EMBO J.* **8,** 483–488.

Fanslow, W., Anderson, D., Grabstein, K., Clark, E., Cosman, D., and Armitage, R. (1992). Soluble forms of CD40 inhibit biologic responses of human B cells. *J. Immunol.* **149,** 655–660.

Finkelman , F. D., Katona, I. M., J. F. Urban, J., Snapper, C. M., Ohara, J., and Paul, W. E. (1986). Suppression of in vivo polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1. *Proc. Natl. Acad. Sci. USA* **83,** 9675–9679.

Finkelman, F. D., Katona, I. M., Mosmann, T. R., and Coffman, R. L. (1988). IFN-γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J. Immunol.* **140,** 1022–1030.

Fish, S., Zenowich, E., Fleming, M., and Manser, T. (1989). Molecular analysis of original antigenic sin. I. Clonal selection, somatic mutation and isotype switching. *J. Exp. Med.* **170,** 1191–1209.

Fujieda, S., Zhang, K., and Saxon, A. (1995). IL-4 plus CD40 mAb induces human B cells gamma subclass specific isotype switch: Switching to γ1, γ3, γ4 but not γ2. *J. Immunol.* **155,** 2318–2328.

Fukita, Y., Mizuta, T.-R., Shirozu, M., Ozawa, K., Shimizu, A., and Honjo, T. (1993). The human Sμbp-2, a DNA-binding protein specific to the single-stranded guanine-

rich sequence related to the immunoglobulin μ chain switch region. *J. Biol. Chem.* **268,** 17463–17470.

Fuleihan, R., Ramesh, N., Loh, R., Jabara, H., Rosen, F. S., Chatila, T., Fu, S. M., Stamenkovic, I., and Geha, R. S. (1993). Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc. Natl. Acad. Sci. USA* **90,** 2170–2173.

Fultz, M., J., Barber, S. A., Dieffenbach, C. W., and Vogel, S. N. (1993). Induction of IFN-γ in macrophages by lipopolysaccharide. *Int. Immunol.* **5,** 1383–1392.

Gaff, C., and Gerondakis, S. (1990). RNA splicing generates alternate forms of germline α heavy chain transcripts. *Int. Immunol.* **2,** 1143–1148.

Gaff, C., Grumont, R. J., and Gerondakis, S. (1992). Transcriptional regulation of the germline immunoglobulin Cα and Cε genes: Implications for commitment to an isotype switch. *Int. Immunol.* **4,** 1145–1151.

Gascan, H., Gauchat, J.-F., Aversa, G., Vlasselaer, P. V., and deVries, J. E. (1991). Anti-CD40 monoclonal antibodies or CD4$^+$ T cell clones and IL-4 induce IgG4 and IgE switching in purified human B cells via different signaling pathways. *J. Immunol.* **147,** 8–13.

Gauchat, J.-F., G. Aversio, J. G., and deVries, J. E. (1992). Modulation of IL-4 induced germline ε RNA synthesis in human B cells by tumor necrosis factor-α, anti-CD40 monoclonal antibodies or transforming growth factor-β correlates with levels of IgE production. *Int. Immunol.* **4,** 397–406.

Gauchat, J.-F., Lebmen, D. A., Coffman, R. L., Gascan, H., and deVries, J. E. (1990). Structure and expression of germline ε transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J. Exp. Med.* **172,** 463–473.

Gearhart, P. J., Sigal, N. H., and Klinman, N. R. (1975). Production of antibodies of identical idiotypes but diverse immunoglobulin classes by cells derived from a single stimulated B cell. *Proc. Natl. Acad. Sci. USA* **72,** 1707–1711.

Gerondakis, S. (1990). Structure and expression of murine germ-line immunoglobulin ε heavy chain transcripts induced by interleukin 4. *Proc. Natl. Acad. Sci. USA* **87,** 1581–1585.

Gerstein, R. M., Frankel, W. N., Hsieh, C.-L., Durdik, J. M., Rath, S., Coffin, J. M., Nisonoff, A., and Selsing, E. (1990). Isotype switching of an immunoglobulin heavy chain transgene occurs by DNA recombination between different chromosomes. *Cell* **63,** 537–548.

Goodman, D. J., Gaff, C., and Gerondakis, S. (1993). The IL-4 induced increase in the frequency of resting murine splenic B cells expressing germline Ig heavy chain γ1 transcripts correlates with subsequent switching to IgG_1. *Int. Immunol.* **5,** 199–208.

Goroff, D. K., Holmes, J. M., Bazin, H., Nisol, F., and Finkelman, F. D. (1991). Polyclonal activation of the murine immune system by an antibody to IgD. XI. Contribution of membrane IgD cross-linking to the generation of an in vivo polyclonal antibody response. *J. Immunol.* **146,** 18–25.

Greenspan, N. S., and Cooper, L. J. N. (1992). Intermolecular cooperativity: A clue to why mice have IgG3? *Immunol. Today* **13,** 164–168.

Gritzmacher, C. A. (1989). Molecular aspects of heavy-chain class switching. *Crit. Rev. Immunol.* **9,** 173–200.

Gruss, P., and Walther, C. (1992). Pax in development. *Cell* **69,** 719–722.

Harriman, G. R., Bradley, A., Das, S., Rogers-Fani, P., and Davis, A. C. (1996). IgA class switch in Iα exon deficient mice. Role of germline transcription in class switch recombination. *J. Clin. Invest.* **97.**

Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M., and McKnight, S. L. (1994). An interleukin-4-induced transcription factor: IL-4 Stat. *Science* **265,** 1701–1706.

Hsieh, P., Camerini-Otero, C. S., and Camerini-Otero, R. D. (1990). Pairing of homologous DNA sequences by proteins: Evidence for three-stranded DNA. *Genes Dev.* **4,** 1951–1963.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethamann, H., Kamijo, R., Vilcek, J., Zinkernagel, R. M., and Aguet, M. (1993). Immune response in mice that lack the interferon-γ receptor. *Science* **259,** 1742–1745.

Hummel, M., Kaminshka, J., and Dunnick, W. (1987). Switch region content of hybridomas: The two spleen cell IgH loci tend to rearrange to the same isotype. *J. Immunol.* **138,** 3539–3548.

Hurwitz, J. L., and Cebra, J. J. (1982). Rearrangements between the immunoglobulin heavy chain gene J_H and C_μ regions accompany normal B lymphocyte differentiation in vitro. *Nature* **299,** 742–744.

Hurwitz, J. L., Coleclough, C., and Cebra, J. J. (1980). C_H gene rearrangements in IgM-bearing cells and in the normal splenic DNA component of hybridomas making different isotypes of antibody. *Cell* **22,** 349–359.

Ichiki, T., Takahashi, W., and Watanabe, T. (1992). The effect of cytokines and mitogens on the induction of Cε germline transcripts in a human Burkitt lymphoma B cell line. *Int. Immunol.* **4,** 747–754.

Ichiki, T., Takahashi, W., and Watanabe, T. (1993). Regulation of the expression of human Cε germline transcript: Identification of a novel IL-4 responsive element. *J. Immunol.* **150,** 5408–5417.

Irsch, J., Hendriks, R., Tesch, H., Schuurman, R., and Radbruch, A. (1993). Evidence for a human IgG1 class switch program. *Eur. J. Immunol.* **23,** 481–486.

Isakson, P. (1982). T cell derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* **155,** 734–748.

Islam, K. B., Nilsson, L., Sideras, P., Hammarström, L., and Smith, C. I. E. (1991). TGFβ_1 induces germ-line transcripts of both IgA subclasses in human B lymphocytes. *Int. Immunol.* **3,** 1099–1106.

Iwasato, T., Arakawa, H., Shimizu, A., Honjo, T., and Yamagishi, H. (1992). Biased distribution of recombination sites within S regions upon immunoglobulin class switch recombination induced by transforming growth factor β and lipopolysaccharide. *J. Exp. Med.* **175,** 1539–1546.

Iwasato, T., Shimizu, A., Honjo, T., and Yamagishi, H. (1990). Circular DNA is excised by immunoglobulin class switch recombination. *Cell* **62,** 143–149.

Jabara, H. H., Fu, S. M., Geha, R. S., and Vercelli, D. (1990). CD40 and IgE: Synergism between anti-CD40 mAB and IL-4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* **172,** 1861–1864.

Jacob, J., Kassir, R., and Kelsoe, G. (1991). In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of the responding cell populations. *J. Exp. Med.* **173,** 1165–1175.

Jumper, M. D., Splawski, J. B., Lipsky, P. E., and Meek, K. (1994). Ligation of CD40 induces sterile transcripts of multiple H chain isotypes in human B cells. *J. Immunol.* **152,** 438–454.

Jung, S., Rajewsky, K., and Radbruch, A. (1993). Shutdown of class switch recombination by deletion of a switch region control element. *Science* **259,** 984–987.

Jung, S., Siebenkotten, G., and Radbruch, A. (1994). Frequency of immunoglobulin E class switching is autonomously determined and independent of prior switching to other classes. *J. Exp. Med.* **179,** 2023–2026.

Kataoka, T., Miyata, T., and Honjo, T. (1981). Repetitive sequences in class-switch recombination regions of immunoglobulin heavy chain genes. *Cell* **23,** 357–368.

Katzenberg, D. R., Tilley, S. A., and Birshtein, B. K. (1989). Nucleotide sequence of an unequal sister chromatid exchange site in a mouse myeloma cell line. *Mol. Cell. Biol.* **9,** 1324–1326.

Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T., and Kikutani, H. (1994). The immune responses in CD40-deficient mice: Impaired immunoglobulin class switching and germinal center formation. *Immunity* **1,** 167–178.

Kearney, J. F., Cooper, M. D., and Lawson, A. R. (1976). B cell differentiation induced by lipopolysaccharide. IV. Development of immunoglobulin class restriction in precursors of IgG synthesizing cells. *J. Immunol.* **117,** 1567–1572.

Kearney, J. F., and Lawton, A. R. (1975). B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J. Immunol.* **115,** 671–676.

Keeton, M. R., Curriden, S. A., vanZonneveld, A.-J., and Loskutoff, D. J. (1991). Identification of regulatory sequences in the type I plasminogen activator inhibitor gene responsive to TGFβ. *J. Biol. Chem.* **266,** 23048–23052.

Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B., and Fauci, A. S. (1986a). Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* **137,** 3855–3860.

Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M. B., and Fauci, A. S. (1986b). Production of transforming growth factor β by human lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* **163,** 1037–1050.

Kenter, A. L., Wuerffel, R., Sen, R., Jamieson, C. E., and Merkulov, G. V. (1993). Switch recombination breakpoints occur at nonrandom positions in the Sγ tandem repeat. *J. Immunol.* **151,** 4718–4731.

Kenter, A. L., and Birstein, B. K. (1981). Chi, a promoter of generalized recombination in λ phage, is present in immunoglobulin genes. *Nature* **293,** 402–404.

Kenter, A. L., and Hu, B. The immunoglobulin Sγ specific DNA binding protein SNAP is related to the HLH transcription factors E47/E12. Submitted for publication.

Kenter, A. L., and Watson, J. V. (1987). Cell cycle kinetics model for LPS-stimulated spleen cells correlates switch region rearrangements with S phase. *J. Immunol. Methods* **97,** 111–117.

Kenter, A. L., and Wuerffel, R. A. (1995). Detection of DS breaks in immunoglobulin Sγ3 DNA from normal activated B cells—Implications for switch recombination. *In* "Mechanisms of B Cell Neoplasia 1995." Editiones Roche, Basel, Switzerland.

Kepron, M. R., Chen, Y.-W., Uhr, J W., and Vitetta, E. (1989). IL-4 induces the specific rearrangement of γ1 genes on the expressed and unexpressed chromosomes of lipopolysaccharide activated normal murine B cells. *J. Immunol.* **143,** 334–339.

Kim, K.-M., Adachi, T., Nielson, P. J., Terashima, M., Lamers, M. C., Kohler, G., and Reth, M. (1994). Two new proteins preferentially associated with membrane IgD. *EMBO J.* **13,** 3793–3800.

Kim, K.-M., and Reth, M. (1995). The B cell antigen receptor of class IgD induces a stronger and more prolonged protein tyrosine phosphorylation than that of class IgM. *J. Exp. Med.* **181,** 1005–1014.

Kim, P.-H., and Kagnoff, M. F. (1990). Transforming growth factor β1 increases IgA isotype switching at the clonal level. *J. Immunol.* **145,** 3773–3778.

Kimata, H., and Fujimoto, M. (1994). Vasoactive intestinal peptide specifically induces human IgA1 and IgA2 production. *Eur. J. Immunol.* **24,** 2262–2265.

Kipps, T. J., and Herzenberg, L. A. (1986). Homologous chromosome recombination generating immunoglobulin allotype and isotype switch variants. *EMBO J.* **5,** 263–268.

Kitani, A., and Strober, W. (1993). Regulation of Cγ subclass germ-like transcripts in human peripheral blood B cells. *J. Immunol.* **151,** 3478–3488.

Kitani, A., and Strober, W. (1994). Differential regulation of Cα1 and Cα2 germ-line and mature mRNA transcripts in human peripheral blood B cells. *J. Immunol.* **153,** 1466–1477.

Knapp, M. R., Liu, C.-P., Newell, N., Ward, R. B., Tucker, P. W., Strober, S., and Blattner, F. (1982). Simultaneous expression if immunoglobulin μ and δ heavy chains by a cloned B-cell lymphoma: A single copy of the V_H gene is shared by two adjacent C_H genes. *Proc. Natl. Acad. Sci. USA* **79,** 2996–3000.

Knight, K., Kingzette, M., Crane, M. A., and Zhai, S.-K. (1995). Transchromosomally derived Ig heavy chains. *J. Immunol.* **155,** 684–691.

Knight, K. L., Malek, T., and Hanley, W. C. (1974). Recombinant rabbit secretory immunoglobulin molecules: α-Chains with maternal (paternal) variable region allotypes and paternal (maternal) constant region allotypes. *Proc. Natl. Acad. Sci. USA* **71,** 1169–1173.

Kohler, I., and Rieber, E. P. (1993). Allergy-associated Iε and Fcε receptor II (CD23b) genes activated via binding of an interleukin-4-induced transcript factor to a novel responsive element. *Eur. J. Immunol.* **23,** 3066–3071.

Korthauer, U., Graf, D., Mages, H. W., Briere, F., Padayachee, M., Malcolm, S., Ugazio, A. G., Notarangelo, L. D., Levinsky, R. J., and Kroczek, R. A. (1993). Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* **361,** 539–541.

Kotani, H., and Kmiec, E. B. (1994). A role for RNA synthesis in homologous pairing events. *Mol. Cell. Biol.* **14,** 6097–6106.

Kotanides, H., and Reich, N. C. (1993). Requirement of tyrosine phosphorylation for rapid activation of a DNA binding factor by IL-4. *Science* **262,** 1265–1267.

Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol. Cell. Biol.* **12,** 2662–2672.

Kraal, G., Weissman, I. L., and Butcher, E. C. (1982). Germinal center cells: Antigen specificity and changes in heavy chain class expression. *Nature* **298,** 377–379.

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374,** 546–549.

Kühn, R., Rajewsky, K., and Müller, W. (1991). Generation and analysis of interleukin-4 deficient mice. *Science* **254,** 707–710.

Lane, P., Brocker, T., Hubele, S., Padovan, E., Lanzavecchia, A., and McConnell, F. (1993). Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. *J. Exp. Med.* **177,** 1209–1213.

Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A., and Gray, D. (1992). Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur. J. Immunol.* **22,** 2573–2580.

Layton, J. E., Vitetta, E. S., Uhr, J. W., and Krammer, P. H. (1984). Clonal analysis of B cells induced to secrete IgG by T cell-derived lymphokines. *J. Exp. Med.* **160,** 1850–1863.

Lebman, D., and Coffman, R. (1988). Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J. Exp. Med.* **168,** 853–862.

Lebman, D. A., Lee, F. D., and Coffman, R. L. (1990a). Mechanism for transforming growth factor β and Il-2 enhancement of IgA expression in lipopolysaccharide-stimulated B cell cultures. *J. Immunol.* **144,** 952–959.

Lebman, D. A., Nomura, D. Y., Coffman, R. L., and Lee, F. D. (1990b). Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching. *Proc. Natl. Acad. Sci. USA* **87,** 3962–3966.

Lebman, D. A., Park, M. J., Hansen-Bundy, S., and Pandya, A. (1993). Mechanism for TGFβ regulation of α mRNA in LPS-stimulated B cells. *Int. Immunol.* **6,** 113–119.

Lennon, G. G., and Perry, R. P. (1985). Cμ-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5′-nontranslatable exon. *Nature* **318,** 475–478.

Lepse, C. L., Kumar, R., and Ganea, D. (1994). Extrachromosomal eukaryotic DNA substrates for switch recombination: analysis of isotype and cell specificity. *DNA Cell Biol.* **13,** 1151–1161.

Leung, H., and Maizels, N. (1992). Transcriptional regulatory elements stimulate recombination in extrachromosomal substrates carrying immunoglobulin switch-region sequences. *Proc. Natl. Acad. Sci. USA* **89,** 4154–4158.

Leung, H., and Maizels, N. (1994). Regulation and targeting of recombination in extrachromosomal substrates carrying immunoglobulin switch region sequences. *Mol. Cell. Biol.* **14,** 1450–1458.

Li, S. C., Rothman, P. B., Zhang, J., Chan, C., Hirsh, D., and Alt, F. W. (1994). Expression of Iμ-Cγ hybrid germline transcripts subsequent to immunoglobulin heavy chain class switching. *Int. Immunol.* **6,** 491–497.

Liao, F., Birshtein, B., Busslinger, M., and Rothman, P. (1994). The transcription factor BSAP (NF-HB) is essential for immunoglobulin germ-line ε transcription. *J. Immunol.* **152,** 2904–2911.

Liao, F., Giannini, S. L., and Birshstein, B. (1992). A nuclear DNA-binding protein expressed during early stages of B cell differentiation interacts with diverse segments within and 3′ of the Ig H chain gene cluster. *J. Immunol.* **148,** 2909–2917.

Lieberson, R., Giannini, S. L., Birshtein, B. K., and Eckhardt, L. (1991). An enhancer at the 3′ end of the mouse immunoglobulin heavy chain locus. *Nucleic Acids Res.* **19,** 933–937.

Lin, F. L., Sperle, K., and Sternberg, N. (1984). Intermolecular recombination during transfer of DNA into mouse L cells: Role for DNA ends in the recombination process. *Mol. Cell. Biol.* **4,** 1020–1034.

Lin, S. C., and Stavnezer, J. Induction of expression of the promoter for germline γ1 switch transcripts by CD40 signaling involves 3 tandem binding sites for NF-κB. Submitted for publication.

Lin, Y.-C. A., and Stavnezer, J. (1992). Regulation of transcription of the germline Igα constant region gene by an ATF element and by a novel transforming growth factor-β1-responsive elements. *J. Immunol.* **149,** 2914–2925.

Lorenz, M., Jung, S., and Radbruch, A. (1995). Switch transcripts in immunoglobulin class switching. *Science* **267,** 1825–1828.

Louie, S. W., Ramirez, L. M., Krieg, A. M., Maliszewski, C. R., and Bishop, G. A. (1993). Endogeneous secretion of IL-4 maintains growth and thy-1 expression of a transformed B cell clone. *J. Immunol.* **150,** 399–406.

Lundgren, M., Strom, L., Bergqvist, L. O., Skog, S., Heiden, T., Stavnezer, J., and Severinson, E. (1995). Cell cycle regulation of germline immunoglobulin transcription: Potential role of Ets family members. *Eur. J. Immunol.* **25,** 2042–2051.

Lundgren, M., Larrson, C., Femino, A., Xu, M., Stavnezer, J., and Severinson, E. (1994). Activation of the immunoglobulin germ line γ1 promoter: Involvement of C/EBP transcription factors and their possible interaction with a NF-IL4 site. *J. Immunol.* **153,** 2983–2995.

Lundgren, M., Persson, U., Larsson, P., Magnusson, C., Smith, C., Hammerstrom, L., and Severinson, E. (1989). Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. *Eur. J. Immunol.* **19,** 1311–1315.

Lutzker, S., and Alt, F. W. (1988). Structure and expression of germline immunoglobulin γ2b transcripts. *Mol. Cell. Biol.* **8,** 1849–1852.

Lutzker, S., Rothman, P., Pollock, R., Coffman, R., and Alt, F. W. (1988). Mitogen- and IL-4-regulated expression of germline Igγ2b transcripts: Evidence for directed heavy chain class switching. *Cell* **53,** 177–184.

Lycke, N., Severinson, E., and Strober, W. (1990). Cholera toxin acts synergistically with IL-4 to promoter IgG1 switch differentiation. *J. Immunol.* **145,** 3316–3324.

Madisen, L., and Groudine, M. (1994). Identification of a locus control region in the immunoglobulin heavy-chain locus that deregulates c-*myc* expression in plasmacytoma and Burkitt's lymphoma cells. *Genes Dev.* **8,** 2212–2226.

Maki, R., Roder, W., Traunecker, A., Sidman, C., Wabl, M., Raschke, W., and Tonegawa, S. (1981). The role of DNA rearrangement and alternative RNA processing in the expression of IgD genes. *Cell* **24,** 353–365.

Mandler, R., Chu, C. C., Paul, W., Max, E. E., and Snapper, C. M. (1993a). Interleukin 5 induces Sμ-Sγ1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: A three component for Ig class switching. *J. Exp. Med.* **178,** 1577–1586.

Mandler, R., Finkelman, F. D., Levine, A. D., and Snapper, C. M. (1993b). IL-4 induction of IgE class switching by lipopolysaccharide-activated murine B cells occurs predominantly through sequential switching. *J. Immunol.* **150,** 407–418.

Marcu, K. B., Lang, R. B., Stanton, L. W., and Harris, L. J. (1982). A model for the molecular requirements of immunoglobulin heavy chain class switching. *Nature* **298,** 87–89.

Matsuoka, M., Yoshida, K., Maeda, T., Usuda, S., and Sakano, H. (1990). Switch circular DNA is formed in cytokine-treated mouse splenocytes: Evidence for intramolecular DNA deletion in immunoglobulin class switching. *Cell* **62,** 135–142.

McHeyzer-Williams, M. G., McLean, M. J., Lalor, P. A., and Nossal, G. J. V. (1993). Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* **178,** 295–307.

McIntyre, T. M., Kehry, M. R., and Snapper C. M. (1995). Novel in vitro model for high-rate IgA class switching. *J. Immunol.* **154,** 3156–3161.

McIntyre, T. M., Klinman, D. R., Rothman, P., Lugo, M., Dasch, J. R., Mond, J. J., and Snapper, C. M. (1993). Transforming growth factor β_1 selectively stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J. Exp. Med.* **177,** 1031–1037.

Mellon, I., Spivak, G., and Hanawalt, P. C. (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell* **51,** 241–249.

Mezard, C., Pompon, D., and Nicolas, A. (1992). Recombination between similar but not identical DNA sequences during yeast transformation occurs with short stretches of identity. *Cell* **70,** 659–670.

Mills, F. C., Mitchell, M. P., Harindranath, N., and Max, E. E. (1995). Human Ig Sγ regions and their participation in sequential switching to IgE. *J. Immunol.* **155,** 3021–3036.

Mills, F. C., Thyphronitis, G., Finkelman , F. D., and Max, E. E. (1992). Ig μ–ε isotype switch in IL-4 treated human B lymphoblastoid cells. Evidence for a sequential switch. *J. Immunol.* **149,** 1075–1085.

Mills, F. C., Brooker, J. S., and Camerini-Otero, R. D. (1990). Sequences of human immunoglobulin switch regions: Implications for recombination and transcription. *Nucleic Acids Res.* **18,** 7305–7311.

Mizuta, T.-R., Fukita, Y., Miyoshi, T., Shimizu, A., and Honjo, T. (1993). Isolation of cDNA encoding a binding protein specific to 5′-phosphorylated single-stranded DNA with G-rich sequences. *Nucleic Acids Res.* **21,** 1761–1766.

Moore, K. W., Rogers, J., Hunkapiller, T., Early P., Nottenburg, C., Weissman, I., Bazin, H., Wall, R., and Hood, L. E. (1981). Expression of IgD may use both DNA rearrangement and RNA splicing mechanisms. *Proc. Natl. Acad. Sci. USA* **78,** 1800–1804.

Mowatt, M. R., and Dunnick, W. A. (1986). DNA sequence of the murine γ1 switch segment reveals novel structural features. *J. Immunol.* **136,** 2674–2683.

Murre, C., and Baltimore, D. (1992). The helix–loop–helix motif: Structure and function. *In* "Transcriptional Regulation," pp. 861–879. Cold Spring Harbor Press, Plainview, NY.

Nelson, K. J., Haimovich, J., and Perry, R. P. (1983). Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: Processing of μ_m and μ_s mRNA. *Mol. Cell. Biol.* **3,** 1317–1332.

Neurath, M. F., Max, E. E., and Strober, W. (1995). Pax5 (BSAP) regulates the murine immunoglobulin 3′α enhancer by suppressing binding of NF-αP, a protein that controls heavy chain transcription. *Proc. Natl. Acad. Sci. USA* **92,** 5336–5340.

Neurath, M. F., Strober, W., and Wakatsuki, Y. (1994). The murine Ig 3′α enhancer is a target site with repressor function for the B cell lineage-specific transcription factor BSAP (NF-HB, Sα-BP). *J. Immunol.* **153,** 730–742.

Nikaido, T., Nikai, S., and Honjo, T. (1981). Switch region of immunoglobulin Cμ gene is composed of simple tandem repetitive sequences. *Nature* **292,** 845–848.

Nikaido, T., Yamawaki-Kataoka, Y., and Honjo, T. (1982). Nucleotide sequences of switch regions of immunoglobulin Cε and Cγ genes and their comparison. *J. Biol. Chem.* **257,** 7322–7329.

Nilsson, L., Islam, K. B., Olafsson, O., Zalcberg, I., Samakovlis, C., and Hammerstrom, L., Smith, C. I. E., and Sideras, P. (1991). Structure of TGF-β1 induced human immunoglobulin Cα1 and Cα2 germ-line transcripts. *Int. Immunol.* **3,** 1107–1115.

Nilsson, L., and Paschalis, S. (1993). The human Iα1 and Iα2 germline promoter elements: Proximal positive and distal negative elements may regulate the tissue specific expression of Cα1 and Cα2 germline transcripts. *Int. Immunol.* **5,** 271–282.

Nitschke, L., Kosco, M. H., Kohler, G., and Lamers, M. C. (1993). IgD-deficient mice can mount normal immune responses to thymus-independent and -dependent antigens. *Proc. Natl. Acad. Sci. USA* **90,** 1887–1891.

Noelle, R. J., Roy, M., Shepherd, D. M., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992a). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA* **89,** 6550–6554.

Noelle, R. J., Shepherd, D. M., and Fell, H. P. (1992b). Cognate interaction between T helper cells and B cells VII. Role of contact and lymphokines in the expression of germline and mature γ1 transcripts. *J. Immunol.* **149,** 1164–1169.

Nolan-Willard, M., Berton, M. T., and Tucker, P. (1992). Coexpression of μ and γ1 heavy chains can occur by a discontinuous transcription mechanism from the same unrearranged chromosome. *Proc. Natl. Acad. Sci. USA* **89,** 1234–1238.

Obata, M., Kataoka, T., Nakai, S., Yamagishi, H., Takahashi, N., Yamawaki-Kataoka, Y., Nikaido, T., Shimizu, A., and Honjo, T. (1981). Structure of a rearranged γ1 chain gene and its implication to immunoglobulin class-switch mechanism. *Proc. Natl. Acad. Sci. USA* **78,** 2437–2441.

Okabe, T., Watanabe, T., and Kudo, A. (1992). A pre B and B cell-specific DNA-binding protein EBB-1, which binds to the promoter of the VpreB1 gene. *Eur. J. Immunol.* **23,** 37.

Ott, D. E., Alt, F. W., and Marcu, K. B. (1987). Immunoglobulin heavy chain switch region recombination within a retroviral vector in murine-pre-B cells. *EMBO J.* **6,** 577–584.

Ott, D. E., and Marcu, K. B. (1989). Molecular requirements for immunoglobulin heavy chain constant region gene switch-recombination revealed with switch-substrate retroviruses. *Int. Immunol.* **1,** 582–591.

Owens, J. D., Jr., Finkelman, F. D., Mountz, J. D., and Mushinski, J. F. (1991). Nonhomologous recombination at sites within mouse J_H -Cδ locus accompanies Cμ deletion and switch to IgD secretion. *Mol. Cell. Biol.* **11,** 5660–5670.

Perlmutter, A. P., and Gilbert, W. (1984). Antibodies of the secondary response can be expressed without switch recombination in normal mouse B cells. *Proc. Natl. Acad. Sci. USA* **81,** 7189–7193.

Perlmutter, R. M., Hansburg, D., Briles, D. E., Nicolotti, R. A., and Davie, J. M. (1978). Subclass restriction of murine anti-carbohydrate antibodies. *J. Exp. Med.* **121,** 566–576.

Petrini, J., Shell, B., Hummel, M., and Dunnick, W. (1987a). The immunoglobulin heavy chain switch: Structural features of γ1 recombinant switch regions. *J. Immunol.* **138,** 1940–1949.

Pettersson, S., Cook, G. P., Burggermann, M., Williams, G. T., and Neuberger, M. S. (1990). A second B cell-specific enhancer 3′ of the immunoglobulin heavy-chain locus. *Nature* **344,** 165–168.

Plaut, M., Pierce, J., Watson, C., Hanley, H., Nordan, R., and Paul, W. (1989). Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature* **339,** 64–67.

Punnonen, J., Aversa, G., Cocks, B. G., McKenzie, A. N. J., Menon, S., Zurawski, G., De Waal Malefyt, R., and Devries, J. E. (1993). Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. USA* **90,** 3730–3734.

Purkerson, J. M., and Isakson, P. C. (1992). Interleukin 5 (IL-5) provides a signal that is required in addition to IL-4 for isotype switching to IgG1 and IgE. *J. Exp. Med.* **175,** 973–982.

Purkerson, J. M., Newberg, M., Wise, G., Lynch, K. R., and Isakson, P. C. (1988). Interleukin 5 and interleukin 2 cooperate with interleukin 4 to induce IgG1 secretion from anti-Ig-treated B cells. *J. Exp. Med.* **168,** 1175–1180.

Qiu, G., Gauchat, J., Vogel, M., Mandaliaz, M., Wreck, A. D., and Stadler, B. (1990). Human IgE mRNA expression by peripheral blood lymphocytes stimulated with interleukin-4 and pokeweed mitogen. *Eur. J. Immunol.* **20,** 2191–2199.

Rabbitts, T. H., Forster, A., Dunnick, W., and Bentley, D. L. (1980). The role of gene deletion in the immunoglobulin heavy chain switch. *Nature* **283,** 351–356.

Radbruch, A., Liesegang, B., and Rajewsky, K. (1980). *Proc. Natl. Acad. Sci. USA* **77,** 2909–2913.

Radbruch, A., and Sablitsky, F. (1983). Deletion of Cμ genes in mouse B lymphocytes upon stimulation with LPS. *EMBO J.* **2,** 1929–1935.

Radcliffe, G., Lin, Y.-C., Julius, M., Marcu, K., and Stavnezer, J. (1990). Structure of germline immunoglobulin α heavy-chain RNA and its location on polysomes. *Mol. Cell Biol.* **10,** 382–386.

Reaban, M. E., and Griffin, J. A. (1990). Induction of RNA-stabilized DNA conformers by transcription of an immunoglobulin switch region. *Nature* **348,** 342–344.

Reaban, M. E., Lebowitz, J., and Griffin, J. A. (1994). Transcription induces the formation of a stable RNA–DNA hybrid in the immunoglobulin α switch region. *J. Biol. Chem.* **269,** 21850–21857.

Renshaw, B. R., III, Fanslow, W. C., Armitage, R. J., Campbell, K. A., Liggitt, D., Wright, B., Davison, B. L., and Maliszewski, C. R. (1994). Humoral immune response in CD40 ligand-deficient mice. *J. Exp. Med.* **180,** 1889–1900.

Roes, J., and Rajewsky, K. (1993). Immunoglobulin D-deficient mice reveal an auxiliary receptor function for IgD in antigen-mediated recruitment of B cells. *J. Exp. Med.* **177,** 45–55.

Roper, R. L., Brown, D. M., and Phipps, R. P. (1995). Prostaglandin E_2 promotes B lymphocyte Ig isotype switching to IgE. *J. Immunol.* **154,** 162–170.

Rothman, P., Li, S. C., Gorham, B., Glimcher, L., Alt, F., and Boothby, M. (1991). Identification of a conserved LPS/IL-4 responsive element located at the promoter of germline ε transcripts. *Mol. Cell. Biol.* **11,** 5551–5561.

Rothman, P., Chen, Y.-Y., Lutzker, S., Li, S. C., Stewart, V., Coffman, R., and Alt, F. W. (1990a). Structure and expression of germline immunoglobulin heavy chain ε transcripts: Interleukin-4-plus lipopolysaccharide-directed switching to Cε. *Mol. Cell. Biol.* **10,** 1672–1679.

Rothman, P., Lutzker, S., Gorham, B., Stewart, V., Coffman, R., and Alt, F. W. (1990b). Structure and expression of germline immunoglobulin γ3 heavy chain gene transcripts: Implications for mitogen and lymphokine directed class-switching. *Int. Immunol.* **2,** 621–627.

Rothman, P., Lutzker, S., Cook, W., Coffman, R., and Alt, F. W. (1988). Mitogen plus interleukin 4 induction of Cε transcripts in B lymphoid cells. *J. Exp. Med.* **168,** 2385–2389.

Sablitsky, F., Radbruch, A., and Rajewsky, K. (1982). Spontaneous immunoglobulin class switching in myeloma and hybridoma cell lines differs from physiological class switching. *Immunol. Rev.* **67,** 59–73.

Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1980). Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature* **286,** 676–683.

Sandler, M. A., Zhang, J.-N., D. R. Westerhausen, J., and Billadello, J. J. (1994). A novel protein interacts with the major TGFβ responsive element in the plasminogen activator inhibitor type-1 gene. *J. Biol. Chem.* **269,** 21500–21504.

Savelkoul, H. F. J., Lebman, D. A., Benner, R., and Coffman, R. (1988). Increase of precursor frequency and clonal size of murine IgE secreting cells by IL-4. *J. Immunol* **141,** 749–755.

Schmitz, J., and Radbruch, A. (1989). An interleukin 4-induced DNase I hypersensitive site indicates opening of the γ1 switch region prior to switch recombination. *Int. Immunol.* **1,** 570–575.

Schultz, D. L., Rothman, P., Kühn, R., Kehry, M., Muller, W., Rajewsky, K., Alt, F., and Coffman, R. L. (1992). T helper cell membranes promote IL-4-independent expression of germ-line Cγ1 transcripts in B cells. *J. Immunol.* **149,** 60–67.

Severinson, E., Fernandez, C., and Stavnezer, J. (1990). Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination. *Eur. J. Immunol.* **20,** 1079–1984.

Severinson, E., Sideras, P., and Bergstedt-Lindqvist, S. (1987). IgG1 induction factor. *Int. Rev. Immunol.* **2,** 143.

Severinson-Gronowicz, E., Doss, C., and Schroder, J. (1979). Activation to IgG secretion by lipopolysaccharide requires several proliferation cycles. *J. Immunol.* **123,** 2057–2062.

Sha, W. C., Liou, H.-C., Tuomanen, E. I., and Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. *Cell* **80,** 321–330.

Shapira, S. K., Vercelli, D., Jabara, H. H., Fu, S. M., and Geha, R. S. (1992). Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J. Exp. Med.* **175,** 289–292.

Shi, M. J., and Stavnezer, J. Regulation of the promoter for mouse germline α switch transcripts by TGFβ-responsive elements, by an ets consensus element, and by an ATF-1 binding site. Submitted for publication.

Shimizu, A., Nussenzweig, M. C., Han, H., Sanchez, M., and Honjo, T. (1991). Trans-splicing as a possible molecular mechanism for the multiple isotype expression of the immunoglobulin gene. *J. Exp. Med.* **173,** 1385–1393.

Shimizu, A., Takahashi, N., Yaoita, Y., and Honjo, T. (1982). Organization of the constant region gene family of the mouse immunoglobulin heavy chain. *Cell* **28,** 499–506.

Shockett, P., and Stavnezer, J. (1991). Effect of cytokines on switching to IgA and α germline transcripts in the B lymphoma I.29μ: Tranforming growth factor-β activates transcription of the unrearranged Cα gene. *J. Immunol.* **147,** 4374–4383.

Shockett, P., and Stavnezer, J. (1993). Inhibitors of poly(ADP-ribose) polymerase increase antibody class switching. *J. Immunol.* **151,** 6962–6976.

Shparago, N., Zelazowski, P., Jim, L., McIntyre, T. M., Stuber, E., Pecanha, L. M. T., Kehry, M. R., Mond, J. J., Max, E. E., and Snapper, C. M. Interleukin-10 selectively regulates murine isotype switching but does not alter steady-state levels of germline C_H RNA. Submitted for publication.

Sideras, P., Mizuta, T.-R., Kanamori, H., Suzuki, N., Okamoto, M., Kuze, K., Ohno, H., Doi, S., Fukuhara, S., Hassan, M. S., Hammarstrom, L., Smith, E., Shimizu, A., and Honjo, T. (1989). Production of sterile transcripts of Cγ genes in an IgM-producing human neoplastic B cell line that switches to IgG-producing cells. *Int. Immunol.* **1,** 631–642.

Siebenkotten, G., Esser, C., Wabl, M., and Radbruch, A. (1992). The murine IgG1/IgE class switch program. *Eur. J. Immunol.* **22,** 1827–1834.

Singh, M., and Birshtein, B. K. (1993). NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3'α enhancer at early stages of B-cell differentiation. *Mol. Cell. Biol.* **13,** 3611–3622.

Snapper, C. M., and Finkelman, F. D. (1993). Immunoglobulin class switching. *In* Fundamental Immunology," 3rd ed., pp. 837–864. Raven Press, New York.

Snapper, C. M., and Mond, J. J. (1993). Towards a comprehensive view of Ig class switching. *Immunol. Today* **14,** 15–17.

Snapper, C. M., and Paul, W. E. (1987). Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236,** 944–947.

Snapper, C. M., Kehry, M. R., Castle, B. E., and Mond, J. J. (1995a). Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. *J. Immunol.* **154,** 1177–1187.

Snapper, C. M., Zelazowski, P., Rosas, F. R., Kehry, M. R., Tian, M., Baltimore, D., and Sha, W. C. (1996). B cells from p50/NF-κB knockout mice have selective defects in proliferation, differentiation, germline C_H transcription, and Ig class switching. *J. Immunol.* **156,** in press.

Snapper, C. M., McIntyre, T. M., Mandler, R., Pecanha, L. M. T., Finkelman, F. D., Lees, A., and Mond, J. J. (1992). Induction of IgG3 secretion by interferon γ: A model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J. Exp. Med.* **175,** 1367–1371.

Snapper, C. M., Pecanha, L. M. T., Levine, A. D., and Mond, J. J. (1991). IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. *J. Immunol.* **147,** 1163–1170.

Sonoda, E., Hitoshi, Y., Yamaguch, N., Ishii, T., Tominaga, A., Araki, S., and Takatsu, K. (1992). Differential regulation of IgA production by TGFβ and IL-5: TGFβ induces IgA-positive cells bearing IL-5 receptor, whereas IL-5 promotes their survival and maturation into IgA-secreting cells. *Cell. Immunol.* **140,** 158–172.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N., and Takatsu, K. (1989). Transforrming growth factor β induces IgA

production and acts additively with interleukin 5 for IgA production. *J. Exp. Med.* **170,** 1415–1420.

Spira, G., Gregor, P., Aguila, H. L., and Scharff, M. D. (1994). Clonal variants of hybridoma cells that switch isotype at a high frequency. *Proc. Natl. Acad. Sci. USA* **91,** 3423–3427.

Splawski, J. B., Fu, S. M., and Lipsky, P. E. (1993). Immunoregulatory role of CD40 in human B cell differentiation. *J. Immunol.* **150,** 1276–1285.

Stanton, L. W., and Marcu, K. B. (1982). Nucleotide sequence and properties of the murine γ3 immunoglobulin heavy chain gene switch region: implications for successive Cγ gene switching. *Nucleic Acids Res.* **10,** 5993–6006.

Stavnezer, J. (1995). Regulation of antibody production and class switching by TGFβ. *J. Immunol.* **155,** 1647–1651.

Stavnezer, J., Radcliffe, G., Lin, Y.-C., Nieutupski, J., Berggren, L., Sitia, R., and Severinson, E. (1988). Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. *Proc. Natl. Acad. Sci. USA* **85,** 7704–7708.

Stavnezer, J., Sirlin, S., and Abbott, J. (1995). Induction of immunoglobulin isotype switching in cultured I.29 B lymphoma cells: Characterization of the accompanying rearrangements of heavy chain genes. *J. Exp. Med.* **161,** 577–601.

Stavnezer, J., Abbott, J., and Sirlin, S. (1984). Immunoglobulin heavy chain switching in cultured I.29 murine B lymphoma cells: Commitment to an IgA or IgE switch. *Curr. Topics Microbiol. Immunol.* **113,** 109–116.

Stavnezer, J., Marcu, K. B., Sirlin, S., Alhadeff, B., and Hammerling, U. (1982). Rearrangements and deletions of immunoglobulin heavy chain genes in the double-producing B cell lymphoma I.29 *Mol. Cell. Biol.* **2,** 1002–1013.

Stavnezer-Nordgren, J., and Sirlin, S. (1986). Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J.* **5,** 95–102.

Stein, S. H., and Phipps, R. P. (1991). Antigen-specific IgG2a production in response to prostaglandins E_2, immune complexes and IFN-γ. *J. Immunol.* **147,** 2500–2506.

Strachan, T., and Read, A. P. (1994). PAX genes. *Curr. Opin. Genet. Dev.* **4,** 427–438.

Szotak, J. W., Weaver, T. L., Rothstein, R. J., and Stahl, F. W. (1983). The double-strand-break repair model for recombination. *Cell* **33,** 25–35.

Szurek, P., Petrini, J., and Dunnick, W. (1985). Complete nucleotide sequence of the murine γ3 switch region and analysis of switch recombination sites in two γ3-expressing hybridomas. *J. Immunol.* **135,** 620–626.

Tanaka, T., Chu, C. C., and Paul, W. E. (1992). An antisense oligonucleotide complementary to a sequence in Ig2b increases γ2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion. *J. Exp. Med.* **175,** 597–607.

Teale, J. M., and Klinman, N. R. (1984). Control of the production of different classes of antibody. *In* "Fundamental Immunology," pp. 519–535. Raven Press, New York.

Terashima, M., Kim, K.-M., Adachi, T., Nielson, P. J., Reth, M., Kohler, G., and Lamers, M. C. (1994). The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *EMBO J.* **13,** 3782–3792.

Thornton, B. P., Vetcicka, V., and Ross, G. D. (1994). Natural antibody and complement-mediated antigen processing and presentation. *J. Immunol.* **152,** 1727–1737.

Tilley, S. A., and Birshtein, B. K. (1985). Unequal sister chromatid exchange. A mechanism affecting Ig gene arrangement and expression. *J. Exp. Med.* **162,** 675–694.

Turaga, P. S. D., Berton, M. T., and Teale, J. M. (1993). Frequency of B cells expressing germ-line γ_1 transcripts upon IL-4 induction. *J. Immunol.* **151,** 1383–1390.

Urbanek, P., Wang, Z.-Q., Fetka, I., Wagner, E. F., and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* **79,** 901–912.

van der Stoep, N., Korver, W., and Logtenberg, T. (1994). In vivo and in vitro IgE isotype switching in human B lymphocytes: Evidence for a predominantly direct IgM to IgE class switch program. *Eur. J. Immunol.* **24,** 1307–1311.

Venkitaraman, A. R., Williams, G. T., Dariavach, P., and Neuberger, M. S. (1991). The B-cell antigen receptor of the five immunoglobulin classes. *Nature* **352,** 777–781.

Vercelli, D., Jabara, H. H., Arai, K.-I., and Geha, R. S. (1989). Induction of human IgE synthesis requires interleukin 4 and T/B interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J. Exp. Med.* **169,** 1295–1307.

Vitetta, E., Ohara, J., Meyers, C., Layton, J., Krammer, P., and Paul, W. (1985). Serological biochemical and functional identity of B cell stimulatory factor 1 and B cell differentiation factor for IgG1. *J. Exp. Med.* **162,** 1726–1731.

von-Schwedler, U., Jack, H. M., and Wabl, M. (1990). Circular DNA is a product of the immunoglobulin class switch rearrangement. *Nature* **345,** 452–456.

Wabl, M., Meyer, J., Beck-Engeser, G., Tenkhoff, M., and Burrows, P. D. (1985). Critical test of a sister chromatid exchange model for the immunoglobulin heavy-chain class switch. *Nature* **313,** 687–689.

Wakatsuki, Y., Neurath, M. F., Max, E. E., and Strober, W. (1994). The B cell-specific transcription factor BSAP regulates B cell proliferation. *J. Exp. Med.* **179,** 1099–1108.

Wakatsuki, Y., and Strober, W. (1993). Effect of downregulation of germline transcripts on immunoglobulin A isotype differentiation. *J. Exp. Med.* **178,** 129–138.

Waldman, A. S., and Waldman, B. C. (1991). Stimulation of intrachromosomal recombination in mammalian cells by an inhibitor of poly(ADP-ribosylation). *Nucleic Acids Res.* **19,** 5943–5947.

Waters, S. H., Saikh, K. U., and Stavnezer, J. (1989). A B-cell-specific nuclear protein that binds to DNA sites 5′ to immunoglobulin Sα tandem repeats is regulated during differentiation. *Mol. Cell. Biol.* **9,** 5594–5601.

Weinstein, P. D., and Cebra, J. J. (1991). The preference for switching to IgA expression by Peyers patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. *J. Immunol.* **147,** 4126–4135.

Weiss, E. A., Michael, A. , and Yuan, D. (1989). Role of transcriptional termination in the regulation of μ mRNA expression in B lymphocytes. *J. Immunol.* **143,** 1046–1052.

White, M. B., Word, C. J., Humphries, C. G., Blattner, F. R., and Tucker, P. W. (1990). Immunoglobulin D switching can occur through homologous recombination in human B cells. *Mol. Cell. Biol.* **10,** 3690–3699.

Whitmore, A. C., Prowse, D. M., Haughton, G., and Arnold, L. W. (1991). Ig isotype switching in B lymphocytes. The effect of T cell-derived interleukins, cytokines, cholera toxin, and antigen on isotype switch frequency of a cloned B cell lymphoma. *Int. Immunol.* **3,** 95–103.

Williams, M., Hanakahi, L. A., and Maizels, N. (1993). Purification and properties of LR1, and inducible DNA binding protein from mammalian B lymphocytes. *J. Biol. Chem.* **268,** 13731–13737.

Williams, M., and Maizels, N. (1991). LR1, a lipopolysaccharide-responsive factor with binding sites in the immunoglobulin switch regions and heavy-chain enhancer. *Genes Dev.* **5,** 2353–2361.

Winter, E., Krawinkel, U., and Radbruch, A. (1987). Directed Ig class switch recombination in activated murine B cells. *EMBO J.* **6,** 1663–1671.

Wortis, H. H., Teutsch, M., Higer, M., Zheng, J., and Parker, D. C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. *Proc. Natl. Acad. Sci. USA* **92,** 3348–3352.

Wu, T. T., Reid-Milller, M., Perry, H. M., and Kabat, E. A. (1984). Long identical repeats in the mouse γ2b switch region and their implications for the mechanism of class switching. *EMBO J.* **3,** 2033–2040.

Wuerffel, R., Jamieson, C. E., Morgan, L., Merkulov, G. V., Sen, R., and Kenter, A. L. (1992). Switch recombination breakpoints are strictly correlated with DNA recognition motifs for immunoglobulin Sγ3 DNA-binding proteins. *J. Exp. Med.* **176,** 339–349.

Wuerffel, R. A., Nathan, A. T., and Kenter, A. L. (1990). Detection of an immunoglobulin switch region-specific DNA binding protein in mitogen-stimulated mouse splenic B cells. *Mol. Cell. Biol.* **10,** 1714–1718.

Xing, Y., Johnson, C. V., Dobner, P., and Lawrence, J. B. (1993). Differential nuclear distribution of intron and exon sequences for endogenous RNAs revealed by fluorescence in situ hybridization. *Science* **259,** 1326–1330.

Xu, B., and Clayton, D. A. (1995). A persistent RNA–DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. *Mol. Cell. Biol.* **15,** 580–589.

Xu, J., Foy, T. M., Laman, J. D., Elliot, E. A., Dunn, J. J., Waldschmidt, T. J., Elsemore, J., Noelle, R. J., and Flavell, R. A. (1994). Mice deficient for the CD40 ligand. *Immunity* **1,** 423–432.

Xu, L., Kim, M. G., and Marcu, K. B. (1992). Properties of B cell stage specific and ubiquitous factors binding to immunoglobulin heavy chain gene switch regions. *Int. Immunol.* **4,** 875–887.

Xu, L., and Rothman, P. (1994). IFN-γ represses ε germline transcription and subsequently down-regulates switch recombination to ε. *Int. Immunol.* **6,** 515–521.

Xu, M., and Stavnezer, J. (1992). Regulation of transcription of immunoglobulin germ-line γ1 RNA: Analysis of the promoter/enhancer. *EMBO J.* **11,** 145–155.

Yancopolous, G., DePihno, R., Zimmerman, K., Lutzker, S., Rosenberg, N., and Alt, F. (1986). Secondary rearrangement events in pre B cells: V_HDJ_H replacement by LINE-1 sequence and directed class switching. *EMBO J.* **5,** 3259–3266.

Yaoita, Y., and Honjo, T. (1980). Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome. *Nature* **286,** 850–853.

Yasui, H., Akahor, Y., Hiran, M., Yamada, K., and Kurosawa, Y. (1989). Class switch from μ to δ is mediated by homologous recombination between σμ and Σμ sequences in human immunoglobulin gene loci. *Eur. J. Immunol.* **19,** 1399–1403.

Yuan, D. (1986). Regulation of IgD synthesis in murine neonatal B lymphocytes. *Mol. Cell. Biol.* **6,** 1015–1022.

Yuan, D., and Tucker, P. W. (1984). The transcriptional regulation of the μ–δ heavy chain locus in normal murine B lymphocytes. *J. Exp. Med.* **160,** 564–583.

Yuan, D., and Witte, P. L. (1988). Transcriptional regulation of μ and δ gene expression in bone marrow pre-B and B lymphocytes. *J. Immunol.* **140,** 2808–2814.

Zelazowski, P., Collins, J. T., Dunnick, W., and Snapper, C. M. (1995). Antigen receptor cross-linking differentially regulates germ-line C_H ribonucleic acid expression in murine B cells. *J. Immunol.* **154,** 1223–1231.

Zhang, J., Bottaro, A., Li, S., Stewart, V., and Alt, F. W. (1993). A selective defect in IgG_{2b} switching as a result of targeted mutation of the $I_{\gamma 2b}$ promoter and exon. *EMBO J.* **12,** 3529–3537.

Zwollo, P., and Desiderio, S. (1994). Specific recognition of the *blk* promoter by the B-lymphoid transcription factor B-cell-Specific Activator Protein. *J. Biol. Chem.* **21,** 15310–15317.

ADVANCES IN IMMUNOLOGY, VOL. 61

Interleukin-2 Receptor Signaling Mechanisms

LARRY M. KARNITZ AND ROBERT T. ABRAHAM

Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905

I. Introduction

It has been three decades since the first descriptions of T-cell growth factor activities appeared in the literature (Kasakura and Lowenstein, 1965; Gordon and MacLean, 1965). These growth-promoting activities were released into culture media from allogeneic mixed lymphocyte reactions, and a significant fraction of the mitogenic activity present in these lymphocyte-conditioned supernatants was surely attributable to the cytokine we now know as interleukin-2 (IL-2). Although the initial descriptions of soluble T-cell growth factors were received with limited enthusiasm (antigen was considered to be a sufficient mitogenic stimulus at this time), several pioneering investigators doggedly pursued the characterization of soluble mediators released from mononuclear cells following stimulation with antigens or mitogenic lectins (see Smith, 1988, for review). Subsequent studies quickly identified activated T cells as the source of the growth factor. The derivation of a murine cytolytic T lymphocyte line that displayed continuous dependence on IL-2 for growth in culture permitted the development of a sensitive bioassay for the T-cell growth factor. Indeed, the bioassay was a crucial advance that led to the biochemical purification of IL-2, and ultimately to the isolation of a full-length cDNA clone encoding the long-sought T-cell growth factor.

Prior to the molecular cloning of IL-2, studies of the biology and biochemistry of IL-2 action were severely handicapped by the minute quantities of purified material obtainable from T-cell-conditioned media. The expression of the IL-2 cDNA in bacteria allowed the production of purified, recombinant cytokine in virtually unlimited quantities. For the first time, immunologists around the world had access to highly purified and standardized preparations of recombinant IL-2. As a result, our understanding of the biology and biochemistry of IL-2 actions has progressed at a dizzying pace during the past decade. A high-affinity multisubunit receptor for IL-2 has been molecularly characterized, and much has been learned concerning the contribution of each subunit to the overall function of this receptor. Recent research has considerably advanced our understanding of the proximal signaling elements and downstream signaling cascades engaged as a consequence of IL-2 receptor (IL-2R) occupancy.

This chapter summarizes our current knowledge of IL-2R signaling. Although we shall concentrate mainly on the "party line" regarding the

biochemistry and function of these signals, we will attempt to highlight what we perceive as crucial questions in the areas of IL-2 signaling and T-cell growth regulation. Finally, we will briefly discuss recent advances regarding the mechanism of action of the potent immunosuppressant rapamycin, which have provided some new and rather unexpected insights into the process of mitogenic signaling in IL-2-stimulated T cells.

A. The T-Cell Activation Program

The exposure of mature, resting T cells to their cognate antigen initiates a complex program of cellular proliferation and differentiation that is an essential prelude to the expression of a suitably coordinated and protective immune response. It has been estimated that execution of this program by an effective antigenic stimulus is accompanied by the activation of more than 100 genes (Ullman *et al.*, 1990). In this context, an "effective" stimulus comprises at least two receptor-linked signal transduction cascades emanating from the T-cell plasma membrane (Crabtree and Clipstone, 1994). The first stimulus derives from the multimeric engagement of T-cell antigen receptors (TCR) with ligands consisting of small peptide fragments lodged in the binding cleft of a self major histocompatibility complex (MHC)-encoded molecule expressed on the surface of an antigen-presenting cell (APC). The interaction between the TCR and the MHC–antigen complex is physically and functionally facilitated by the concomitant binding of the nonpolymorphic CD4 and CD8 coreceptors to invariant regions of MHC class II or class I molecules, respectively. The second stimulus is not antigen-specific, and can be delivered through several classes of receptors on the T cell. A physiologically important transducer of this costimulatory signal is CD28, which recognizes the B-7 family of ligands expressed by APC.

The synergistic activating signals triggered by antigen-induced clustering of TCRs and engagement of CD28 initiate the entry of noncycling (G_0 phase) T cells into G_1 phase of the cell cycle. Although many phenotypic alterations accompany the G_0- to G_1-phase transition, two responses are of particular relevance to the present review (Smith, 1984). First, the activated T cell becomes competent to undergo proliferation due to the *de novo* expression of high-affinity surface receptors for T-cell growth factors. Although the most well-studied of these T-cell growth factors (and the focus of this review) is IL-2, a growing family of cytokines exhibits growth-promoting activities toward various T-cell subpopulations. In the case of helper T cells, TCR engagement triggers a second phenotypic response that is crucial for the expansion of activated T-cell populations, i.e., the transcriptional activation of a number of cytokine genes, including the IL-2 gene. The production of IL-2 and the binding of this cytokine

to its high-affinity receptor then drive the progression of activated T cells through G_1 into S phase and, ultimately, into mitosis. Under physiologic conditions, the activities of IL-2 and other T-cell growth factors are restricted to the microenvironment, and, therefore, these function primarily as localized autocrine or paracrine inducers of T-cell-cycle progression.

In addition to its mitogenic activity, IL-2 drives the differentiation of helper, delayed-type hypersensitivity, and cytolytic T cell precursors into mature effector and memory cells. Whether the signaling events that mediate proliferation and differentiation lie within identical or bifurcating pathways remains unclear. Similar considerations apply to survival-promoting activities of IL-2 in certain factor-dependent T cell lines, and in normal T cells stimulated with anti-TCR antibodies in the absence of CD28. Due to the paucity of information regarding differentiation-specific and antiapoptotic signals, much of the discussion will revolve around the potential roles of various IL-2R-linked signaling events in the mitogenic response. Nonetheless, it is important to bear in mind that IL-2 is a pleiotropic stimulatory factor for T cells, and it cannot be assumed *a priori* that the cascade of signals provoked by IL-2 are devoted solely to the control of cell-cycle progression.

II. IL-2R Structure: Composition of the IL-2R

The discovery of IL-2 initiated a search for the growth factor's receptor. Early receptor binding studies demonstrated that IL-2 exhibited low-affinity ($K_d = 10^{-8}$), intermediate-affinity ($K_d = 10^{-9}$), and high-affinity ($K_d = 10^{-11}$) interactions with various lymphoid cell lines, which suggested the existence of multiple receptors or receptor subunits (see Smith, 1988, for review). The first to be discovered was a 55-kDa glycoprotein now known as IL-2Rα. TCR-triggered T-cell activation induces IL-2Rα expression and allows the activated T cell to respond to physiologic (i.e., subnanomolar) concentrations of IL-2. IL-2Rα was initially purified by immunoaffinity chromatography, and was molecularly cloned and sequenced in 1984 (Cosman *et al.*, 1984; Nikaido *et al.*, 1984; Leonard *et al.*, 1984). IL-2Rα possesses a short intracellular region of only 13 amino acids and, when expressed in nonlymphoid cells, binds ligand with only low affinity (Greene *et al.*, 1985). These results predicted that at least one other receptor subunit contributes to the formation of intermediate- and high-affinity IL-2Rs. This prediction was borne out when chemical crosslinking experiments identified a novel 70- to 75-kDa protein (now known as IL-2Rβ) in the receptor–ligand complex (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987; Bich-Thuy *et al.*, 1987), which, although constitutively expressed on quiescent T cells, is also induced during T-cell activation. The

development of monoclonal antibodies to IL-2Rβ permitted the expression cloning of this subunit in 1989 (Hatekeyama *et al.*, 1989b). Based on the predicted amino acid sequence of IL-2Rβ, the mature IL-2Rβ protein is 525 amino acids, with a 214-amino-acid extracellular domain, a 25-amino-acid transmembrane segment, and a 286-amino-acid cytoplasmic region. Expression of IL-2Rβ and IL-2Rα in hemopoietic cell lines generated functional high-affinity IL-2R (Hatekeyama *et al.*, 1989a,b), whereas expression of IL-2Rβ in hemopoietic cells lacking IL-2Rα reconstituted only the intermediate-affinity IL-2R (Hatekeyama *et al.*, 1989b). These results suggested that IL-2Rβ itself was the intermediate-affinity IL-2R and that the high-affinity IL-2R is composed of IL-2Rα and IL-2Rβ. However, a much different result was obtained when IL-2Rβ was expressed in nonhemopoietic cells (Minamoto *et al.*, 1990; Tsudo *et al.*, 1990). In these cells, IL-2Rβ expression did not generate intermediate-affinity IL-2Rs. Additionally, when ectopically coexpressed in nonhemopoietic cells, the combination of IL-2Rα and IL-2Rβ bound IL-2 with greater affinity than IL-2Rα alone; however, the receptor complexes could not trigger IL-2-induced receptor internalization. These results suggested that although IL-2Rα and IL-2Rβ can bind IL-2, these subunits are not sufficient to elicit intracellular signals, and that a third, hemopoietic cell-specific component was also required. Indeed, chemical crosslinking studies demonstrated that in addition to IL-2Rβ, a previously unidentified 64-kDa membrane protein was also present in the ligand–receptor complex (Saito *et al.*, 1991). In 1992, this 64-kDa chain was biochemically purified and cloned (Takeshita *et al.*, 1992). The deduced amino acid sequence predicts that the mature 64-kDa receptor subunit consists of a 232-amino-acid extracellular domain, a 29-amino-acid transmembrane spanning sequence, and a relatively short 86-amino-acid intracellular region. Because this third subunit is shared among other cytokine receptors (see below for discussion), it has been termed the γ-common chain (γ_c). Heterologous expression studies definitively demonstrated that coexpression of IL-2Rβ and γ_c in nonhemopoietic cells generated intermediate-affinity IL-2Rs, whereas coexpression of all three subunits resulted in formation of high-affinity IL-2Rs (Takeshita *et al.*, 1992).

Although IL-2Rα is required to form a high-affinity IL-2R, IL-2Rβ and γ_c alone are the signal-transducing components of the IL-2R, and heterodimerization of the intracellular domains of IL-2Rβ with γ_c triggers rapid biochemical signaling events as well as long-term proliferation of T cell lines (Nakamura *et al.*, 1994; Nelson *et al.*, 1994). Both IL-2Rβ and γ_c, but not IL-2Rα, are members of the expanding superfamily of hemopoietin receptors (see Cosman, 1993, for review). The extracellular domains of these receptors are typified by conservatively spaced cysteine

residues (Cys–X_{9-10}–Cys–X–Trp–X_{26-32}–Cys–X_{10-15}–Cys, where X represents any amino acid) that may form intrasubunit disulfide linkages in the amino-terminal portion of the receptor subunits and a juxtamembrane region that also contains a Trp–Ser–X–Trp–Ser motif that is required for receptor folding, cell surface expression, and ligand binding (Miyazaki *et al.*, 1991; Yoshimura *et al.*, 1992). However, at least in the case of the growth hormone receptor, this motif does not appear to play a direct role in ligand binding (Cunningham *et al.*, 1991).

A central paradigm that has emerged from the study of hemopoietin receptors is that ligand-triggered receptor subunit oligomerization initiates cellular responses. In recent years, a much better understanding of the subunit compositions of these ligand-induced complexes has emerged, and a comparison between these receptor systems and the IL-2R is useful. Some hemopoietins induce dimerization of identical subunits, whereas others mediate the formation of heterodimeric signaling complexes. In the well-studied example of the growth hormone receptor, a single growth hormone molecule mediates dimerization of two identical signal-transducing receptor subunits (Cunningham *et al.*, 1991). However, in many cases, ligand heterodimerizes nonidentical receptor subunits that are shared among several different cytokine ligands. This concept has been elegantly demonstrated with the GM-CSF/IL-3/IL-5 subfamily of hemopoietin receptors. In this family, each cytokine utilizes a specificity-determining α subunit and a common signal transducing β_c subunit (see Lopez *et al.*, 1992, for review). Therefore, the ability of a β_c-expressing cell to respond to any of these cytokines is dictated by the expression of the α subunit. The receptors for the CNTF/LIF/OSM/IL-6 subfamily follow a similar, but slightly more complicated, paradigm (see Stahl and Yancopoulos, 1993, for review). The signal transducing subunits for this family are either gp130 or LIFR β. In the case of the CNTFR, a specificity-determining α subunit (CNTFR α) is required along with both gp130 and LIFR β to form a functional CNTFR (Davis *et al.*, 1993). Likewise, formation of a functional IL-6R also requires a specificity-conferring α subunit (IL-6Rα) that binds IL-6 with low affinity, but rather than heterodimerizing gp130 and LIFR β, IL-6 homodimerizes two gp130 molecules in the presence of IL-6R α (Taga *et al.*, 1989; Hibi *et al.*, 1990). In contrast, LIF and OSM also utilize both gp130 and LIFR β, and ligand triggers heterodimerization of these signal-transducing subunits without the apparent need for a third α-like subunit to bind either cytokine (Gearing *et al.*, 1992; Baumann *et al.*, 1993).

In the last few years, the receptor subunits for IL-2 have been found to follow a paradigm most similar to that of the CNTF/LIF/OSM/IL-6 subfamily. In addition to being a part of the high-affinity IL-2R, γ_c is also

required to form fully functional IL-4 (Kondo *et al.*, 1993; Russell *et al.*, 1993), IL-7 (Noguchi *et al.*, 1993a; Kondo *et al.*, 1994), IL-9 (Russell *et al.*, 1994), and IL-15 receptors (Giri *et al.*, 1994). For IL-4, IL-7, and IL-9, ligand induces heterodimerization of a specificity-determining subunit (i.e., IL-4R, IL-7R, or IL-9R) with γ_c. Based on the available data, these specificity-conferring subunits appear to have functions analogous to the IL-2Rβ subunit, in that their cytoplasmic domains are essential for signal transduction. However, like the CNTF and IL-6 receptors, a third specificity-determining subunit (IL-2Rα) is required to form a fully functional high-affinity IL-2R. In all these examples, the α subunits either lack or have very short cytoplasmic tails, suggesting that they function solely to bind ligand and assemble signaling-competent receptor oligomers.

In the case of IL-15, the subunit stoichiometry has not yet been fully described. Both IL-2Rβ and γ_c subunits are required to form a functional receptor, and it appears that IL-15 also utilizes an unidentified specificity-determining α subunit (Giri *et al.*, 1994). These results suggest that IL-15R and IL-2R will transduce identical signals and that α subunit expression will define the cytokine responsiveness of the cell.

The discovery that γ_c was an essential component of other cytokine receptors reconciled a previously unexplained observation. If the γ_c subunit served only as a component of the IL-2R, then mice lacking IL-2 would be predicted to have the same phenotype as mice or humans lacking functional γ_c subunit. However, this is not the case. Mice lacking IL-2, although immunologically compromised, have relatively intact immune systems (Schorle *et al.*, 1991), whereas the loss of γ_c has far more profound effects on immune system development. Nature and genetic engineering have provided both human and murine models of γ_c deficiency. Patients with X-linked severe combined immunodeficiency (XSCID) harbor naturally occurring premature truncations of the cytoplasmic domain of γ_c (Noguchi *et al.*, 1993b), whereas gene "knockout" technology has generated mice that lack γ_c (Cao *et al.*, 1995; DiSanto *et al.*, 1995). Although the phenotypes of the γ_c-deficient humans and mice are somewhat different, both exhibit severely perturbed immune system development. These findings suggest that while IL-2 is an important mediator of immune function, other cytokines, which also utilize γ_c, can perform partially redundant functions in T-cell development. However, loss of γ_c and the resultant inability to respond to IL-2, IL-4, IL-7, IL-9, and IL-15 prevents the development of a fully functional immune system.

Shortly after identification of IL-2Rβ, an elegant functional analysis of this subunit was undertaken by Taniguchi's research group. Analysis of the predicted amino acid sequence of the cytoplasmic portion of IL-2Rβ identified three distinct regions (Hatekeyama *et al.*, 1989a,b). The region

closest to the plasma membrane is termed the serine-rich region (amino acids 267–322). Directly following the serine-rich portion of the receptor is the acidic region (amino acids 313–382), which is followed by the carboxyl-terminal proline-rich region (amino acids, 378–525). Subsequently, IL-2Rβ mutants lacking each of these regions were prepared. Although delineation of these domains was originally based on sequence analysis, investigation of the signaling pathways triggered by the above-described IL-2Rβ mutants revealed that these regions may also comprise functional domains (Hatekeyama *et al.*, 1989a). As the other signal-transducing component of the IL-2R, the γ_c subunit has a much shorter, 86-amino acid intracellular domain. An analysis of the predicted protein sequence of γ_c identified a region bearing limited homology to a Src homology 2 (SH2) domain within the membrane-proximal portion of γ_c (Takeshita *et al.*, 1992). SH2 domains, which specifically bind phosphotyrosyl (pTyr)-containing peptide sequences, are active participants in the assembly of signal-transducing complexes (for reviews see Cohen *et al.*, 1995; Pawson, 1995). Although the ability of a receptor to associate with tyrosine-phosphorylated signaling molecules via the receptor's SH2 domain would be an attractive model for signal transduction, there is no evidence to date that the putative SH2 subdomain of γ_c binds pTyr-containing peptides.

Although the extracellular domains of the hemopoietin receptors display several homologous motifs, the intracellular portions of these receptors appeared at first glance to have little in common. However, reevaluation of the intracellular domains of the hemopoietin receptors identified short, membrane-proximal regions of homology termed box 1/box 2 motifs (Fukunaga *et al.*, 1991; Murakami *et al.*, 1991), and these motifs are located within the serine-rich region of IL-2Rβ. As will be described later, the box 1/box 2 motifs, which have been genetically and biochemically implicated in mitogenic signaling by the cytokine receptors, have recently been shown to associate with signaling elements that play crucial roles in the initiation of receptor-triggered signaling.

III. IL-2R-Triggered Proximal Signaling Events

A. IL-2R Couples to Src-Family Kinases

Like many other growth factor receptors, ligation of the IL-2R induces cell proliferation. A common theme among receptors that trigger mitogenesis is the activation of protein tyrosine kinases (PTKs). An initial search for signaling pathways activated by the IL-2R in T cells revealed that, like the receptor tyrosine kinase (RTK) growth factor receptors, IL-2R ligation also provoked tyrosine phosphorylation of multiple protein substrates (Saltzman *et al.*, 1988; Farrar and Ferris, 1989; Sharon *et al.*, 1989; Augus-

tine *et al.*, 1990; Merida and Gaulton, 1990). Further evidence that PTK activation was linked to IL-2-induced responses was provided by pharmacological inhibitors, which revealed that PTKs were requisite for both IL-2-induced substrate phosphorylation and proliferation (Otani *et al.*, 1992). Because the IL-2R subunits possess no intrinsic PTK activity, research efforts focused on the identification of nonreceptor PTKs coupled to the IL-2R subunits. At that time, the frontrunners for the rapidly expanding group of nonreceptor PTKs were the Src-family kinases, which have been implicated in a wide variety of signal-transducing functions initiated by *m*ultichain *i*mmune *r*ecognition *r*eceptors (MIRR), growth factor RTKs, and many of the hemopoietin receptors.

There are currently nine members of the Src family, and these PTKs exhibit both widespread and cell-type-specific patterns of expression (see Bolen *et al.*, 1991, for review). T cells typically express Lck, Fyn, and Yes, whereas other IL-2-responsive cells such as B cells preferentially express Fyn, Lyn, and Blk. Based on sequence homologies found among the Src family members, these PTKs contain four domains. The extreme amino-terminal sequences target the PTK for myristylation at a conserved glycine residue (see Resh, 1994, for review). In addition, certain Src-family PTKs contain a nearby Cys residue that is modified by palmitylation (Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993; Alland *et al.*, 1994). These lipid modifications, along with neighboring basic amino acids, anchor the PTKs to the plasma membrane (Silverman and Resh, 1992; Sigal *et al.*, 1994). Although the modifications are not required for catalytic activity, they are requisite for normal subcellular localization and biological function. The region located adjacent to the myristylation-specifying tail is the "unique" domain. This region confers kinase-specific functions to each enzyme (Pleiman *et al.*, 1993; Carrera *et al.*, 1995) and may also influence substrate selection and coupling of receptors to downstream signaling pathways. As an example, the unique region of Lck allows it to specifically interact with the T-cell coreceptors CD4 and CD8, whereas other Src-family kinases are unable to interact with these transmembrane proteins (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Barber *et al.*, 1989). The next identified domain, which consists of approximately 60 amino acids, is the Src homology 3 domain (SH3). These domains bind specific proline-rich sequences found in other signal-transducing and cytoskeletal proteins and participate in substrate selection and the assembly of signaling complexes (for reviews see Cohen *et al.*, 1995; Pawson, 1995). However, in many instances SH3 domains function in concert with SH2 domains to mediate protein–protein interactions (Panchamoorthy, 1994), and in Src-family kinases an SH2 domain is located directly adjacent to the carboxyl-terminus of the SH3 domain. SH2 domains consist of about 100 amino acids and bind pTyr

residues embedded within an appropriate target amino acid sequence (for reviews see Cohen *et al.*, 1995; Pawson, 1995). The specificity of the SH2–pTyr interaction is primarily dictated by the three residues carboxyl-terminal to the pTyr groups. Although all SH2 domains exhibit sequence homology, amino acid substitutions within the pTyr-binding pocket of the SH2 domain alter the binding specificity of SH2 domains found within the Src family PTKs, as well as other signaling molecules (Marengere *et al.*, 1994). Because tyrosine phosphorylation is an inducible event, the SH2–pTyr interactions can reversibly regulate the assembly of multiprotein signaling complexes that transmit downstream signals. The carboxyl-terminal half of the molecule contains the conserved kinase domain and a functional catalytic domain is required for most, although perhaps not all (Xu and Littman, 1993), biological functions. A conserved tyrosyl residue is located at the extreme carboxyl terminus of the enzyme. When this tyrosine is phosphorylated, the PTK is folded upon itself and its catalytic activity is repressed by an intramolecular interaction between the carboxyl-terminal pTyr residue and the enzyme's SH2 and SH3 domain. Mutation of this tyrosine prevents the PTK from assuming a repressed conformation, rendering it constitutively active and, in some cases, unmasking a transforming activity (see Cooper and Howell, 1993, for review).

Soon after the identification of the IL-2Rβ subunit, Taniguchi and coworkers demonstrated that the Src-family kinase, Lck, was both physically and functionally coupled to IL-2Rβ (Hatekeyama *et al.*, 1991; Horak *et al.*, 1991). Using the IL-2Rβ mutants that lack either the serine-rich, acidic, or proline-rich regions (Fig. 1), mapping experiments demonstrated that the acidic portion of IL-2Rβ interacted with an amino-terminal region of Lck's kinase domain (Hatekeyama *et al.*, 1991). However, studies soon demonstrated that, in addition to Lck, the Src-family kinases Fyn and Lyn could also interact physically and functionally with IL-2Rβ (Torigoe *et al.*, 1992; Koboyashi *et al.*, 1993; Minami *et al.*, 1993). Further evidence that Src family members other than Lck might mediate proliferative signaling was provided by IL-2-responsive cell lines that do not express Lck. One such cell line was derived from CTLL-2 cells, which are an IL-2-dependent murine T cell line that normally expresses both Lck and Fyn. A spontaneous somatic mutant derived from these cells proliferated normally in response to IL-2 and retained IL-2-dependence, and yet did not express detectable Lck (Karnitz *et al.*, 1992). Additionally, a murine myeloid progenitor cell line expressing transfected IL-2Rβ proliferated in response to IL-2, and yet did not express Lck (Otani *et al.*, 1992). Taken together, these results suggest that Lck is not requisite for IL-2-induced proliferation and that Fyn, or other Src-family kinases, may subserve partially or fully redundant roles in coupling IL-2Rβ to downstream signaling pathways.

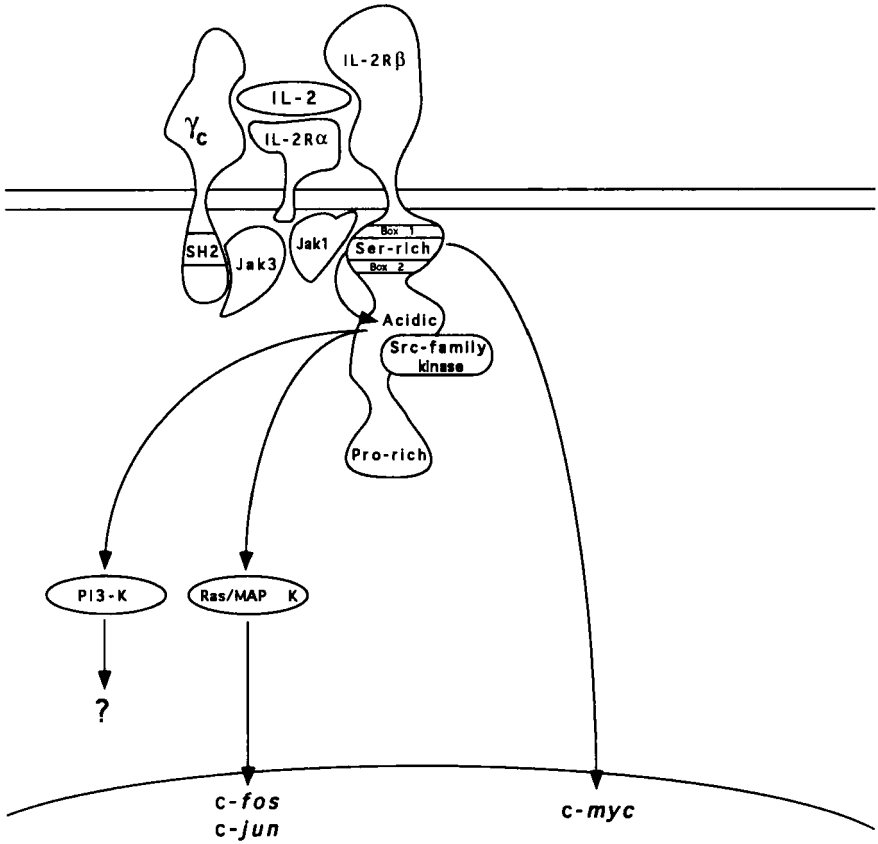


FIG. 1. IL-2Rβ, receptor-associated PTKs, and IL-2-induced signaling pathways. IL-2 induces the coapproximation of the signal-transducing receptor subunits IL-2Rβ and γ_c, and their associated PTKs. PTK juxtaposition triggers activation of initiates signaling to downstream effector pathways. The serine-rich region of IL-2Rβ, which contains the box 1/box 2 motifs, mediates association with the PTKs, Jak1 and Syk. The acidic region, which associates with the Src-family kinase members, and the proline-rich region of IL-2Rβ are also indicated. The γc subunit, which stably interacts with Jak3, also contains an SH2-like subdomain. Induction of c-*myc* mRNA requires only an intact serine-rich region of IL-2Rβ as well as γ_c, whereas expression of c-*fos* and c-*jun* and activation of the PI3-K and Ras/MAP kinase pathways requires both the serine-rich and acidic regions of IL-2Rβ. For a more detailed presentation of IL-2-triggered activation of the Ras/MAP kinase and PI3-K pathways see Figs. 2 and 3, respectively.

The development of a genetically manipulatable experimental system to study IL-2R signaling allowed a dissection of the pathways activated by the IL-2R. These studies relied heavily on BAF/B03 cells, which are a derivative of the IL-3-dependent pre-B cell line, BA/F3. The BAF/B03

cell line constitutively expresses IL-2Rα and γ_c, and ectopic expression of IL-2Rβ in BAF/B03 cells permits the transfected cells to proliferate in response to IL-2 (see Minami *et al.*, 1992), making this cell line a useful model system to analyze the signaling pathways and proliferative phenotypes triggered by various IL-2Rβ mutants. An initial dissection of the signaling pathways activated by receptors lacking the acidic region (Satoh *et al.*, 1992; Minami *et al.*, 1993) revealed that these IL-2Rβ mutants did not induce detectable protein tyrosine phosphorylation in response to IL-2 (Table I and Fig. 1). Additionally, IL-2Rβ mutants lacking the acidic region did not activate the Ras pathway (Satoh *et al.*, 1992) or the phosphatidylinositol-3-kinase (PI3-K) pathway (Williamson *et al.*, 1994) nor did they induce the proto-oncogenes c-*fos* and c-*jun* (Hatekeyama *et al.*, 1992; Minami *et al.*, 1993). These results suggested that because the acidic region associates with Src-family kinases, these PTKs may link IL-2Rβ to downstream signaling pathways. Although IL-2Rβ lacking the acidic region failed to activate many signaling pathways commonly associated with proliferation, this receptor mutant nonetheless supported IL-2 induced proliferation when expressed in the BAF/B03 cell line (Hatekeyama *et al.*, 1989a). It should be borne in mind, however, that the acidic region may have essential functions that are masked in the BAF/B03 cell line (see Section IV). The inescapable message from these studies was that the full spectrum of IL-2-dependent mitogenic signals was not channeled exclusively through the Src-family kinase-binding acidic region. In fact, when analyzed in more detail, these IL-2Rβ mutants induced normal accumulation of c-*myc* mRNA in response to IL-2 (Satoh *et al.*, 1992), suggesting that although the acidic region is critical for the induction of many signaling pathways, signals leading to proliferation and c-*myc* induction emanate from other portions of IL-2Rβ (Fig. 1 and Table I). One of these regions, the proline-rich portion of IL-2Rβ, may participate in IL-2-triggered signal transduction (see Section VI). However, IL-2Rβ subunits that lack the proline-rich region also induced BAF/B03 proliferation (Hatekeyama *et*

TABLE I
SIGNALING PATHWAYS ACTIVATED BY IL-2Rβ CYTOPLASMIC DELETION MUTANTS

	Activated Pathway				
IL-2Rβ Cytoplasmic Domain	PTK	PI3-K	Ras/MAP K	c-*jun*/c-*fos*	c-*myc*
Wild-type	+	+	+	+	+
Serine-rich deletion	−	−	−	−	−
Acidic deletion	−	−	−	−	+
Proline-rich deletion	+	+	+	+	+

al., 1989a), suggesting that these regions of IL-2Rβ and the signaling pathways they engage are not essential for receptor-triggered proliferation.

A much different set of results emerged with IL-2Rβ mutants lacking the serine-rich domain. In BAF/B03 cells expressing these receptor mutants, IL-2 failed to support proliferation (Hatekeyama *et al.*, 1989a). Although this IL-2Rβ mutant did associate with Lck, it did not trigger any known signaling pathways. These results suggested that the serine-rich portion of the receptor engages critical receptor-proximal signaling element(s) that may initiate all IL-2R-triggered signaling events, including those that also require an intact acidic region of IL-2Rβ. Although the serine-rich region was sufficient for induction of c-*myc*, both the serine-rich and acidic regions of IL-2Rβ cooperate to trigger the activation of Src-family kinases (Minami *et al.*, 1993), Ras (Satoh *et al.*, 1992), and PI3-K (Merida *et al.*, 1993; Williamson *et al.*, 1994), as well as the induction of c-*jun* and c-*fos* (Satoh *et al.*, 1992). These findings suggested that the serine-rich region associates with signal-transducing elements that may function upstream of the signaling pathways activated by the acidic region of IL-2Rβ. A clue to the signal transducer(s) that couples to the serine-rich region of IL-2Rβ came from studies demonstrating that two other classes of PTKs also couple to this portion of IL-2Rβ.

B. IL-2R Subunits Are Linked to Jak PTKs

The first PTK found to be associated with the serine-rich region was a member of the Jak family. There are currently four members in the Jak family: Jak1, Jak2, Jak3, and Tyk2. The Jak PTKs were originally identified during efforts to clone novel PTKs using low-stringency hybridizations and the polymerase chain reaction (see Ihle *et al.*, 1994, for review). Therefore, these PTKs initially surfaced as cDNAs with no known functions. However, the discovery that IFN-αβ- and IFN-γ-induced gene expression required Jak-family members (Velazquez *et al.*, 1992; Muller *et al.*, 1993; Watling *et al.*, 1993) led many investigators to analyze whether Jaks might also couple to other hemopoietin receptors. In the case of the IFN receptors and other members of the hemopoietin receptor family, Jaks are required for the phosphorylation and activation of the DNA-binding Signal *t*ransducers and *a*ctivators of *t*ranscription (Stat) factors (see Darnell *et al.*, 1994 for review). However, because Jaks are associated with the portions of hemopoietin receptors that are critical for all aspects of signaling, these PTKs likely have additional roles in hemopoietin receptor-triggered signaling.

Analysis of the predicted protein sequences of Jaks shows that they are structurally quite different from the Src-family PTKs (see Ihle *et al.*, 1994, for review). Most notably, the Jaks do not contain SH2 or SH3 domains.

Surprisingly, within the carboxyl-terminal half of the PTK, they also have two regions that exhibit overall homology to other protein kinase domains. However, the most amino-terminal domain lacks amino acid residues critical for catalytic activity in other protein kinases and consequently the function of this domain remains enigmatic. The deduced amino acid sequence of the most carboxyl-terminal domain exhibits all the characteristics of a functional PTK domain and is predicted to be catalytically active. The presence of the dual kinase domain engendered the acronym, Jak, which is derived from *Ja*nus *k*inase and refers to the two-faced Roman god of doors and gates.

Soon after the demonstration that Jaks were linked to IFN-α/β- and IFN-γ-mediated signaling. IL-2Rβ and γ_c were shown to constitutively and inducibly associate with Jak1 and Jak3, respectively (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). The availability of deletion and point mutants of many different hemopoietin receptor subunits allowed a rapid mapping of the Jak–receptor interaction and a paradigm quickly emerged. The Jaks were found to associate with the short membrane-proximal portions of receptors that contain the box 1/box 2 motifs (Ihle and Kerr, 1995). Although awaiting more detailed mapping studies, these motifs are intimately involved in receptor–Jak associations. Strikingly, the box 1/box 2 motifs are located within the serine-rich region of IL-2Rβ that has previously been shown to be essential for signal transduction. In fact, for all cytokine receptors studied to date, these membrane-proximal sequences are indispensable for receptor-induced signaling (Ihle *et al.*, 1994). Taken together, these results suggest that the association of Jaks with cytokine receptors, including IL-2Rβ, is essential for their signaling competency. Although γ_c associates with Jak3, the box 1/box 2 motifs have not been unequivocally identified in γ_c. Two independent groups have performed γ_c–Jak3 mapping studies, which demonstrated that a region within the carboxyl terminus and a portion of the SH2 subdomain of γ_c are required for Jak3 interaction (Asao *et al.*, 1994; Miyazaki *et al.*, 1994). In the case of the IL-2R, the role of Jak3 has been dramatically underscored. Fibroblasts that express all three IL-2R subunits cannot initiate DNA synthesis in response to IL-2. However, coexpression of Jak3 confers IL-2-triggered DNA synthesis, demonstrating the crucial role these PTKs play in the transmission of proliferative signals (Miyazaki *et al.*, 1994).

C. Yet Another Kinase?

Although the roles of the Src- and Jak-family PTKs in IL-2R function have not yet been clarified, recent evidence suggests that another PTK is also linked to the IL-2R. Syk, a PTK that plays an integral role in signaling from Fc receptors and B cell antigen receptors, is both physically and

functionally coupled to the IL-2R (Minami *et al.*, 1995). Syk and its related family member ZAP-70 are composed of several widely conserved domains (see Chan *et al.*, 1994, for review). Unlike the Src-family PTKs, Syk and ZAP-70 do not undergo lipid modification, suggesting that other mechanisms target these PTKs to membrane receptors. In the amino-terminal half of the PTK, tandem SH2 domains are found, whereas the carboxyl terminus contains a single kinase domain. However, the carboxyl-terminal tyrosine that mediates repression of Src-family PTKs is absent and the mechanisms controlling activation of Syk and ZAP-70 are obscure. Like Jak1, Syk also associates with the serine-rich region of IL-2Rβ (Minami *et al.*, 1995) and may participate in the functions of this domain, one of which is the accumulation of c-*myc* mRNA. When expressed in BA/F3 cells, crosslinking of chimeric receptors containing the extracellular and transmembrane portions of CD16 linked to Syk on the cytoplasmic side activates Syk and triggers an increase in c-*myc* mRNA levels (Minami *et al.*, 1995). By analogy, the coapproximation of IL-2Rβ-bound Syk with other IL-2R-bound PTKs may also activate Syk and transduce downstream signals leading to c-*myc* mRNA accumulation. However, because IL-2Rβ mutants that selectively associate with only Jak1 or Syk have not yet been reported, the relative contribution of each PTK to signal transduction initiated by the serine-rich region of IL-2Rβ is unknown.

D. Initition of Receptor-Proximal Signaling

Signaling by growth factor RTKs and cytokine receptors is initiated by ligand-induced subunit oligomerization. For the growth factor RTKs, ligand-induced dimerization triggers RTK activation and autophosphorylation of the receptor subunits (see Ullrich and Schlessinger, 1990, for review). The pTyr residues in the receptor's cytoplasmic region function as docking sites for SH2-domain-containing signaling proteins and serve as nuclei to assemble and colocalize multiprotein signaling complexes at the plasma membrane. In some instances, the pTyr–SH2 interaction directly activates the associated signaling enzymes. However, in other cases, binding to the receptor aligns these target proteins for receptor-mediated tyrosine phosphorylation and concomitant activation. By analogy with the growth factor RTKs, ligand-induced subunit dimerization of hemopoietin receptor subunits, including IL-2Rβ and γ_c, would serve to coapproximate receptor-bound PTKs. Because all IL-2-triggered signaling events require the portions of both IL-2Rβ and γ_c that associate with and activate the Jak PTKs, the formation of IL-2Rβ–γ_c heterodimers may initiate the transphosphorylation and activation of the associated Jaks. Activated Jaks might then phosphorylate IL-2Rβ and γ_c, providing pTyr sites to initiate the assembly of signaling complexes. In support of this, deletion of the Jak-binding

portion of γ_c abrogates ligand-induced IL-2Rβ tyrosine phosphorylation (Asao *et al.*, 1993). Upon receptor-mediated Jak activation, receptor-bound Jaks may then relay activating signals to molecules associated with other portions of the receptor. The acidic region of IL-2Rβ may be one target of such upstream signals. Although not associated via an SH2 domain-mediated interaction, Src-family kinases may amplify signals initiated by other signaling pathways that lead to proliferation, differentiation, or survival. Two pathways whose activation is dependent upon the acidic domain of IL-2Rβ are the Ras/MAP kinase pathway and the PI3-K pathway. Both have been directly implicated in mitogenic responses, and as will be discussed next, their functions may be intimately intertwined.

IV. The Ras/MAP Kinase Cascade

A. A Path to the Nucleus

With the exception of the IL-4R (Satoh *et al.*, 1991; Duronio *et al.*, 1992; Welham *et al.*, 1994a,b), all hemopoietin receptors activate Ras. In the last few years, a series of reports has illuminated a highly conserved signaling cascade leading from PTK-dependent receptors to the nucleus (see Guan, 1994; Marshall, 1994). This pathway, which will be referred to as the Ras/MAP kinase pathway, has been best described for the growth factor PTKs. This section outlines information from the RTK-based systems and will integrate these findings with the available data for the IL-2R-activated pathway. The central player in this pathway is the membrane-localized, GTP-binding protein, Ras, which is active when bound to GTP and inactive when GDP-bound.

For the IL-2R, initiation of the pathway (Fig. 2) occurs when IL-2-triggered PTK activation induces tyrosine phosphorylation of Shc (Burns *et al.*, 1993; Cutler *et al.*, 1993; Ravichandran and Burakoff, 1993; Liu *et al.*, 1994; Zhu *et al.*, 1994). Shc and other signal-transducing proteins that lack known enzyme activity and possess protein–protein interaction domains are commonly referred to as adaptor proteins. These proteins typically contain at least one pTyr-binding domain that reversibly links upstream signaling molecules to downstream effectors (for review see Cohen *et al.*, 1995; Pawson, 1995). In the case of Shc, this adaptor protein contains two identified pTyr-binding domains. One pTyr-binding domain is located at the amino terminus of Shc and has no homology to SH2 domains. This novel domain has been variably termed *p*rotein *i*nteraction *d*omain (PID (Blaikie *et al.*, 1994), *S*H2 *a*nd *I*RS1 *N*PXY (SAIN)-binding domain (Gustafson *et al.*, 1995), or *p*hospho*t*yrosine-*b*inding (PTB) domain (Kavanaugh and Williams, 1994). For simplicity, we will refer to it as the PTB domain in this chapter. Unlike an SH2–pTyr interaction, in which

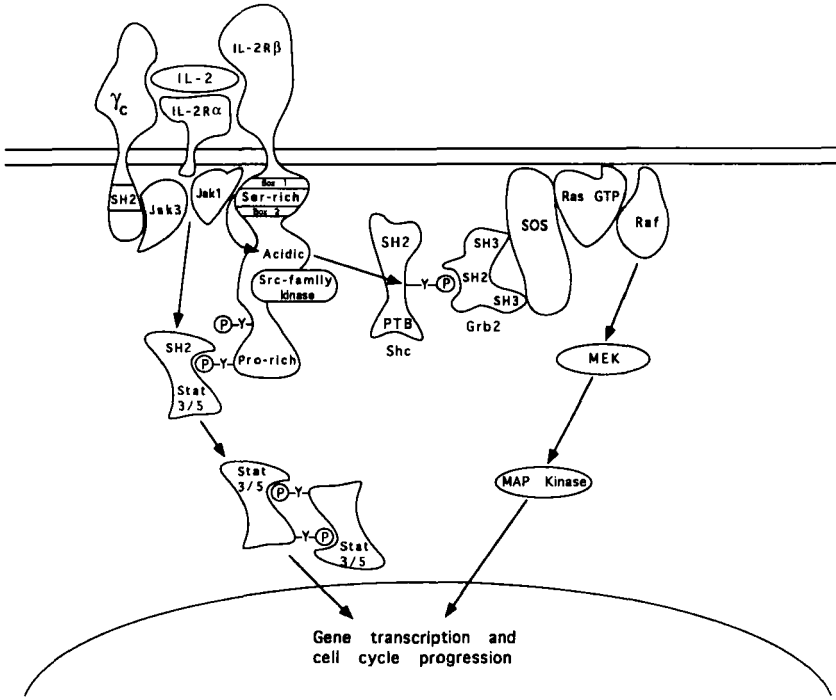


FIG. 2. IL-2 induced Ras and Stat activation. Activation of the Ras/MAP kinase pathway requires cooperation between the serine-rich and acidic regions of IL-2Rβ. In one possible model, Jaks relay an undefined signal to the acidic portion of IL-2Rβ that recruits Shc to the receptor. Activated Jaks and/or Src-family kinases then phosphorylate Shc, leading to formation of a trimolecular Shc–Grb2–SOS complex that activates Ras by inducing the exchange of GDP for GTP. Activated Ras then interacts with and activates Raf, which, in turn, triggers MEK and ultimately MAP kinase activation. MAP kinase translocates to the nucleus and phosphorylates certain transcription factors, modulating their trans-activating potential, or phosphorylates and activates other serine/threonine kinases. In addition to initiating the PI3-K and Ras/MAP kinase pathways, Jaks also transduce signals directly to the nucleus by phosphorylating Stat3 and/or Stat5. Phosphorylation induces dimerization via an SH2–pTyr interaction that also triggers nuclear translocation. In the nucleus, the Stat activity may be further affected by serine/threonine phosphorylation by proline-directed kinases such as MAP kinase.

the binding specificity is determined by amino acid residues carboxyl-terminal to the pTyr (i.e., pTyr–X–X–X, where X represents any amino acid), the binding specificity of a PTB domain is defined by the peptide sequence amino-terminal to the pTyr residue (i.e., X–X–X–pTyr). Several PTB interaction sites have been identified, including those within c-Erb B2 (Kavanaugh *et al.*, 1995) the epidermal growth factor (EGF) receptor

(Blaikie *et al.*, 1994), and the insulin receptor β subunit (Gustafson *et al.*, 1995). These sites are composed of an Asn–Pro–X–Tyr motif that, when tyrosine-phosphorylated, directs the PTB–pTyr interaction. At the carboxyl terminus of Shc is a canonical SH2 domain. The availability of two separate and different pTyr-binding domains within a single Shc molecule suggests that Shc may reversibly assemble other tyrosine-phosphorylated proteins into signal-transducing complexes. Sandwiched between the amino-terminal PTB domain and the carboxyl-terminal SH2 domain is a region rich in glycine and proline that exhibits homology to collagen. This domain contains Tyr^{317} that is phosphorylated in response to stimuli and is critical for Shc function. However, no other function has yet been ascribed to the collagen domain.

Shc phosphorylation at Tyr^{317} creates a consensus binding site (Tyr–Val–Asn–Val) for the SH2 domain of another adaptor protein, Grb2, which is composed of an SH2 domain flanked on each side by an SH3 domain (see Downward, 1994, for review). The SH3 domains of Grb2 mediate a constitutive, and possibly inducible (Ravichandran *et al.*, 1995), interaction between this adaptor and the guanine nucleotide exchange factor *S*on *o*f *S*evenless (Sos), via the SH3 domains of Grb2 interacting with proline-rich sequences of Sos. By an unknown mechanism, the formation of the trimolecular Shc–Grb2–Sos complex triggers the exchange of GDP for GTP onto Ras, resulting in Ras activation. GTP-bound Ras activates a serine/threonine kinase cascade that is initiated when the effector domain of activated Ras interacts with the regulatory amino terminus of the serine/threonine kinase Raf, resulting in Raf recruitment to the plasma membrane (see Daum *et al.*, 1994, for review). Although the Ras–Raf interaction is obligatory for Raf activation, the interaction does not activate Raf catalytic activity, and an undefined Ras-independent activation event is also required (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Activated Raf phosphorylates and activates the dual-specificity threonine/tyrosine kinase MEK which, in turn, phosphorylates closely spaced threonyl and tyrosyl residues in MAP kinase. These phosphorylations activate MAP kinase, which relays the signal to downstream effector systems by phosphorylating multiple cytoplasmic substrates, including other serine/threonine kinases. In addition, activated MAP kinase translocates to the nucleus, where one known target is the ternary complex factor ($p62^{elk}$/TCF) of the *s*erum *r*esponse *f*actor (SRF) (see Treisman, 1994, for review). The DNA target of the phosphorylated SRF complex is the *s*erum *r*esponse *e*lement (SRE), which plays a critical role in the activation of the c-*fos* gene triggered by IL-2 and other growth factors (Trouche *et al.*, 1991; Hatekeyama *et al.*, 1992). Therefore, this pathway links Ras activation to receptor-triggered expres-

sion of a proto-oncogene whose function appears related to cell cycle progression.

B. IL-2R Coupling to the Ras/MAP Kinase Pathway

Tyrosine phosphorylation of Shc provokes the initial stimulus for the Ras/MAP kinase pathway, and IL-2 has been shown to activate all aspects of the pathway, including Ras (Satoh *et al.*, 1991; Duronio *et al.*, 1992; Welham *et al.*, 1994a,b), Raf (Turner *et al.*, 1991), MEK (Karnitz *et al.*, 1995), and MAP kinase (Perkins *et al.*, 1993; Karnitz *et al.*, 1995). However, a paucity of information is available describing how the IL-2R choreographs this reaction. As alluded to in Section III, the acid region of IL-2Rβ, which mediates the induction of PTK activity and c-*fos* and c-*jun* mRNA, also participates in the activation of the Ras/MAP kinase pathway (Fig. 1 and Table I), and IL-2Rβ mutants that lack the acidic region cannot activate Ras in response to IL-2 (Satoh *et al.*, 1992). However, these IL-2Rβ mutants can activate the Jak PTKs in reponse to IL-2R ligation (Witthuhn *et al.*, 1994), demonstrating that Jak activation alone is not sufficient to mediate Ras activation. Additionally, IL-2Rβ subunits that lack the serine-rich region also fail to activate Ras, suggesting that the Jaks also participate in Shc phosphorylation and Ras activation. A similar division of receptor cytoplasmic regions into functional domains has been reported for the GM-CSFR β_c subunit, and an analysis of the parallels between the IL-2R and GM-CSFR systems is informative. As observed for IL-2Rβ, a box 1/box 2 motif is found within the membrane-proximal portion of GM-CSFR β_c, and is required for GM-CSF-induced Jak activation (Quelle *et al.*, 1994) and c-*myc* induction (Sato *et al.*, 1993). In addition, a distal 140-amino acid region of GM-CSFR β_c has an analogous function to the acidic region of IL-2Rβ. Like the acidic region of IL-2Rβ, the distal portion of GM-CSFR β_c, which mediates Src family kinase activation and the induction of c-*jun* and c-*fos* mRNA accumulation, is essential for the activation of the Ras/MAP kinase pathway and PI3-K (Sato *et al.*, 1993). A further parallel between the cytokine receptor subunits is also evident. All signaling events that require the distal region of β_c also require the Jak-binding, membrane-proximal portion of GM-CSFR β_c. These results suggest that activation of the Ras/MAP kinase pathway by both cytokine receptor subunits requires the coordinated cooperation between two regions of these hemopoietin receptors.

C. Shc Phosphorylation and Ras Activation

How do signals emanating from these disparate receptor regions collaborate to trigger Shc phosphorylation and Ras activation? Shc is one of the most prominent tyrosine-phosphorylated substrate proteins in IL-2-

stimulated T cells (Burns *et al.*, 1993). Although the acidic portion of IL-2Rβ is clearly required for Ras activation, the function of the acidic region in Shc phosphorylation is currently unclear (Fig. 2). In one scenario, upon γ_c and IL-2Rβ juxtaposition and corresponding Jak activation, one or more of the tyrosyl residues found within the acidic region is phosphorylated by the receptor-bound Jaks and recruits Shc to IL-2Rβ via either a PTB- or SH2 domain-mediated interaction. Shc would then be anchored to the receptor and tyrosine-phosphorylated by receptor-associated Jak, Src, or Syk-family kinases. In support of this possibility, Shc is coprecipitated with tyrosine-phosphorylated IL-2Rβ (Ravichandran and Burakoff, 1993); however, whether this interaction requires tyrosine residues found within the acidic region is not known. The amino acid sequences surrounding the tyrosines in the acidic region of IL-2Rβ do not conform to known binding sites for either the SH2 or PTB domain of Shc, suggesting that the sequence elements directing the interaction between IL-2Rβ and Shc may be novel. On the other hand, the binding of Shc to IL-2Rβ may not be direct, and other mechanisms may be employed by the IL-2R subunits to target Shc for phosphorylation. In support of this idea, EGF (Decker, 1993; Soler *et al.*, 1994) and erythropoietin (He *et al.*, 1995) receptors that lack receptor tyrosine phosphorylation sites retain the ability to phosphorylate Shc and activate the Ras pathway, suggesting that Shc can be recruited to and phosphorylated by both RTK and cytokine receptors without binding to receptor pTyr residues.

One indirect mechanism for Shc recruitment to the IL-2R involves the Src-family kinases associated with the acidic region of the receptor. These PTKs may simultaneously localize Shc to the activated receptor complex and phosphorylate the adaptor protein. The IL-2Rβ-associated PTK Fyn coprecipitates with Shc in T cells (L. Karnitz, unpublished observations), and expression of v-Src in fibroblasts induces efficient tyrosine phosphorylation of Shc and the attendant association of Grb2 (McGlade *et al.*, 1992). These observations suggest that Shc may be a physiological substrate for a Src-family kinase. In one such model, activation of PTKs associated with the serine-rich region of IL-2Rβ might relay an activating signal to the acidic region of IL-2Rβ, resulting in Src-family kinase activation and Shc phosphorylation. However, it should be noted that no definitive role for any Src-family kinase in IL-2-triggered activation of the Ras/MAP kinase pathway has been demonstrated.

The role of γ_c in the activation of Shc is a matter of conjecture. The γ_c subunit is required for induction of c-*fos* and c-*jun* (Asao *et al.*, 1993), which is a surrogate marker for the activation of the Ras/MAP kinase pathway. However, γ_c cannot directly mediate Shc phosphorylation and activation of the Ras/MAP kinase pathway. This is most convincingly dem-

onstrated by the IL-4R. The high-affinity signal-transducing IL-4R contains γ_c, and yet IL-4 does not activate Ras (Duronio *et al.*, 1991; Satoh *et al.*, 1991; Welham *et al.*, 1994a,b), suggesting that γ_c merely serves to juxtapose receptor-bound Jak3 to IL-2Rβ and its associated signaling molecules. IL-2Rβ then specifies the selection and/or phosphorylation of downstream signaling moieties, one of which is Shc.

D. The Role of Ras in Mitogenic Signaling

A looming question in the field of IL-2 signaling research is the role of the Ras/MAP kinase pathway in IL-2-induced proliferation, differentiation, and survival. Much of the work addressing the role of the Ras/MAP kinase pathway has centered on fibroblast cell systems, and, due in large part to the lack of amenable experimental systems, we currently have little understanding of the role of the pathway in T cells. As described earlier, one of the standard model systems for dissection of IL-2-triggered signaling pathways is the BAF/B03 cell line expressing various IL-2Rβ mutants. Although this cell line has been exceptionally useful in the delineation of IL-2-triggered signaling pathways, recent work has uncovered potential problems with the cell line when interpreting the proliferative phenotypes triggered by these IL-2Rβ mutants. As described in Section III, when IL-2Rβ deletion mutants that lack the acidic region are expressed in BAF/B03 cells, these receptors allow the cells to grow in response to IL-2 (Hatekeyama *et al.*, 1989a), suggesting that the acidic region and the attendant activation of the Ras/MAP kinase pathway are not required for IL-2-induced proliferation. However, in studies that utilized chimeric IL-2Rβ and γ_c molecules expressed in T cell lines, a very different picture emerged. When the acidic region of IL-2Rβ is deleted, these receptors cannot confer IL-2-induced proliferation, whereas chimeric receptors with a full-length intracellular IL-2Rβ domain can induce mitogenesis (personal communication, M.A. Goldsmith). These results suggest that in T cells, the acidic region of IL-2Rβ plays an essential role in IL-2-triggered proliferation. One possible explanation of these disparate results comes from recent findings which utilized various truncations mutants of GM-CSFR β_c expressed in BA/F3 cells to study GM-CSF signaling (Sato *et al.*, 1993; Quelle *et al.*, 1994). GM-CSFR β_c has been functionally divided into two interdependent regions. One is the Jak-binding, membrane-proximal region that, like IL-2Rβ, transduces mitogenic signals and induces c-*myc* (Quelle *et al.*, 1994). GM-CSFR β_c also possesses a distal 140-amino-acid region, which is required for GM-CSF-triggered Shc phosphorylation and activation of the Ras/MAP kinase pathway. Like the acidic portion of IL-2Rβ, this distal portion is not essential for growth of BA/F3 cells in the presence of serum (Sato *et al.*, 1993). However, further work revealed that

in the absence of serum, GM-CSF could not induce long-term proliferation in BA/F3 cells expressing these GM-CSFR β_c mutants (Quelle *et al.*, 1994). These studies also suggested that serum activates Ras in BA/F3 cells. Further evidence that Ras is essential for hemopoietin receptor-triggered proliferation came from an extension of the GM-CSFR studies in BA/F3 cells. Under similar culture conditions (i.e., no serum), the GM-CSFR β_c mutants lacking the 140-amino-acid distal region were able to grow in GM-CSF when they were complemented with constitutively activated Ras (Kinoshita *et al.*, 1995). Taken together, the results in T cells using chimeric IL-2Rs in conjunction with the analysis of GM-CSF-triggered signaling pathways in BA/F3 cells suggest that signals emanating from the acidic region of IL-2R β, including those of the Ras/MAP kinase pathway, are essential for IL-2-induced T-cell proliferation. However, as will be seen in the following section, the acidic region is also required to activate another signaling pathway that regulates cell growth and survival.

V. The PI3-K Pathway

PI3-K was one of the first IL-2-activated signaling enzymes to be identified (Augustine *et al.*, 1991; Merida *et al.*, 1991; Remillard *et al.*, 1991). However, unlike the Ras/MAP kinase pathway, PI3-K has not been neatly placed into a linear receptor-to-nucleus signaling cascade in any cell type that has been examined. Multiple proteins with PI3-K activity or homology to the catalytic domain of p110 have been identified, suggesting that a family of related lipid kinases may be components of multiple cell signaling pathways. However, this portion of the chapter will focus on the heterodimeric enzyme, which is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (see Downes and Carter, 1991, for review). The heterodimeric PI3-K is activated by nearly all receptors that trigger proliferation, and has been the target of intense study in many receptor and cell signaling systems. PI3-K catalyzes the phosphorylation of PI on the D-3 position of the inositol ring. In intact cells, PI-4,5-P_2 is the preferred substrate for the lipid kinase and enzyme-catalyzed phosphorylation of this substrate yields PI-3,4,5-P_3 (Auger *et al.*, 1989; Hawkins *et al.*, 1992). However, in addition to being a lipid kinase, PI3-K exhibits serine/threonine kinase activity toward a limited set of substrates, with one substrate being the p85 regulatory subunit itself (Dhand *et al.*, 1994b), suggesting that the lipid kinase may relay signals via two possible mechanisms.

The p85 and p110 subunits are constitutively and tightly associated, and p85 functions as an adaptor protein that both activates and down-regulates p110 catalytic activity. The 85-kDa subunit consists of an array of domains also found in other signal-transducing proteins. Starting at the amino termi-

nus of the molecule, the first recognizable signaling-associated domain is an SH3 domain. Following the SH3 domain is a region that is homologous with the GTPase-activating proteins (GAP) for small G proteins belonging to the Rho/Rac family. Flanking the Rho–GAP domain on each side are proline-rich sequences that are *bona fide* ligands for SH3 domains (Kapeller *et al.*, 1994; Pleiman *et al.*, 1994). The carboxyl-terminal half of the protein comprises tandem SH2 domains with similar pTyr-binding specificities. The SH2 domains are separated by a 104-amino-acid inter-SH2 domain, which directs the stable interaction of p85 with the amino terminus of the p110 subunit (Klippel *et al.*, 1993; Dhand *et al.*, 1994a). Aside from tethering the p110 subunit to the regulatory p85 subunit, association of the inter-SH2 domain of p85 with p110 enhances the catalytic activity of p110 (Hu *et al.*, 1995).

A. PI3-K Activation

Because PI3-K has been implicated in proliferative signaling responses, intense interest has focused on the biochemical mechanisms that regulate PI3-K catalytic activity (see Downes and Carter, 1991, for review). These mechanisms can be divided into two broad categories. In one, protein–protein interactions regulate the enzyme's activity, and in the other, post-translational modifications modulate PI3-K's activity. Most likely, the relative contribution of each regulatory mechanism is determined by both the cell and the receptor type triggering PI3-K activation. As an example, PI3-K activation mediated by platelet-derived growth factor (PDGF) RTKs may utilize a combination of these methods to regulate activity. The PDGF receptor autophosphorylation sites include two tyrosines that are embedded within canonical sites (Tyr–X–X–Met) that bind the tandem p85 SH2 domains (Fantl *et al.*, 1992). Binding of p85 to Tyr–X–X–Met sites tethers the dimeric lipid kinase near the cell membrane and triggers a conformational change that up-regulates the catalytic activity of p110 (Myers *et al.*, 1992; Soltoff *et al.*, 1992; Backer *et al.*, 1992; Shoelson *et al.*, 1993). However, in addition to associating with the activated RTK, PI3-K may also be regulated by GTP-bound Ras (Rodriguez-Viciana *et al.*, 1994; Kodaki *et al.*, 1994). The effector domain of GTP- but not GDP-bound Ras associates with the p110 subunit. This interaction may directly increase PI3-K activity, or, as for Raf, may provide a partial activating signal for PI3-K. Additional regulatory mechanisms for PI3-K have also been identified. For example, the binding of proline-rich sequences found within p85 to SH3 domains derived from the Src-family kinases Fyn and Lyn also activates PI3-K (Pleiman *et al.*, 1994), suggesting that receptor-bound Src-family kinases may participate in the activation of PI3-K. All of these PI3-K regulatory mechanisms involve protein–protein interactions

that recruit and assemble PI3-K into receptor-triggered signaling complexes. However, post-translational modifications of PI3-K also regulate the enzyme. Both RTKs and Src-family kinases can phosphorylate the p85 subunit (Cohen *et al.*, 1990; Hayashi *et al.*, 1992; Kavanaugh *et al.*, 1992; Soltoff *et al.*, 1992; Hayashi *et al.*, 1993), and this post-translational alteration has been proposed to up-regulate enzyme activity (Ruiz-Larrea *et al.*, 1993). If this mode of regulation actually contributes to the regulation of PI3-K in intact cells, then it appears to be less prevalent than the allosteric mechanisms outlined above. In addition to tyrosine phosphorylation, the p85 subunit also undergoes stimulus-induced serine phosphorylation of Ser^{608}, which is within the inter-SH2 domain of p85 that mediates binding to p110 (Dhand *et al.*, 1994a). Surprisingly, p110-mediated phosphorylation of p85 inhibits both the lipid and protein kinase activities, suggesting that the enzyme catalyzes its own down-regulation and signal attenuation. Clearly, the above findings indicate that a complex set of regulatory controls impinge on PI3-K, and further suggest that this enzyme plays key roles in multiple cell signaling pathways.

B. IL-2-Induced PI3-K Activation

The molecular mechanisms that couple the IL-2R to PI3-K activation are less well documented than for the growth factor RTKs. However, as with the RTKs, IL-2-triggered PI3-K activation is blocked by pharmacological PTK inhibitors (Merida *et al.*, 1991). When IL-2Rβ mutants are analyzed for their ability to activate PI3-K, a pattern similar to that found for activation of the Ras/MAP kinase pathway emerged (Fig. 1 and Table I). Like IL-2R-induced Ras activation, PI3-K activation requires a cooperation between the serine-rich and acidic regions of IL-2Rβ (Merida *et al.*, 1993; Williamson *et al.*, 1994). Because of the close parallels between IL-2-induced PI3-K and Ras activation, and the fact that Ras has previously been linked to PI3-K activation, these results suggested that Ras may play a role in IL-2-induced PI3-K activation. However, the role of Ras in the regulation of PI3-K in IL-2-stimulated T cells is not yet clear, and an example of Ras-independent PI3-K activation in T cells is provided by IL-4. Like IL-2, IL-4 also elicits PI3-K activation (Wang *et al.*, 1992), and yet, unlike IL-2, IL-4 does not activate Ras (Satoh *et al.*, 1991; Duronio *et al.*, 1992; Welham *et al.*, 1994a,b), raising the possibility that Ras-independent PI3-K activation mechanisms also exist in T cell lines.

Other reports suggest that IL-2-regulated PTKs may directly mediate PI3-K activation. IL-2 triggers the rapid tyrosine phosphorylation of p85 in both T cell lines (Karnitz *et al.*, 1994) and BAF/B03 cells expressing IL-2Rβ (Merida *et al.*, 1993), suggesting that this post-translational modification may participate in PI3-K activation. An initial clue to the identity

of the PTK(s) that phosphorylates PI3-K was provided by the IL-2Rβ mutants. The fact that PI3-K tyrosine phosphorylation and activation required the acidic region of IL-2Rβ (Williamson *et al.*, 1994), which binds and activates Src-family kinases, suggested that these PTKs might be involved in PI3-K regulation (Fig. 3). Further work revealed that PI3-K coprecipitates with Fyn isolated from T-cell lysates, suggesting a direct connection between the two signaling enzymes (Augustine *et al.*, 1991).

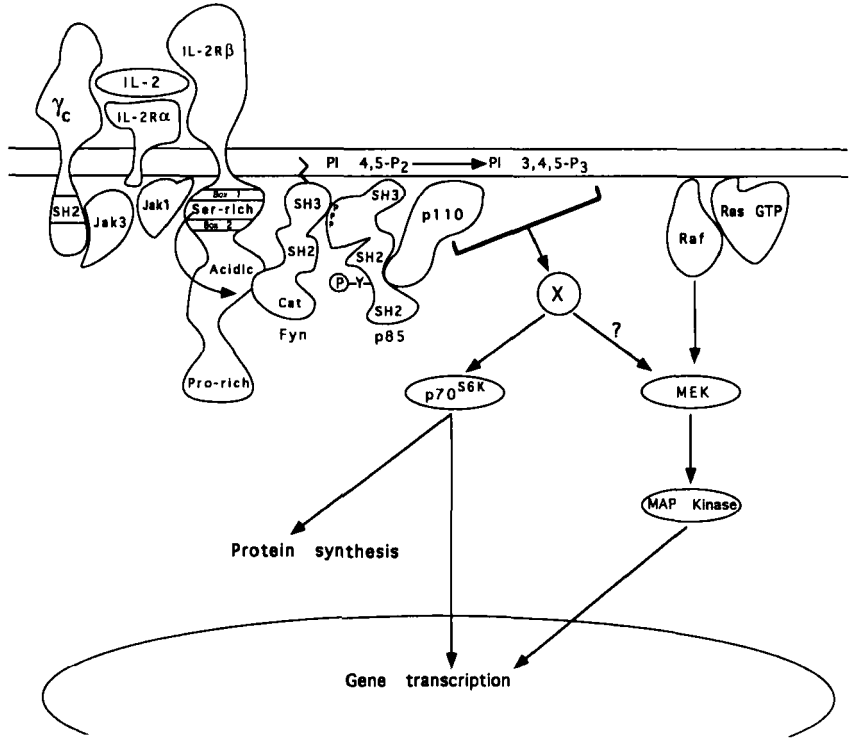


FIG. 3. IL-2-induced PI3-K activation and downstream effectors. In a simplified scheme, IL-2 triggers Jak activation, which relays an activating signal to the acidic region of IL-2Rβ. One possible candidate for coupling the acidic portion of IL-2Rβ to PI3-K may be Fyn, which is composed of SH3 and SH2 domains and a catalytic domain (cat). Heterodimeric PI3-K is associated with Fyn via proline-rich sequences (indicated as PPP) of p85 associating with the SH3 domain of Fyn, targeting PI3-K to the activated IL-2R complex. Activated PI3-K phosphorylates phosphatidylinositol (PI)-4,5-P_2 to yield a potential lipid second messenger, PI-3,4,5-P_3, which directly activates unknown downstream effectors (represented by "X"). Alternatively, the intrinsic serine/threonine kinase activity may modify and activate downstream signal transducers (represented by "X"). Two distal targets of PI3-K catalytic activity are the serine/threonine kinase $p70^{S6K}$, which may regulate both gene transcription and protein synthesis, and MEK, an upstream activator of MAP kinase.

Several groups soon demonstrated a direct association between the Fyn SH3 domain and two proline-rich regions within p85 (Prasad *et al.*, 1993; Karnitz *et al.*, 1994; Pleiman *et al.*, 1994). Further evidence that Fyn participated in the regulation of PI3-K was provided by the overexpression of Fyn in IL-2-responsive T cells (Karnitz *et al.*, 1994). In these cells, Fyn augmented IL-2-triggered PI3-K activity. These studies demonstrated that immobilized p85 stably associated with T cell-derived Fyn and that p85-bound Fyn tyrosine phosphorylated the p85 subunits in an IL-2-dependent manner. Taken together, these studies suggest that, in response to IL-2, IL-2Rβ-coupled Fyn is activated and this PTK regulates PI3-K activity either by a direct Fyn SH3-p85 association or by enhancing p85 tyrosine phosphorylation resulting in lipid kinase activation and the relay of signals to downstream effectors (Fig. 3).

Other regulatory mechanisms may also impinge upon PI3-K in IL-2-stimulated T cells. PI3-K coprecipitates with ligand-activated IL-2Rβ (Truitt *et al.*, 1994), suggesting that a pTyr residue(s) on IL-2Rβ may mediate the receptor-kinase association. Indeed, fusion protein studies demonstrated that the interaction is mediated by the tandem SH2 domains of p85 interacting with phosphorylated IL-2Rβ. The interaction between tyrosine-phosphorylated IL-2Rβ and p85 SH2 domains was blocked by a peptide that encompassed tyrosine-phosphorylated Tyr^{392} of IL-2Rβ (Truitt *et al.*, 1994). Surprisingly, the amino acid sequence surrounding Tyr^{392}, which is in the acidic region of IL-2Rβ, is not predicted to be a binding site for p85 SH2 domains, suggesting that another intermediary may direct this interaction. However, unlike the PDGF receptor–PI3-K interaction, which is required for PDGF-induced PI3-K activation, IL-2Rβ deletion mutants that lack Tyr392 activate PI3-K as well as full-length IL-2Rβ (Williamson *et al.*, 1994), indicating that the association is not essential for PI3-K activation by the IL-2R.

C. Downstream Effectors for PI3-K

While a tentative mechanism for IL-2-triggered PI3-K activation can be traced (Fig. 3), a more puzzling question is which IL-2-activated signaling pathways and cellular responses require PI3-K. Clues to this question have been gleaned from the study of PI3-K in a variety of cell types and receptor systems using several disparate techniques, including the use of pharmacological inhibitors of PI3-K (Cheatham *et al.*, 1994; Okada *et al.*, 1994; Powis *et al.*, 1994; Vlahos *et al.*, 1994; Karnitz *et al.*, 1995; Monfar *et al.*, 1995), dominant negative versions of the p85 regulatory subunit (Chuang *et al.*, 1993; Muslin *et al.*, 1993; Jhun *et al.*, 1994), anti-p85 antibodies (Jhun *et al.*, 1994), receptor mutants that cannot activate PI3-K (Shurtleff *et al.*, 1990; Joly *et al.*, 1994), and receptor mutants that specifically activate

PI3-K (Valius and Kazlauskas, 1993). Taken together, these studies have provided some provocative insights into the functions of PI3-K in an already broad array of cellular responses. Two areas of intense and overlapping interest include the identification of both proximal and distal effectors for PI3-K as well the biological functions of PI3-K.

Although some of the downstream effectors of PI3-K are now being identified, it is not yet known how PI3-K relays receptor-initiated signals to downstream effectors. Two possibilities are tenable. In one, PI3-K-generated 3-phosphorylated PIs may function as second messengers. The highly charged lipid product of PI3-K (PI-3,4,5-P_3) is not a substrate for the well-characterized PI-specific phospholipase C (Serunian *et al.*, 1989; Lips and Majerus, 1991), suggesting that the 3-phosphorylated lipids may themselves be second messengers. Biochemical evidence suggests that PI-3,4,5-P_3, is a potent activator of the nonclassical, Ca^{2+}-independent protein kinase C isozyme δ, ε, and η (Toker *et al.*, 1994), suggesting that PI3-K-activating stimuli may trigger the activation of these protein kinases (Dhand *et al.*, 1994b). However, PI3-K is also a serine/threonine protein kinase, which may provide a second mechanism to couple activated PI3-K to downstream effectors. In addition to phosphorylating p85, the protein kinase activity of PI3-K phosphorylates the insulin receptor substrate 1 (IRS1) (Lam *et al.*, 1994), a key element in the propagation of insulin-mediated signals, suggesting that other signaling proteins may be downstream targets for the lipid/protein kinase. However, it is not yet known whether lipid second messengers, protein kinase activity, or a combination of both triggers the activation of downstream effectors (Fig. 3). Although the mechanism(s) used by PI3-K to relay signals is not known, several IL-2-activated signaling pathways have been found to be downstream of PI3-K.

The discovery of the potent pharmacological PI3-K inhibitor wortmannin (Okada *et al.*, 1994; Powis *et al.*, 1994) provided an invaluable tool to dissect the role of PI3-K in IL-2- and other receptor-triggered signaling and cellular responses. Addition of wortmannin to an exponentially growing, IL-2-responsive CTLL-2 cell line resulted in accumulation in the G_1 phase of the cell cycle, with a concomitant reduction in cells in S phase (Karnitz *et al.*, 1995), suggesting that PI3-K regulates an IL-2-triggered signaling pathway(s) that is required for proliferation. The first PI3-K-regulated signaling enzyme to be identified in IL-2-dependent T cells (Fig. 3) was the mitogen-activated, serine/threonine kinase, p70 S6 kinase ($p70^{S6K}$) (Karnitz *et al.*, 1995; Monfar *et al.*, 1995). Activation of $p70^{S6K}$ does not require the Ras/MAP kinase pathway (Ming *et al.*, 1994), suggesting that it represents an independent signaling pathway, and a variety of studies in both hemopoietic and nonhemopoietic cells suggest that $p70^{S6K}$ is required for mitogenesis (Price *et al.*, 1992; Lane *et al.*, 1993). The major intracellular

target of $p70^{S6K}$ in mitogen-stimulated cells is the S6 protein of the 40S ribosomal subunit, and phosphorylation of the 40S subunit may regulate protein synthesis (see Section VIII). However, $p70^{S6K}$ also phosphorylates another less well-characterized target, the DNA-binding transcription factor, cAMP-response element (CRE) modulator τ (CREMτ), which is a member of the CRE-binding (CREB) protein family of trans-activators (de Groot *et al.*, 1994). Thus, PI3-K may regulate the activation of CRE-dependent gene transcription via $p70^{S6K}$.

Multiple studies in nonhemopoietic cell systems have suggested that PI3-K also interfaces with the Ras/MAP kinase pathway (Muslin *et al.*, 1993; Yamuachi *et al.*, 1993; Kodaki *et al.*, 1994; Rodriguez-Viciano *et al.*, 1994; Hu *et al.*, 1995), which is an essential component of RTK-triggered proliferative responses. Analysis of the Ras/MAP kinase pathway in IL-2-stimulated T cells revealed that in addition to inhibiting $p70^{S6K}$ activation, wortmannin also attenuated IL-2-induced MAP kinase activation (Karnitz *et al.*, 1994), suggesting that PI3-K may play a role in the activation of the Ras/MAP kinase pathway in T lymphocytes (Fig. 3). In nonhemopoietic cell systems, several studies have generated conflicting data regarding the interplay between Ras and PI3-K, and PI3-K has been shown to activate Ras in some systems (Hu *et al.*, 1995) and be activated by Ras in others (Rodriguez-Viciana *et al.*, 1994). However, an analysis of the Ras/MAP kinase pathway in T cells revealed that wortmannin did not affect IL-2-triggered Ras activation. These findings suggest that PI3-K does not function upstream of Ras in T cells. Further dissection of the Ras/MAP kinase pathway revealed that IL-2-triggered MEK activation was also inhibited by wortmannin, whereas activation of Raf, the canonical activator of MEK, was not affected by PI3-K inhibition (Karnitz *et al.*, 1995). These results suggest that in addition to Raf, a PI3-K-dependent activation pathway also impinges upon MEK in T lymphocytes. In addition, these findings imply that the Ras/MAP kinase pathway is not a linear trail leading from receptor to nucleus. Rather, upon receptor ligation, a complex web-like signaling network is engaged that ultimately regulates MAP kinase activation and the concomitant IL-2-induced expression of proto-oncogenes such as c-*fos*. However, in addition to activating PI3-K and the Ras/MAP kinase pathway, as we describe in the following sections, IL-2 also triggers other signaling pathways that may interface with the PI3-K and Ras/MAP kinase pathways and whose roles in IL-2-induced proliferation remain elusive.

VI. IL-2R Signaling: Jaks and Stats

The studies discussed above indicate that IL-2 triggers the heterodimerization and functional activation of Jak1 and Jak3, and receptor mutagenesis

and transfection experiments have shown that stimulation of Jak PTK activities is a proximal and essential event for the initiation of signal output from the ligand-bound IL-2R. However, the intracellular substrates for the IL-2R-coupled Jak1 and Jak3 PTKs remain largely speculative. As alluded to in Section III, seminal experiments performed in the IFN-α/β and IFN-γ receptor systems have strongly implicated the Jaks as critical upstream regulators of a family of latent cytoplasmic transcription factors termed Stats (for reviews see Darnell *et al.*, 1994; Shuai *et al.*, 1994). Studies of IFN-α-responsive genes led to the identification of a 12- to 15-base-pair enhancer element that was both necessary and sufficient to confer IFN-α inducibility on a heterologous reporter gene. This sequence was designated the *i*nterferon-*s*timulated *r*esponse *e*lement (ISRE) (Israel *et al.*, 1986; Vogel *et al.*, 1986; Reid *et al.*, 1989). Similarly, IFN-γ-induced transcription of certain immediate response genes was dependent on a highly related enhancer element, the GAS (gamma-activated sequence) site (Lew *et al.*, 1991). With the specific enhancer sequences in hand, it was possible to fish out the cognate transcription factors that bound to these sequences in IFN-stimulated cells (Fu, 1992; Schindler *et al.*, 1992; Shuai *et al.*, 1992; Shuai, 1994). An elegant combination of biochemical techniques, molecular cloning, and somatic cell genetics eventually yielded the first three members of a novel family of transcriptional trans-activators, Stat1α, Stat1β, and Stat2 (Table II).

A series of reports subsequently showed that the purported linkage between Jaks and Stats was not restricted to the receptors for IFNs. Stimulation with numerous cytokines, including IL-3, IL-4, IL-6, and, surprisingly, EGF, induced the rapid appearance of intranuclear DNA-binding activities that recognized Stat-responsive elements in gel-shift assays (Kotanides and Reich, 1993; Larner *et al.*, 1993; Sadowski *et al.*,

TABLE II
CYTOKINE-INDUCED ACTIVATION OF JAK AND STAT ISOFORMS

Cytokine	Jak	Stat
IL-2	Jak1/Jak3	Stat 3 /Stat 5
IL-4	Jak1/Jak3	STF-IL4/Stat4
IL-6	Jak1/Jak2/Tyk2	Stat1/Stat3
GM-CSF	Jak2	Stat5
Erythropoietin	Jak2	Stat5
Interferon-α/β	Jak1/Tyk2	Stat1α, Stat1β/Stat2
Interferon-γ	Jak2/Jak1	Stat1α

Note. The Jak and Stat isoforms activated by representative members of the hemopoietin receptor family (see Ihle *et al.*, 1994; Ihle and Kerr, 1995; Karnitz and Abraham, 1995).

1993, Silvennoinen *et al.*, 1993; Rothman *et al.*, 1994; Stahl *et al.*, 1995). These results provoked a flurry of activity aimed at the characterization of the Stat proteins activated by these and other cytokines. Additional cDNA clones encoding novel Stat family members were soon isolated, and specific subsets of Stat proteins were assigned to different ligand-stimulated cytokine receptor–Jak complexes (Table II). At the present time, seven Stats encoded by six distinct but evolutionarily related genes have been characterized, and it seems likely that additional family members await discovery.

The deduced amino acid sequence of the most well-studied Stat, Stat1α, immediately suggested that this latent transcription factor participated in a PTK-dependent pathway of nuclear signaling (Shuai *et al.*, 1993, 1994). The carboxyl-terminal region of Stat1α contains sequences that align fairly well with SH2 and SH3 domains found in other PTK-regulated signaling proteins. These domains are conserved in all Stat family members described to date. Subsequent experiments showed that Stat1α undergoes rapid phosphorylation on a single tyrosyl residue (Tyr^{701}) that lies immediately carboxyl-terminal of the SH2 domain (Shuai *et al.*, 1993). Tyrosine phosphorylation of Stat1α triggers the dimerization of this protein, either with another Stat1α protein or with other phosphorylated Stat proteins. In these dimers, the pTyr residue from each Stat monomer interacts with the SH2 domain of the partner (Shuai *et al.*, 1994). Presumably, the array of Stat dimers formed in response to cytokine stimulation is determined in part by the amino acid sequences surrounding the pTyr residues and the binding specificities of the Stat SH2 domains. The SH2 domain also plays a determinant role in the selection of specific Stat proteins for phosphorylation by different cytokine receptor-Jak complexes. Ligand stimulation leads to the phosphorylation of tyrosines in the cytoplasmic domain of one or more receptor subunits, and certain of these pTyr residues mediate the recruitment of Stats to the receptor and its associated PTKs (Heim *et al.*, 1995; Lin *et al.*, 1995; Stahl *et al.*, 1995). When variations in the levels of Stat protein expression are taken into account, it becomes clear that the combinatorial assembly of Stat protein dimers confers considerable plasticity on the spectrum of Stat factors that can be assembled in response to cytokine stimulation.

The tyrosine phosphorylation and subsequent dimerization of Stats trigger the translocation of these proteins to the nucleus, where they bind DNA in a sequence-specific fashion (Shuai *et al.*, 1994). Whether dimerization alone is sufficient for the transport of Stats into the nucleus remains unclear. Once in the nucleus, different Stat factors bind to distinct enhancer elements, although the DNA recognition sequences are frequently related to the IFN-γ-responsive GAS site [consensus sequence, TTNCNNNAA

(Shuai, 1994)]. Again, although dimerization plays a crucial role in the DNA-binding activities of Stat factors, other post-translational modifications of the component Stat monomers, e.g., serine/threonine phosphorylation, may regulate both the DNA-binding and trans-activating potentials of these factors (see below). As discussed above, the discovery of this novel and relatively distinct pathway of nuclear signaling dramatically increases the range of transcriptional responses that can be induced by a particular cytokine in various cell lineages, or within the same cell lineage at various stages of differentiation. Moreover, the abilities of sets of cytokines, such as IL-3, GM-CSF, and IL-5 to evoke both redundant and specific responses from their target cells may reflect in part the assembly of both overlapping and distinct arrays of Stat factors induced by the respective receptor-Jak complexes (Lin *et al.*, 1995).

A. IL-2-Activated Stat Factors

In the wake of the series of reports describing the activation of Stats by hematopoietin receptor superfamily members, we and others postulated that IL-2 would also trigger the appearance of Stat factors in the nuclei of activated T cells. Our interest in this pathway was further heightened by data suggesting that a Ras-independent pathway of nuclear signaling played a central role in transducing growth-stimulatory signals from the IL-2R. As described in Section III, mutagenesis studies by Taniguchi and co-workers identified a serine-rich, membrane-proximal region of the IL-2Rβ cytoplasmic domain that associated with Jak1 and transduced a Src-family kinase- and Ras-independent mitogenic signal leading to c-*myc* gene expression, an event that is highly correlated with T-cell proliferation (Fig. 2). Furthermore, the observation that EGF-induced Stat activation in A431 epidermoid carcinoma cells was unaffected by expression of a dominant-negative Ras mutant (Silvennoinen *et al.*, 1993) suggested that the IL-2-triggered Ras-independent pathway of mitogenic signaling might be mediated through the nuclear translocation of Stat factors in T cells. While ensuing studies by ourselves and others quickly proved that the IL-2R activates specific Stat proteins in T cells (Nielsen *et al.*, 1994; Brunn *et al.*, 1995; Hou *et al.*, 1995; Lin *et al.*, 1995), it will become clear from the discussion below that many key issues surrounding the functions of these transcription factors have yet to be resolved.

Our initial studies used the murine factor-dependent T-cell lines CTLL-2 and HT-2 as model systems. Nuclear extracts prepared from cells stimulated with either IL-2 or IL-4 contained inducible DNA-binding activities that bound to a high-affinity Stat-binding site. Further characterizations of the IL-2- and IL-4-induced Stat-like DNA-binding activities revealed that the induction of these activities was blocked by the PTK inhibitor

herbimycin A. Finally, the Stat dimers found in the nuclei of IL-2- or IL-4-stimulated T cells were functionally active, as transient transfection assays revealed that tandem Stat-binding sites placed upstream of a basal promoter and reporter gene construct conferred IL-2- and IL-4-inducible expression on this synthetic transcription unit.

The Stat isoforms activated by IL-2 were subsequently identified by both immunologic and biochemical techniques. Antibody supershift and immunoprecipitation experiments indicated that the major Stat protein activated by IL-2 in CTLL-2 and HT-2 cells was Stat3, while the minor species was Stat1α (Brunn *et al.*, 1995). The appearance of three Stat complexes in IL-2-stimulated CTLL-2 and HT-2 cells was explained by combinatorial associations among the activated Stat3 and Stat1α monomers, which would be predicted to yield two homodimers and one heterodimer. The most abundant Stat complex induced by IL-2 was the Stat3 homodimer, which was originally characterized as the IL-6-inducible *a*cute *p*hase *r*esponse *f*actor (APRF), due to its involvement in the transcriptional activation of the IL-6-inducible acute-phase genes (Akira *et al.*, 1994; Lutticken *et al.*, 1994; Wegenka *et al.*, 1994; Zhong *et al.*, 1994).

Two recent reports have added both complexity and some controversy to the above findings. Using freshly isolated peripheral blood lymphocytes (PBL) as responder cells, one group (Lin *et al.*, 1995) found that IL-2 induced the nuclear translocation of Stat5, which was initially described as a prolactin-responsive mammary gland transcription factor in sheep. However, similar experiments performed with preactivated (i.e., mitogenic lectin-stimulated) PBL yielded an intriguing finding. Whereas Stat5 was by far the most abundant Stat isoform found in the nuclei of freshly isolated PBL after exposure to IL-2, both Stat3 and Stat5 were activated at roughly similar levels in IL-2-stimulated, preactivated PBL. In contrast, an independent report provided definitive biochemical evidence that the Stat5 protein was activated by IL-2, but failed to confirm the nuclear translocation of Stat3 in preactivated PBL (Hou *et al.*, 1995). This discrepancy is not explained by any obvious differences in the cell populations or oligonucleotide probes used in these studies. The IL-2-dependent modification of Stat3 in preactivated PBL is consistent with our own findings that Stat3 phosphorylation is triggered by IL-2 in factor-dependent murine T-cell lines, whose phenotype most closely resembles that of activated T cells.

In addition to their studies with IL-2, Lin *et al.* (1995) also analyzed the Stat proteins activated in T cells by other cytokines that utilize the γc receptor subunit. IL-2, IL-4, IL-7, and IL-15 activated Stat5, whereas IL-4, which also utilizes γc, triggered Stat6 activation. These observations suggest some important conclusions. First, the set of Stat proteins activated by a particular cytokine receptor is not determined by the types of Jaks

coupled to the receptor subunits. Although the receptors for IL-2, IL-4, IL-7, and IL-15 uniformly activate Jak1 and Jak3, IL-2, IL-7, and IL-15 activate different Stat proteins than does IL-4. Because all these receptors share γc, the β subunit must specify the Stat isoform which is activated. This notion is supported by recent findings that the two most carboxyl-terminal tyrosines of IL-2Rβ, which are located within the proline-rich region, are required for Stat5 activation (Taniguchi, 1995). Thus, the most straightforward model is that tyrosine phosphorylation of IL-2Rβ creates binding motifs that dictate the Stat isoforms recruited by the activated receptor. The second conclusion is that the coupling of the IL-2R and presumably other cytokine receptors to specific Stat proteins displays an unexpected plasticity. Although controversial, the notion that IL-2 triggers differential Stat activation in naive versus preactivated PBLs is certainly provocative. Moreover, IL-2 induces two electrophoretically distinct forms of Stat5 in preactivated PBL, whereas only one Stat5 isoform is affected in naive PBL (Hou *et al.*, 1995). These results suggest that prior antigen or cytokine exposure strongly influences the array of Stat proteins activated by the IL-2R and related receptors, possibly by altering Stat isoform expression or modulating the molecular mechanisms that recruit Stats to the activated receptor. Although the regulatory mechanisms that govern the activation of Stats by these cytokine receptors may seem hopelessly confusing, they begin to provide a molecular framework for understanding the pleiotropic and redundant actions of cytokines that bind these receptors.

As stated at the beginning of this section, our understanding of the function of Stat factors in IL-2 signaling lags considerably behind the identification of the players in this pathway. Whether Stats are involved in the transmission of mitogenic signals is obviously of paramount importance. At present, evidence for a function of Stats in the expression of essential growth-regulatory genes is largely negative. EGF receptor, IL-4R, and IL-6R (gp130) mutants that cannot interact with and activate Stat proteins retain the ability to transmit mitogenic signals (Silvennoinen *et al.*, 1993; Hou *et al.*, 1994; Stahl *et al.*, 1995). On the other hand, the activation of Stat5 by both IL-2 and prolactin is intriguing (Gouilleux *et al.*, 1995; Hou *et al.*, 1995; Lin *et al.*, 1995), because prolactin possesses T-cell growth factor activity (Clevenger *et al.*, 1990). Additionally, the only intracellular tyrosine residues of IL-2Rβ that are essential for IL-2-induced mitogenesis are the carboxyl-terminal sites that bind Stat5 (Goldsmith *et al.*; 1994; Taniguchi, 1995), suggesting that Stat5 activation may be required for T-cell proliferation. Finally, a recent study has shown that T-cell transformation by the human T-cell lymphocytotropic virus-I (HTLV-I) is accompanied by constitutive activation of both Jaks (Jak1 and Jak3) and Stats (Stat3 and Stat5) (Migone *et al.*, 1995). Obviously, these findings do not

establish a causal relationship between a nuclear translocation of Stat proteins and T-cell proliferation. It remains possible that Stat-dependent nuclear signaling is segregated from those IL-2-stimulated pathways that govern cell-cycle progression. For example, Stat factors may play important roles in IL-2-induced expression of genes that stimulate T-cell effector functions, such as cytokine secretion by T-helper cells or granzyme expression by cytolytic T cells. If this notion proves correct, then the events leading to Stat factor activation and function could prove an interesting area for pharmacologic interventions in certain immunopathologic states.

Upon reading the above statements, most students of signal transduction would eagerly point out that postreceptor signaling pathways rarely, if ever, work in isolation. Rather, the norm would be that Stat factor activation and function would be intertwined with other, seemingly independent regulatory events triggered by IL-2. The Jak–Stat pathway appears to be no exception. Ongoing studies are revealing an intriguing link between the Jak–Stat pathway and a signaling cascade involved in cell growth control. In our original studies of Stat3 activation by IL-2, we were surprised to find a significant amount of tyrosine-phosphorylated Stat3 in the nuclei of IL-2-deprived T cells. The working model at that time predicted that this phosphorylated Stat3 would be functionally active; however, transient transfection assays revealed that the factor-deprived cells contained virtually no Stat-dependent transcriptional activity. These results suggest that IL-2-dependent tyrosine phosphorylation is not sufficient to fully activate the Stat3 homodimer as a transcription factor. A possible solution to this problem was provided by the recent finding that serine phosphorylation of Stat3 strongly influences the stability of the Stat3–Stat3 · DNA complex (Zhang *et al.*, 1995). Although the serine/threonine kinase(s) responsible for this modulatory phosphorylation event is unknown, it is interesting that Stat3, as well as Stat1α and Stat4, contain a conserved, –Pro–Met–Ser–Pro– sequence which is a near-perfect consensus site for phosphorylation by proline-directed kinases. Thus, IL-2-induced Stat3 activation in T cells may receive critical inputs from seemingly unrelated signaling pathways that activate proline-directed kinases such as MAP kinases, *J*un *N*-terminal *k*inases (Jnks), or *c*yclin-*d*ependent *k*inase (cdks). Clearly, the potential interplay between the Jak–Stat pathway and other signaling cascades should prove a fertile ground for future studies of the molecular mechanism of IL-2 action in T lymphocytes.

VII. IL-2-Dependent G_1-Phase Progression

Some of the most exciting advances in modern cell biology have taken place in the field of cell-cycle control. These studies have profoundly

altered our concepts of the mechanisms through which growth factor receptor occupancy induces mammalian cells to initiate and complete one cell division cycle. The biochemical machinery that orchestrates the orderly progression of cycling cells through G_1, S, G_2, and M phases has been the subject of numerous excellent reviews (Kirschner, 1992; Sherr, 1993; Hartwell and Kastan, 1994; Hunter and Pines, 1994; Sherr, 1994). It is not unexpected that this new knowledge, much of which was gleaned from model systems as diverse as yeast, flies, and invertebrate eggs, has intensified interest in the biochemical events that govern the proliferation of antigen-activated T cells. Indeed, both cell biologists and immunologists are beginning to recognize that the T cell is a very useful model for studies of the mammalian cell cycle. This recognition is certainly long overdue, as Cantrell and Smith (1984) proposed the human T lymphocyte as a powerful cell growth model nearly a decade ago. One additional event that has added both depth and breadth to molecular analyses of IL-2-dependent T-cell proliferation is the discovery of rapamycin, a potent immunosuppressant whose mechanism of action targets this phase of the T-cell activation program. Ongoing studies using rapamycin as a pharmacologic probe are revealing some previously unrecognized intermediate steps in the signaling pathway that couples IL-2R occupancy to the cell-cycle machinery in activated T cells.

Rapamycin is a macrocyclic lactone derived from the filamentous bacterium *Streptomyces hygroscopicus* (see Sigal and Dumont, 1992, for review). This drug was initially characterized as a potent antifungal agent; however, the pharmaceutical interest in rapamycin intensified when its chemical structure was shown to resemble that of a powerful immunosuppressant, FK506. Although structurally similar, FK506 and rapamycin interfere with entirely distinct steps in the T-cell activation program. Early studies demonstrated that the antiproliferative effects of FK506 on TCR-stimulated T cells should be overcome by addition of exogenous IL-2. Additional studies have since demonstrated that FK506 inhibits a TCR-mediated signaling event required for the transcription of the IL-2 and other cytokine genes. In contrast, rapamycin effectively inhibits the proliferation of activated T cells by blocking the ability of IL-2 to drive the progression of these cells through G_1 and into S phase.

Surprisingly, FK506 and rapamycin lack any intrinsic immunosuppressive activities. Rather, extensive studies have uncovered a gain-of-function model in which the binding of these drugs to intracellular receptors generates an immunosuppressive drug–protein complex in T cells (for reviews see Schreiber, 1992; Schreiber and Crabtree, 1992). Both FK506 and rapamycin bind to a highly conserved and widely expressed family of intracellular receptors, the *FK*506-*b*inding *p*roteins (FKBPs) (Schreiber,

1991; Sigal and Dumont, 1992). Although at least seven FKBPs have been biochemically isolated and molecularly cloned, the relevant receptor for both drugs is the 12-kDa FKBP, FKBP12. Like the other members of this family, FKBP12 is a peptidyl–prolyl isomerase (PPIase) that catalyzes *cis–trans* interconversions at proline-containing peptide bonds and is inhibited with nearly equivalent potencies by FK506 and rapamycin. However, the disparate immunosuppressive actions of FK506 and rapamycin on T-cell activation and proliferation were clearly not explained by their shared inhibitory effects on the PPIase activity of FKBP12, and an explanation for this mystery ultimately emerged from a convergence of genetic studies in yeast and from biochemical and pharmacologic evidence obtained in mammalian cells. These studies conclusively demonstrated that the complex formed between FK506 and FKBP12 was the proximal inhibitor of TCR-triggered T-cell signaling. The FKBP12-FK506 complex binds with high affinity to and inhibits the activity of the Ca^{2+}-regulated serine/threonine phosphatase calcineurin (Schreiber, 1992). Calcineurin plays a crucial role in coupling TCR-triggered increases in cytoplasmic free Ca^{2+} to the activation of IL-2 gene transcription in the nucleus. In contrast, the binding of rapamycin to FKBP12 generates a distinct complex that neither binds to nor inhibits calcineurin. This observation launched an intensive search for the protein ligand(s) of the FKBP12–rapamycin complex in T cells. Within the past year, at least four independent groups have reported the biochemical isolation and molecular cloning of an identical FKBP12–rapamycin-binding protein (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). This gene encoding this protein is a mammalian homolog of the *Saccharomyces cerevisiae* *T*arget *o*f *R*apamycin-1 (*TOR1*) and *TOR2* genes that confer resistance to the potent antifungal ligand rapamycin. The mammalian homolog has been variably termed RAFT1, FRAP1, RAPT1, and *m*ammalian TOR (mTOR). We will utilize the acronym mTOR throughout this chapter. Although the functional analysis of mTOR is at a relatively preliminary stage, it appears that a previously unrecognized component of the mitogenic signaling machinery engaged by the ligand-bound IL-2R has been isolated. Before delving into the knowledge gained from studies of the molecular mechanism of rapamycin's action in T cells, it may be worth a momentary pause to consider the implications of the gain-of-function model described above. Can it be purely fortuitous that two structurally related, microbial metabolites serve to drive otherwise unfavorable interactions between mammalian FKBP12 and two completely distinct target proteins, both of which play critical roles in the generation of T-cell-dependent immune responses? Is it possible that endogenous FKBP12 ligands, yet to be discovered, subserve physiologic functions in immune homeostasis?

A. Effect of Rapamycin on G_1-Phase Progression

The inhibitory effect of rapamycin on IL-2-stimulated T-cell proliferation was explained by a drug-induced block in cell cycle progression from G_1 into S phase (Dumont *et al.*, 1990; Bierer *et al.*, 1990; Morice *et al.*, 1993b). Although most attention has focused on the suppressive effect of rapamycin on factor-dependent T-cell growth, it should be recognized that the antimitogenic activity is not restricted to the T-cell compartment of the hematopoietic system. Rapamycin is a nearly equipotent inhibitor of the proliferation of myeloid progenitor cells stimulated with IL-3, IL-4, or GM-CSF, as well as of the constitutive growth of numerous leukemic cell lines, including Jurkat, YAC-1, and EL-4 (Dumont *et al.*, 1994; R. T. Abraham, unpublished observations). However, the growth-inhibitory effects of rapamycin on nonhematopoietic cells are far more variable. The serum-dependent growth of Swiss mouse 3T3 fibroblasts and 3T3L1 preadipocytes is only marginally affected by rapamycin (Chung *et al.*, 1992; R. T. Abraham, unpublished observations), whereas the proliferation of serum-stimulated MG-63 osteosarcoma cells or insulin-stimulated BC3H1 muscle cells is highly sensitive to this drug (Albers *et al.*, 1993; Jayaraman and Marks 1993).

To gain further insights into the growth-arrest state induced by rapamycin, cell-cycle distribution studies were performed using the IL-2-dependent murine T-cell line CTLL-2 as the model system (Morice *et al.*, 1993a,b). Growth factor-deprived CTLL-2 cells accumulate in the G_1 phase of the cell cycle. Restimulation of the cells with IL-2 initiates cell-cycle progression, and the cells began to enter S phase approximately 11–12 hr after cytokine addition. This 11- to 12-hr delay therefore defines a minimal length for the G_0/G_1 interval after growth arrest induced by IL-2 starvation. In parallel samples, exponentially growing CTLL-2 cells that were treated with rapamycin similarly accumulated in G_1 phase. However, when these cells were released from growth-arrest by removal of rapamycin, they began to enter S phase within 2 hr. These data indicate that, in contrast to the early G_0/G_1 arrest induced by IL-2 deprivation, rapamycin blocks cell-cycle progression at a much later point in G_1, near the so-called restriction point (Morice *et al.*, 1993b). Prior to the restriction point, the progression of T cells (and other mammalian cells) through G_1 is abruptly halted if growth factors are removed from the culture medium. The biochemical machinery that controls G_1- to S-phase progression requires continuous signals from stimulated growth factor receptors until the events needed for restriction point traverse have been executed (Pardee, 1989). After this point, endogenous regulatory loops take over the cell-cycle machinery, and S, G_2, and M phases are completed in a relatively autonomous fashion. CTLL-2 cells arrest relatively late in G_1 in the presence of rapamycin, but

retain dependence on IL-2 for S-phase entry after release from the drug-induced growth arrest. Thus, rapamycin blocks IL-2-dependent T-cell growth prior to the restriction point. Based on the model for rapamycin's action described above, the most straightforward conclusion would be that mTOR, the target of the FKBP12–rapamycin complex, is required for the passage of mid- to late-G_1-phase T cells through the restriction point.

B. Effect of Rapamycin on cdk Activities

The timing of the cell-cycle arrest state induced by rapamycin suggested that the drug does not interfere with biochemical events associated with early G_1-phase progression. Indeed, IL-2-stimulated protein tyrosine phosphorylation, Ras activation, Stat factor activation, and c-*myc* mRNA accumulation, all of which occur within the first hour after IL-2R occupancy, are unaffected by rapamycin (R. T. Abraham, unpublished observations). The only notable exception to these results was $p70^{S6K}$ activation, which is observed within several minutes after IL-2 exposure and is profoundly inhibited by rapamycin (see Section VIII). These and other data directed the attention of several laboratories further downstream in the mitogenic signaling cascade. The interface between growth factor receptor-initiated proximal signal transduction cascades and the nuclear machinery that controls the cell cycle remains poorly understood. Nonetheless, the successful propagation of a mitogenic signal from the cell surface requires that these early signals ultimately govern the assembly and/or catalytic activities of the G_1 cyclins and their associated cdks (Sherr, 1993, 1994).

The cyclin–cdk complexes represent the heart of the complex biochemical machinery that ensures the orderly progression of cycling cells from G_1 to S phase (Kirschner, 1992). Individual cyclin–cdk activities rise and decline in temporally defined waves during each cell cycle (for reviews see Sherr, 1994; Hunter and Pines, 1994). As discussed earlier, antigenic stimuli trigger the entry of resting T cells into the cell cycle (G_0- to G_1-phase progression), but not progression of these cells into S phase. T-cell activation also leads to the concerted synthesis of three G_1 cyclins: cyclin D_2, cyclin E, and cyclin A (Ajchenbaum *et al.*, 1993; Firpo *et al.*, 1994). Cyclin D_2 associates with one of two catalytic subunits, cdk4 or cdk6, whereas cyclin E and cyclin A form complexes with cdk2. However, these complexes are inactive unless the T cell is exposed to IL-2, thereby guaranteeing that passage through the restriction point and S-phase commitment are contingent on the delivery of mitogenic signals from the IL-2R (Firpo *et al.*, 1994). Although definitive data are not yet available for T lymphocytes, studies in other hemopoietic cell types suggest that IL-2 triggers cyclin D_2-associated cdks in activated T cells within the first few hours after exposure (Matsushima *et al.*, 1994). These complexes then give way to a

second wave of cdk activity that appears in mid-G_1 and peaks as the cells approach the G_1–S phase boundary (Morice *et al.*, 1993b; Firpo *et al.*, 1994). The active complex in this case is cyclin E–cdk2. In normal (nonleukemic) T cells, the sequential activation of the cyclin D–cdk4/cdk6 and cyclin E–cdk2 complexes propels the cell through the restriction point. Finally, cyclin A–cdk2 activity, which is required for S-phase entry and progression, appears in late G_1 and remains elevated throughout S phase (Morice *et al.*, 1993b) (Fig. 4).

Treatment of IL-2-stimulated T cells with rapamycin profoundly affects the sequential activation of the G_1 cyclin–cdk complexes that orchestrate G_1- to S-phase progression. The early increases in cyclin D_2-associated cdk4 and cdk6 activities are strongly reduced by this immunosuppressant drug (R. T. Abraham, unpublished observations). Recent results indicate that a key function of the cyclin D_2-associated cdk activities is to hyperphosphorylate the retinoblastoma protein (pRb) (Kato *et al.*, 1993; Lukas *et al.*, 1995). Hypophosphorylated pRb inhibits G_1- to S-phase progression in part by sequestering transcription factors, such as E2F, which are necessary for the expression of S-phase-associated genes (Weinberg, 1995). Phosphorylation of pRb removes this brake on cell-cycle progression. In keeping with the idea that rapamycin interferes with the activation of cyclin D_2–cdk4/cdk6 complexes, IL-2-dependent hyperphosphorylation of pRb is strongly inhibited in drug-treated T cells (Terada *et al.*, 1993). Rapamycin also interferes with the activation and functions of the more downstream G_1 cyclin–cdk complexes. In the presence of rapamycin, cyclin E–cdk2 complexes were fully assembled during mid- to late-G_1 phase, but these complexes were devoid of catalytic activity (Morice *et al.*, 1993b). These results suggest that the rapamycin-induced G_1 arrest point immediately precedes the peak of cyclin E–cdk2 activity normally observed in late-G_1-phase T cells stimulated with IL-2. Similarly, as would be expected for cells arrested prior to cyclin E–cdk2 activation, cyclin A–cdk2 activity was virtually absent in rapamycin-treated cells (Morice *et al.*, 1993b). Two inhibitory mechanisms prevent the assembly and activation of cyclin A–cdk2 complexes. First, the cyclin A–cdk2 complexes that form fail to become catalytically active. Second, the IL-2-induced expression of additional cyclin A mRNA and protein during G_1 phase is blocked by rapamycin. Although the exact mechanism is not known, previous evidence suggests that cyclin A gene expression is contingent upon the prior assembly of active cyclin E–cdk2 complexes (Guadagno *et al.*, 1993; Slingerland *et al.*, 1994).

The above results indicate that rapamycin exposure negatively affects the assembly and/or function of all three G_1 cyclin–cdk complexes in IL-2-stimulated T cells. Perhaps the most surprising result was that complexes

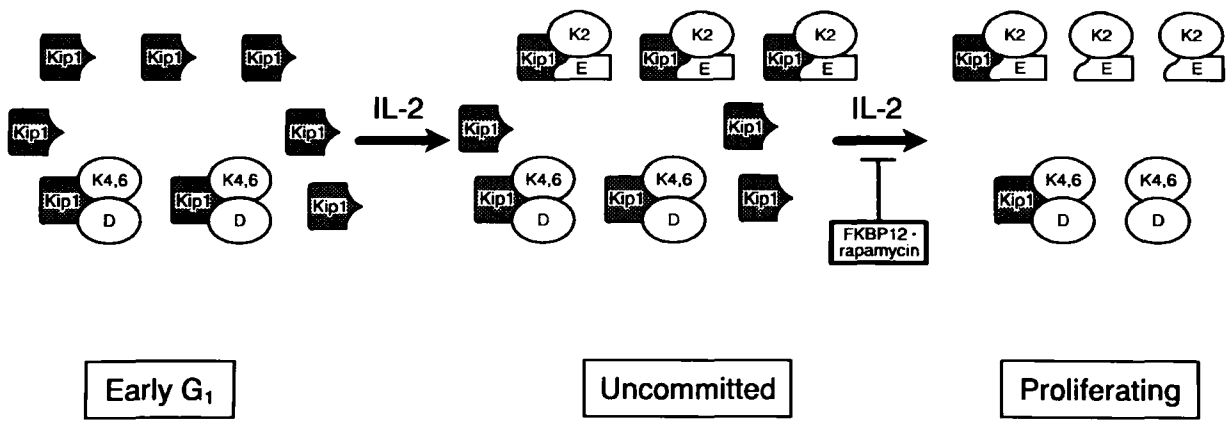


FIG. 4. Role of cdk inhibitor KIP1 in G_1-phase progression. Activated T cells (indicated as "early G_1") contain high levels of KIP1, which inhibits the activities of G1 cyclin-associated cdks, including cyclin D (D)–cdk4 and –cdk6 (K4,6) complexes. IL-2 stimulation induces the further accumulation of cyclin D–cdk complexes, as well as the assembly of cyclin E (E)–cdk2 (K2) complexes as the T cells progress through G_1 phase (indicated as "uncommitted"). Continued IL-2 exposure decreases KIP1 expression, thereby allowing the assembly of active G_1 cyclin–cdk complexes, which, in turn, drive the cell toward S phase (indicated as "proliferating"). The growth-inhibitory FKBP12–rapamycin complex blocks G_1-phase progression by interfering with the IL-2-dependent signaling pathway leading to KIP1 down-regulation.

containing cyclins D, E, and A can be assembled in the presence of rapamycin, but fail to express their respective protein kinase activities. The cdk subunits of these protein kinases are regulated by a series of positive and negative phosphorylation events. However, at least in the case of cyclin E–cdk2, the phosphorylation of the cdk2 subunit suggested that the complex should be fully active in rapamycin-treated T cells (Morice *et al.*, 1993b). A possible explanation for this apparent paradox surfaced with the discovery of a series of inhibitor proteins that targeted the G_1 cyclin–cdk activities (Sherr and Roberts, 1995). These findings provoked speculation that rapamycin might tip the balance between the positive and negative signals for G_1 cyclin–cdk2 activation by favoring the production and/or activity of one of these newly defined cdk inhibitor proteins. The identification and subsequent characterization of a heat-stable 27-kDa *K*inase *I*nhibitor *P*rotein-*1* (KIP1) (Polyak *et al.*, 1994) revealed an elegant mechanism for the regulation of IL-2-dependent G_1-phase progression, and provided a fundamental insight into the impact of rapamycin on this process.

KIP1 binds and inhibits the kinase activities of cyclin–cdk complexes containing cyclins D, E, A, and B, although it clearly interacts most avidly with the first three sets of complexes *in vitro* (Sherr and Roberts, 1995). Titration experiments revealed that KIP1 is a stoichiometric inhibitor of the G_1 cyclin–cdk activities (Firpo *et al.*, 1994; Sherr and Roberts, 1995). Thus, G_1 cyclin–cdk complexes cannot execute their cell-cycle regulatory functions until the overall numbers of such complexes surpass a threshold set by the amount of KIP1. Resting T cells express very high levels of KIP1, and continue to do so after activation by TCR-dependent stimuli (Firpo *et al.*, 1994; Nourse *et al.*, 1994). The persistent expression of KIP1 likely explains the presence of inactive cyclin D_2–, cyclin E–, and cyclin A–cdk complexes in activated T cells, as such complexes are immediately sequestered by the excess of free KIP1. IL-2 plays two important roles in overcoming the block to G_1-phase progression imposed by KIP1. First, by provoking the assembly of additional G_1 cyclin–cdk complexes, IL-2 drives the levels of these complexes closer to the KIP1-determined threshold. Second, IL-2 triggers a prompt decline in the level of KIP1 protein in G_1-phase T cells, which effectively lowers the threshold for activation of each G_1 cyclin–cdk complex (Firpo *et al.*, 1994). The physiologic importance of IL-2-induced KIP1 down-regulation during G_1-phase progression is underscored by the finding that this event is blocked by rapamycin (Nourse *et al.*, 1994). The failure of IL-2 to reduce KIP1 levels in the presence of rapamycin provides a satisfying explanation for the progressive accumulation of inactive cyclin E–cdk2 complexes in IL-2-stimulated CTLL-2 cells. In more global terms, the above results provide a mechanistic basis for understanding the requirement for two distinct sets of receptor-mediated

signals for G_0- to S-phase progression in peripheral blood-derived T cells. Antigenic stimuli render the cell "competent" to proliferate by inducing the expression of G_1 cyclins and their association with preformed cdks. However, the activated T cells cannot commit to S-phase entry until they receive a progression signal for KIP1 down-regulation, which is delivered through the IL-2R. Additional regulatory and checkpoint controls intrinsic to the cell-cycle machinery then govern the orderly activation of cyclin D-, cyclin E-, and cyclin A-associated cdks as the stimulated T cell moves toward the G_1–S boundary. By preventing the IL-2-induced decrease in KIP1, and the subsequent activation of G_1 cyclin–cdk complexes, rapamycin arrests the T cell in mid/late G_1.

VIII. Characterization of the Target Protein of the FKBP12–Rapamycin Complex

As stated in Section VII, the proximal effectors of the immunosuppressive actions of FK506 and rapamycin are the respective complexes formed when these drugs bind to a shared intracellular receptor, FKBP12. Although rapamycin was a potent inhibitor of G_1 cyclin–cdk activities in IL-2-stimulated T cells, it soon became evident that these protein kinases were not direct targets for the FKBP12–rapamycin complex. As described in Section VII, the protein target of the FKBP12–rapamycin complex in mammalian cells has recently been identified as mTOR. The mTOR cDNA encodes a 289-kDa protein that bears a remarkably high degree of amino acid sequence homology with two previously identified proteins, TOR1 and TOR2, from *S. cerevisiae*. The *TOR1* and *TOR2* genes were isolated in a screen for mutant alleles that render yeast resistant to the growth-inhibitory effect of rapamycin. The yeast TOR1 and TOR2 proteins are approximately 67% identical to one another, and nearly 50% identical to mTOR (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). In addition, the carboxyl termini of mTOR, TOR1, and TOR2 bear a striking resemblance to the catalytic domains of mammalian and yeast PI3-Ks, suggesting that these homologs also possess lipid kinase activity.

Genetic studies in yeast indicate that *TOR1* and *TOR2* perform both overlapping and distinct functions related to the control of cell-cycle progression (Cafferkey *et al.*, 1993; Helliwell *et al.*, 1994). Strikingly, double disruptions of the *TOR1* and *TOR2* genes resulted in growth arrest in early G_1, which is identical to the growth-arrest state induced by rapamycin in yeast (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). These results argue strongly that the antifungal, and by interference, the immunosuppressive effects of rapamycin are mediated through the drug-induced "knockout" of TOR protein function, which leads to G_1-phase arrest in both yeast and T cells.

A. Role of mTOR in IL-2 Signaling: The p70^{S6K} Connection

The current lack of knowledge regarding mTOR's enzymatic activity severely handicaps efforts to define the role of this protein in the transduction of mitogenic signals emanating from the IL-2R. Nonetheless, numerous studies using rapamycin as a probe strongly suggest that mTOR is an obligate upstream regulator of p70^{S6K} (Calvo *et al.*, 1992; Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992; Ferrari *et al.*, 1993). Activation of p70^{S6K} is a virtually ubiquitous response of mammalian cells to growth factor receptor stimulation. Exposure of activated, G_1-phase T cells to IL-2 leads to a rapid (minutes) and protracted (hours) increase in p70^{S6K} phosphorylation and catalytic activity (Calvo *et al.*, 1992). In T cells and in all other mammalian cell types examined, growth factor-dependent p70^{S6K} activation is suppressed to basal or even subbasal levels by rapamycin. Moreover, addition of rapamycin to IL-2-stimulated cells containing fully activated p70^{S6K} results in a rapid return of the protein kinase activity to the background level (Chung *et al.*, 1992). The latter result suggests that p70^{S6K} activity is continuously dependent on a positive regulatory input from mTOR, and that loss of mTOR function promptly reverses the activating input, possibly through the action of cellular phosphatases. As mentioned in Section V, IL-2-stimulated p70^{S6K} activation is also sensitive to inhibition by wortmannin. Although the available evidence is not definitive, recent data strongly suggest that wortmannin and rapamycin do not inhibit p70^{S6K} through direct mechanisms of action (Weng *et al.*, 1995). Thus, two classes of lipid kinases, a wortmannin-sensitive PI3-K and a rapamycin-sensitive mTOR, participate in coupling IL-2R stimulation to p70^{S6K} activation. The role of p70^{S6K} in IL-2-stimulated G_1-phase progression remains unclear. However, antibody microinjection experiments in serum-stimulated rat embryo fibroblasts indicate that p70^{S6K} activity is required virtually throughout the G_1 phase interval for entry into S phase of the cell cycle (Lane *et al.*, 1993).

The major substrate specificity of p70^{S6K} in intact cells supports speculation that this protein kinase participates in the stimulation of protein synthesis by T-cell and other mammalian cell growth factors. The most prominent p70^{S6K} substrate in mitogen-stimulated cells is the 40S ribosomal protein S6, which is phosphorylated on multiple serine residues by p70^{S6K} *in vivo* and *in vitro* (Ferrari and Thomas, 1994). Phosphorylated 40S subunits are preferentially incorporated into polysomes, suggesting that S6 phosphorylation might prime the transcriptional machinery for an influx of mRNAs derived from mitogen-responsive genes. S6 phosphorylation may also enhance the efficiency with which the 40S subunit recognizes and initiates the translation of specific mRNAs. Recent studies have shown

that rapamycin selectively blocks the translation of mRNAs containing a polypyrimidine tract immediately 3′ to the N^7-methylguanosine cap (Jefferies *et al.*, 1994; Terada *et al.*, 1994). Although the relationship between the inhibitions of $p70^{S6K}$ and specific mRNA translation remains correlative, it seems plausible that a $p70^{S6K}$-dependent increase in the translation of a polypyrimidine tract mRNA(s) encoding a labile protein might be a rate-limiting step in the process of G_1-phase progression in IL-2-stimulated T cells.

In addition to its potential role in translation initiation, $p70^{S6K}$ may phosphorylate other intracellular substrates involved in cell-cycle control. As described in Section V, one such substrate is the cAMP response element modulator τ (CREMτ), a member of the CREB/ATF family of cAMP response element CRE-binding transcription factors. In serum-stimulated fibroblasts, CREMτ is phosphorylated on a single serine residue by $p70^{S6K}$, and, as would be predicted, rapamycin prevents this modification (de Groot *et al.*, 1994). Phosphorylation of CREMτ by $p70^{S6K}$ greatly enhances the trans-activating function of this protein. Whether IL-2 stimulates rapamycin-sensitive CREMτ phosphorylation or function in T lymphocytes is unknown. However, one group has shown that IL-2 stimulates an increase in CRE-dependent transcription in late-G_1-phase T cells (Feuerstein *et al.*, 1995). This response was suppressed by rapamycin, which suggests a possible link to $p70^{S6K}$ and CREMτ. A potential connection between these events and the cell cycle was revealed by the recent finding that a CRE element is involved in the transcriptional activation of the cyclin A gene in human fibroblasts (Desdouets *et al.*, 1995). As discussed previously, rapamycin abolishes the increase in cyclin A mRNA accumulation that normally accompanies the entry of IL-2-stimulated CTLL-2 cells into late G_1 phase. In addition, wortmannin, which also inhibits IL-2-induced $p70^{S6K}$ activation, also retards transit through G_1, further linking $p70^{S6K}$ to cell-cycle progression (Karnitz *et al.*, 1995). It will be interesting to learn whether the inhibitory effect of rapamycin on IL-2-dependent cyclin A expression is mediated through the inhibition of CRE-dependent transcription.

In summary, the profound inhibitory effect of rapamycin on IL-2-dependent $p70^{S6K}$ activation strongly suggests that mTOR is an obligate component of the signal transduction pathway leading to $p70^{S6K}$ activation in IL-2-stimulated T cells. Other data suggest that $p70^{S6K}$ activation is required for the commitment of growth factor-stimulated, G_1-phase cells to enter S phase. The stimulatory effects of $p70^{S6K}$ on either translation initiation or CREMτ, or both, could represent the rapamycin-sensitive pathway that couples the IL-2R and, in turn, mTOR, to the machinery that controls the activation of the G_1 cyclin–cdk complexes. Although this

model is appealing, at least two important discrepancies should be noted. First, a $p70^{S6K}$ homolog has not been identified in yeast, which suggests that rapamycin-induced G_1 arrest does not involve $p70^{S6K}$ in this cell type. Second, and perhaps more troubling to cell biologists and immunologists, is that rapamycin effectively inhibits mitogen-induced $p70^{S6K}$ activation in all mammalian cell types, whereas the growth-inhibitory effects of the drug vary significantly, particularly among nonhematopoietic cell lineages. Perhaps these results hint that activated T cells and other bone marrow-derived cells are generally more reliant on mTOR function for G_1 progression than are many epithelial or mesenchymal fibroblasts, which, in the presence of rapamycin, might utilize a signaling pathway that bypasses mTOR.

IX. Summary and Perspective

The advent of recombinant DNA technology fostered rapid advances in our understanding of the structure of the high-affinity IL-2R complex. The availability of cDNAs encoding the individual subunits of this receptor established that the IL-2Rβ and γc cytoplasmic domains play essential roles in the transduction of IL-2-mediated regulatory signals across the T-cell plasma membrane. The proximal signaling machinery engaged by these intracellular domains remained a mystery until the discovery that IL-2 provoked a rapid increased in protein tyrosine phosphorylation in activated T cells (Saltzman *et al.*, 1988). The 7 intervening years since this seminal observation have been witness to a remarkable accumulation of knowledge regarding the intracellular signaling events elicited by IL-2 and other cytokines in a variety of bone marrow-derived cells. The IL-2R is proximally linked to at least three classes of nonreceptor PTKs, the Jaks and Syk- and Src-family members. The rapid tyrosine phosphorylation signals provoked by IL-2 trigger several downstream signaling cascades, two of which, the Ras and JAK–Stat pathways, are known to make their way to the nucleus, where they regulate the expression of specific genes related to IL-2-dependent T-cell growth, survival, or differentiation. This unprecedented view of IL-2-induced biochemical events that regulate various T-cell functional responses raises expectations that immunologists will one day modulate immune responses in humans through pharmacologic or genetic manipulations of specific signaling intermediates used by the IL-2R and related members of the cytokine receptor superfamily.

Our ability to achieve this goal will likely hinge on the answers to several challenging questions raised by the advances summarized in this chapter. Given the current state of knowledge regarding the IL-2R-coupled signaling apparatus, it is remarkable that we understand so little concerning the

mechanism by which IL-2R ligation triggers T-cell mitogenesis. Although the Ras pathway assumes a prominent role in most models of growth factor signal transduction in mammalian cells, the requirement for this pathway in IL-2-dependent T-cell proliferation has not been elucidated. Furthermore, a compelling body of data suggest that c-*myc* gene expression is a critical step in the mitogenic response to IL-2; however, the upstream signaling events that lead to increased c-*myc* gene transcription remain unknown. Perhaps the most exciting frontier in this field relates to the roles of the emerging family of lipid kinases, exemplified by PI3-K, mTOR, and the newly described ataxia telangiectasia gene product (Savitski *et al.*, 1995), in the regulation of T-cell-cycle progression from G_1 to S phase. If the current rate of progress is any indication, the answers to these questions will surface within the next few years and, undoubtedly, we will see some unexpected new twists on the current paradigm of IL-2R signaling.

REFERENCES

Ajchenbaum F, Ando K, DeCaprio JA, and Griffin JD: *J. Biol. Chem.* **268:**4113–4119.

Akira S, Nishio Y, Inoue M, Wang X-J, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, and Kishimoto T: *Cell* **77:** 63–71, 1994.

Albers MW, Brown EJ, Tanaka A, Williams RT, Hall FL, and Schreiber SL: *Ann. N.Y. Acad. Sci.* **696:**54–62, 1993.

Alland L, Peseckis SM, Atherton RE, Berthiaume L, and Resh MD: *J. Biol. Chem.* **269:**16701–16705, 1994.

Asao H, Takeshita T, Ishii N, Kumake S, Nakamura M, and Sugamura K: *Proc. Natl. Acad. Sci. USA* **90:**4127–4131, 1993.

Asao H, Tanaka N, Ishii N, Higuchi M, Takeshita T, Nakamura M, Shirasawa T, and Sugamura K: *FEBS Lett.* **351:**201–206, 1994.

Auger KR, Serunian LA, Soltoff SP, Libby P, and Cantley LC: *Cell* **57:**167–175, 1989.

Augustine JA, Schlager JW, and Abraham RT: *Biochim. Biophys. Acta* **1052:**313–322, 1990.

Augustine JA, Sutor SL, and Abraham RT: *Mol. Cell. Biol.* **11:**4431–4440, 1991.

Backer JM, Myers MG Jr, Shoelson SE, Chin DJ, Sun X-J, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, and White MF: *EMBO J.* **11:**3469–3479, 1992.

Barber EK, Dasgupta JD, Schlossman SF, Trevillyan JM, and Rudd CE: *Proc. Natl. Acad. Sci. USA* **86:**3277–3281, 1989.

Baumann H, Ziegler SF, Mosley B, Morella KK, Pajovic S, and Gearing DP: *J. Biol. Chem.* **268:**2414–2417, 1993.

Bich-Thuy LT, Dukovich M, Peffer NJ, Fauci AS, Kehrl JH, and Greene WC: *J. Immunol.* **139:**1550–1556, 1987.

Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G, and Schreiber SL: *Proc. Natl. Acad. Sci. USA* **87:**9231–9235, 1990.

Blaikie P, Immanuel D, Wu J, Li N, Yajnik V, and Margolis B: *J. Biol. Chem.* **269:**32031–32034, 1994.

Bolen JB, Thompson PA, Eiseman E, and Horak ID: *Adv. Cancer Res.* **57:**103–149, 1991.

Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, and Schreiber SL: *Nature* **369:**756–758, 1994.

Brunn GJ, Falls EL, Nilson AE, and Abraham RT: *J. Biol. Chem.* **270:**11628–11635, 1995.

Burns LA, Karnitz LM, Sutor SL, and Abraham RT: *J. Biol. Chem.* **268:**17659–17661, 1993.

Cafferkey R, Young PR, McLaughlin MM, Bergsma DJ, Koltin Y, Sathe GM, Faucette L, Eng W-K, Johnson RK, and Livi GP: *Mol. Cell. Biol.* **13:**6012–6023, 1993.

Calvo V, Crews CM, Vik TA, and Bierer BE: *Proc. Natl. Acad. Sci. USA* **89:**7571–7575, 1992.

Cantrell DA, and Smith KA: *Science* **224:**1312–1316, 1984.

Cao X, Shores EW, Hu-Li J, Anver MR, Kelsall BL, Russell SM, Drago J, Noguchi M, Grinberg A, Bloom ET, Paul WE, Katz SI, Love PE, and Leonard WJ: *Immunity* **2:**223–238, 1995.

Carrera AC, Paradis H, Borlado LR, Roberts TM, and Martinez A-C: *J. Biol. Chem.* **270:** 3385–3391, 1995.

Chan AC, Desai DM, and Weiss A: *Annu. Rev. Immunol.* **12:**555–592, 1994.

Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, and Kahn CR: *Mol. Cell. Biol.* **14:**4902–4911, 1994.

Chiu MI, Katz H, and Berlin V: *Proc. Natl. Acad. Sci. USA* **91:**12574–12578, 1994.

Chuang L-M, Myers MG Jr, Backer JM, Shoelson SE, White MF, Birnbaum MJ, and Kahn CR: *Mol. Cell. Biol.* **13:**6653–6660, 1993.

Chung J, Kuo CJ, Crabtree GR, and Blenis J: *Cell* **69:**1227–1236, 1992.

Clevenger CV, Russell DH, Appasamy PM, and Prystowsky MB: *Proc. Natl. Acad. Sci. USA* **87:**6460–6464, 1990.

Cohen B, Liu Y, Druker B, Roberts RM, and Schaffhausen BS: *Mol. Cell. Biol.* **10:**2909–2915, 1990.

Cohen GB, Ren R, and Baltimore D: *Cell* **80:**237–248, 1995.

Cooper JA, and Howell B: *Cell* **73:**1051–1054, 1993.

Cosman D: *Cytokine* **5:**95–106, 1993.

Cosman D, Cerretti DP, Larsen A, Park L, March C, Dower S, Gillis S, and Urdal D: *Nature* **312:**768–771, 1984.

Crabtree GR, and Clipstone NA: *Annu. Rev. Biochem.* **63:**1045–1083, 1994.

Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, and Wells JA: *Science* **254:**821–825, 1991.

Cutler RL, Liu L, Damen JE, and Krystal G: *J. Biol. Chem.* **268:**21463–21465, 1993.

Darnell JE Jr, Kerr IM, and Stark GR: *Science* **264:**1415–1421, 1994.

Daum G, Eisenmann-Tappe I, Fries H-W, Troppmair J, and Rapp UR: *Trends Biochem. Sci.* **19:**474–480, 1994.

Davis S, Aldrich TH, Stahl N, Pan L, Taga T, Kishimoto T, Ip NY, and Yancopoulos GD: *Science* **260:**1805–1808, 1993.

Decker SJ: *J. Biol. Chem.* **268:**9176–9179, 1993.

de Groot RP, Ballou LM, and Sassone-Corsi P: *Cell* **79:**81–91, 1994.

Desdouets C, Matesic G, Molina CA, Foulkes NS, Sassone-Corsi P, Brehot C, and Sobczak-Thepot J: *Mol. Cell. Biol.* **15:**3301–3309, 1995.

Dhand R, Hara K, Hiles I, Bax B, Gout I, Panayotou G, Fry MJ, Yonezawa K, Kasuga M, and Waterfield MD: *EMBO J.* **13:**511–521, 1994a.

Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, Kasuga M, Courtneidge SA, and Waterfield MD: *EMBO J.* **13:**522–533, 1994b.

DiSanto JP, Muller W, Guy-Grand D, Fischer A, and Rajewsky K: *Proc. Natl. Acad. Sci. USA* **92:**377–381, 1995.

Downes CP, and Carter AN: *Cell. Signal.* **3:**501–513, 1991.

Downward J: *FEBS Lett.* **338:**113–117, 1994.

Dumont FJ, Altmeyer A, Kastner C, Fischer PA, Lemon KP, Chung J, Blenis J, and Staruch MJ: *J. Immunol.* **152:**992–1003, 1994.

Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA, and Sigal NH: *J. Immunol.* **144:**1418–1424, 1990.

Duronio V, Welham MJ, Abraham S, Dryden P, and Schrader JW: *Proc. Natl. Acad. Sci. USA* **89:**1587–1591, 1992.

Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, and Williams LT: *Cell* **69:**413–423, 1992.

Farrar WL, and Ferris DK: *J. Biol. Chem.* **264:**12562–12567, 1989.

Ferrari S, Pearson, RB, Siegmann M, Kozma SC, and Thomas G: *J. Biol. Chem.* **268:**16091–16094, 1993.

Ferrari S, and Thomas G: *Crit. Rev. Biochem. Mol. Biol.* **29:**385–413, 1994.

Feuerstein N, Huang D, and Prystowsky MB: *J. Biol. Chem.* **270:**9454–9458, 1995.

Firpo EJ, Koff A, Solomon MJ, and Roberts JM: *Mol. Cell. Biol.* **14:**4889–4901, 1994.

Fu X-Y: *Cell* **70:**323–335, 1992.

Fukunaga R, Ishizaka-Ikeda E, Pan C-X, Seto Y, and Nagata S: *EMBO J.* **10:**2855–2865, 1991.

Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gillis S, Mosley B, Ziegler SF, and Cosman D: *Science* **255:**1434–1437, 1992.

Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D, and Anderson D: *EMBO J.* **13:**2822–2820, 1994.

Goldsmith MA, Xu W, Amaral MC, Kuczek ES, and Greene WC: *J. Biol. Chem.* **269:**14698–14704, 1994.

Gordon J, and MacLean LD: *Nature* **208:**795–796, 1965.

Gouilleux F, Pallard C, Dusanter-Fourt I, Wakao H, Haldosen L-A, Norstedt G, Levy D, and Groner B: *EMBO J.* **14:**2005–2013, 1995.

Greene WC, Robb RJ, Svetlik PB, Rusk CM, Depper JM, and Leonard WJ: *J. Exp. Med.* **162:**363–368, 1985.

Guadagno TM, Ohtsubo M, Roberts JM, and Assoian RK: *Science* **262:**1572–1575, 1993.

Guan K-L: *Cell. Signal.* **6:**581–589, 1994.

Gustafson TA, He W, Craparo A, Schaub CD, and O'Neill TJ: *Mol. Cell. Biol.* **15:**2500–2508, 1995.

Hartwell LH, and Kastan MB: *Science* **266:**1821–1828, 1994.

Hatakeyama M, Kawahara A, Mori H, Shibuya H, and Taniguchi T: *Proc. Natl. Acad. Sci. USA* **89:**2022–2026, 1992.

Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin SD, Perlmutter RM, and Taniguchi T: *Science* **252:**1523–1528, 1991.

Hatakeyama M, Mori H, Doi T, and Taniguchi T: *Cell* **59:**837–845, 1989a.

Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyaska M, and Taniguchi T: *Science* **244:**551–556, 1989b.

Hawkins PT, Jackson TR, and Stephens LR: *Nature* **358:**157–159, 1992.

Hayashi H, Kamohara S, Nishioka Y, Kanai F, Miyake N, Fukiu Y, Shibasaki F, Takenawa T, and Ebina Y: *J. Biol. Chem.* **267:**22575–22580, 1992.

Hayashi H, Nishioka Y, Kamohara S, Kanai F, Ishii K, Fukiu Y, Shibasaki F, Takenawa T, Kido H, Katsunuma N, and Ebina Y: *J. Biol. Chem.* **268:**7107–7117, 1993.

He T-C, Jiang N, Zhuang H, and Wojchowski DM: *J. Biol. Chem.* **270:**11055–11061, 1995.

Heim MH, Kerr IM, Stark GR, and Darnell JE Jr: *Science* **267:**1347–1349, 1995.

Helliwell SB, Wagner P, Kunz J, Deuter-Reinhard M, Henriquez R, and Hall MN: *Mol. Biol. Cell* **5:**105–118, 1994.

Hibi M, Murakami M, Saito M, Hirano T, Taga T, and Kishimoto T: *Cell* **63:**1149–1157, 1990.

Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA, and Bolen JB: *Proc. Natl. Acad. Sci. USA* **88:**1996–2000, 1991.

Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, and McKnight SL: *Science* **265:**1701–1706, 1994.

Hou J, Schindler U, Henzel WJ, Wong SC, and McKnight SL: *Immunity* **2:**321–329, 1995.

Hu Q, Klippel A, Muslin AJ, Fantl WJ, and Williams LT: *Science* **268:**100–102, 1995.

Hunter T, and Pines J: *Cell* **79:**573–582, 1994.

Ihle JN, and Kerr IM: *Trends Genet.* **11:**69–74, 1995.

Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, and Silvennoinen O: *TIBS* **19:**222–227, 1994.

Israel A, Kimura A, Fournier A, Fellous M, and Kourilski P: *Nature* **322:**743–746, 1986.

Jayaraman T, and Marks AR: *J. Biol. Chem.* **268:**25385–25388, 1993.

Jefferies HBJ, Reinard C, Kozma SC, and Thomas G: *Proc. Natl. Acad. Sci. USA* **91:**4441–4445, 1994.

Jhun BH, Rose DW, Seely BL, Rameh L, Cantley L, Saltiel AR, and Olefsky JM: *Mol. Cell. Biol.* **14:**7466–7475, 1994.

Johnston JA, Kawamura M, Kirken RA, Chen Y-Q, Blake TB, Shibuya K, Ortaldo JR, McVicar DW, and O'Shea JJ: *Nature* **370:**151–153, 1994.

Joly M, Kazlauskas A, Fay FS, and Corvera S: *Science* **263:**684–687, 1994.

Kapeller R, Prasad KVS, Janssen O, Hou W, Schaffhausen BS, Rudd CE, and Cantley LC: *J. Biol. Chem.* **269:**1927–1933, 1994.

Karnitz LM, and Abraham RT: *Curr. Op. Immunol.* **7:**320–326, 1995.

Karnitz LM, Burns LA, Sutor SL, Blenis J, and Abraham RT: *Mol. Cell. Biol.* **15:**3049–3057, 1995.

Karnitz LM, Sutor SL, and Abraham RT: *J. Exp. Med.* **179:**1799–1808, 1994.

Karnitz LM, Sutor SL, Torigoe T, Reed JC, Bell MP, McKean DJ, Leibson PJ, and Abraham RT: *Mol. Cell. Biol.* **12:**4521–4530, 1992.

Kasakura S, and Lowenstein L: *Nature* **208:**794–795, 1965.

Kato J-Y, Matsuchime H, Hiebert SW, Ewen ME, and Sherr CJ: *Genes Devel.* **7:**331–342, 1993.

Kavanaugh WM, Klippel A, Escobedo JA, and Williams LT: *Mol. Cell. Biol.* **12:**3415–3424, 1992.

Kavanaugh WM, Turck CW, and Williams LT: *Science* **368:**1177–1179, 1995.

Kavanaugh WM, and Williams LT: *Science* **266:**1862–1864, 1994.

Kinoshita T, Yokota T, Arai K-i, and Miyajima A: *EMBO J.* **14:**266–275, 1995.

Kirschner MW: *In* DeVita VT Jr, Hellman S, and Rosenberg SA, Eds., Important Advances in Oncology: The Biochemical Nature of the Cell Cycle. J. B. Lippincott, Philadelphia, PA, pp. 3–16, 1992.

Klippel A, Escobedo JA, Hu Q, and Williams LT: *Mol. Cell. Biol.* **13:**5560–5566, 1993.

Kobayashi N, Kono T, Hatakeyama M, Minami Y, Miyazaki T, Perlmutter RM, and Taniguchi T: *Proc. Natl. Acad. Sci USA* **90:**4201–4205, 1993.

Kodaki T, Woscholski R, Hallberg B, Rodriguez-Viciana P, Downward J, and Parker PJ: *Curr. Biol.* **4:**798–806, 1994.

Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S-I, and Sugamura K: *Science* **263:**1453–1454, 1994.

Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K-i, and Sugamura K: *Science* **262:**1874–1877, 1993.

Kotanides H, and Reich NC: *Science* **262:**1265–1267, 1993.

Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, and Hall MN: *Cell* **73:**585–596, 1993.

Kuo CJ, Chung J, Fiorentino DF, Flanagan WM, Blenis J, and Crabtree GR: *Nature* **358:**70–73, 1992.

Lam K, Carpenter CL, Ruderman NB, Friel JC, and Kelly KL: *J. Biol. Chem.* **269:**20648–20652, 1994.

Lane HA, Fernandez A, Lamb NJC, and Thomas G: *Nature* **363:**170–172, 1993.

Larner AC, David M, Feldman GM, Igarashi K-i, Hackett RH, Webb DSA, Sweitzer SM, Petricoin EF III, and Finbloom DS: *Science* **261:**1730–1733, 1993.

Leevers SJ, Paterson HF, and Marshall CJ: *Nature* **369:**411–414, 1994.

Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Pumphrey J, Robb RJ, Kronke M, Svetlik PB, Peffer NJ, Waldmann TA, and Greene WC: *Nature* **311:**626–631, 1984.

Lew D, Decker T, Strehlow I, and Darnell JE: *Mol. Cell. Biol.* **182:**182–191, 1991.

Lin J-X, Migone T-S, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S, and Leonard WJ: *Immunity* **2:**331–339, 1995.

Lips DL, and Majerus PW: *J. Biol. Chem.* **264:**8759–8763, 1989.

Liu L, Damen JE, Cutler RL, and Krystal G: *Mol. Cell. Biol.* **14:**6926–6935, 1994.

Lopez AF, Elliott MJ, Woodcock J, and Vadas MA: *Immunol. Today* **13:**495–500, 1992.

Lukas J, Bartkova J, Rohde M, Strauss M, and Bartek J: *Mol. Cell. Biol.* **15:**2600–2611, 1995.

Lutticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yashikawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC, and Horn F: *Science* **263:**89–91, 1994.

Marengere LEM, Songyang Z, Gish GD, Schaller MD, Parsons JT, Stern MJ, Cantley LC, and Pawson T: *Nature* **369:**502–505, 1994.

Marshall CJ: *Curr. Biol.* **4:**82–89, 1994.

Matsushima H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, and Kato J-Y: *Mol. Cell. Biol.* **14:**2066–2076, 1994.

McGlade J, Cheng A, Pelicci G, Pelicci PG, and Pawson T: *Proc. Natl. Acad. Sci. USA* **89:**8869–8873, 1992.

Merida I, Diez E, and Gaulton GN: *J. Immunol.* **147:**2202–2207, 1991.

Merida I, and Gaulton GN: *J. Biol. Chem.* **265:**5690–5694, 1990.

Merida I, Williamson P, Kuziel WA, Greene WC, and Gaulton GN: *J. Biol. Chem.* **268:**6765–6770, 1993.

Migone T-S, Lin J-X, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G, and Leonard WJ: *Science* **269:**79–81, 1995.

Minami Y, Kono T, Yamada K, Kobayashi N, Kawahara A, Perlmutter RM, and Taniguchi T: *EMBO J.* **12:**759–768, 1993.

Minami Y, Kono T, Yamada K, and Taniguchi T: *Biochim. Biophys. Acta* **1114:**163–177, 1992.

Minami Y, Nakagawa Y, Kawahara A, Miyazaki T, Sada K, Yamamura H, and Taniguchi T: *Immunity* **2:**89–100, 1995.

Minamoto S, Mori H, Hatakeyama M, Kono T, Doi T, Ide T, Uede T, and Taniguchi T: *J. Immunol.* **145:**2177–2182, 1990.

Ming X-F, Burgering BMT, Wennstrom S, Claesson-Welsh L, Heldin C-H, Bos JL, Kozma SC, and Thomas G: *Nature* **371:**426–429, 1994.

Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu Z-J, Oishi I, Silvennoinen O, Witthuhn BA, Ihle JN, and Taniguchi T: *Science* **266:**1045–1047, 1994.

Miyazaki T, Maruyama M, Yamada G, Hatakeyama M, and Taniguchi T: *EMBO J.* **10:**3191–3197, 1991.

Monfar M, Lemon KP, Grammer TC, Cheatham L, Chung J, Vlahos CJ, and Blenis J: *Mol. Cell. Biol.* **15:**326–337, 1995.

Morice WG, Brunn GJ, Wiederrecht G, Siekierka JJ, and Abraham RT: *J. Biol. Chem.* **268:**3734–3738, 1993a.

Morice WG, Wiederrecht G, Brunn GJ, Siekierka JJ, and Abraham RT: *J. Biol. Chem.* **268:**22737–22745, 1993b.

Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, Silvennoinen O, Harpur AG, Barbieri G, Witthuhn BA, Schindler C, Pellegrini S, Wilks AF, Ihle JN, Stark GR, and Kerr IM: *Nature* **366:**129–135, 1993.

Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T, and Kishimoto T: *Proc. Natl. Acad. Sci. USA* **88:**11349–11353, 1991.

Muslin AJ, Klippel A, and Williams LT: *Mol. Cell. Biol.* **13:**6661–6666, 1993.

Myers MG Jr, Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, and White MF: *Proc. Natl. Acad. Sci. USA* **89:**10350–10354, 1992.

Nakamura Y, Russell SM, Mess SA, Friedman M, Erdos M, Francois C, Jacques Y, Adelstein S, and Leonard WJ: *Nature* **369:**330–333, 1994.

Nelson BH, Lord JD, and Greenberg PD: *Nature* **369:**333–336, 1994.

Nielsen M, Svejgaard A, Skov S, and Odum N: *Eur. J. Immunol.* **24:**3082–3086, 1994.

Nikaido T, Shimizu A, Ishida N, Sabe H, Teshigawara K, Maeda M, Uchiyama T, Yodoi J, and Honjo T: *Nature* **311:**631–635, 1984.

Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, and Leonard WJ: *Science* **262:**1877–1880, 1993a.

Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW, and Leonard WJ: *Cell* **73:**147–157, 1993b.

Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee M-H, Massague J, Crabtree GR, and Roberts JM: *Nature* **372:**570–573, 1994.

Okada T, Sakuma L, Fukui Y, Hazeki O, and Ui M: *J. Biol. Chem.* **269:**3563–3567, 1994.

Otani H, Siegel JP, Erdos M, Gnarra JR, Toledano MB, Sharon M, Mostowski H, Feinberg MB, Pierce JH, and Leonard WJ: *Proc. Natl. Acad. Sci. USA* **89:**2789–2793, 1992.

Paige LA, Nadler MJS, Harrison ML, Cassady JM, and Geahlen RL: *J. Biol. Chem.* **268:**8669–8674, 1993.

Panchamoorthy G, Fukazawa T, Stolz L, Payne G, Reedquist K, Shoelson S, Songyang Z, Cantley L, Walsh C, and Band H: *Mol. Cell. Biol.* **14:**6372–6385, 1994.

Pardee AB: *Science* **246:**603–608, 1989.

Pawson T: *Nature* **373:**573–580, 1995.

Perkins GR, Marvel J, and Collins MKL: *J. Exp. Med.* **178:**1429–1434, 1993.

Pleiman CM, Clark MR, Gauen LKT, Winitz S, Coggeshall KM, Johnson GL, Shaw AS, and Cambier JC: *Mol. Cell. Biol.* **13:**5877–5887, 1993.

Pleiman CM, Hertz WM, and Cambier JC: *Science* **263:**1609–1612, 1994.

Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, and Massague J: Cell **78:**59–66, 1994.

Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, Zalkow L, Matter WF, Dodge J, Grindey G, and Vlahos CJ: *Cancer Res.* **54:**2419–2423, 1994.

Prasad KV, Janssen O, Kapeller R, Raab M, Cantley LC, and Rudd CE: *Proc. Natl. Acad. Sci. USA* **90:**7366–7370, 1993.

Price DJ, Grove JR, Calvo V, Avruch J, and Bierer BE: *Science* **257:**973–977, 1992.

Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD, and Ihle JN: *Mol. Cell. Biol.* **14:**4335–4341, 1994.

Ravichandran KS, and Burakoff SJ: *J. Biol. Chem.* **269:**1599–1602, 1994.

Ravichandran KS, Lorenz U, Shoelson SE, and Burakoff SJ: *Mol. Cell. Biol.* **15:**593–600, 1995.

Reid LE, Brasnett AH, Gilbert CS, Porter ACG, Gewert DR, Stark GR, and Kerr IM: *Proc. Natl. Acad. Sci. USA* **86:**840–844, 1989.

Remillard B, Petrillo R, Maslinski W, Tsudo M, Strom TB, Cantley L, and Varticovski L: *J. Biol. Chem.* **266:**14167–14170, 1991.

Resh MD: *Cell* **76:**411–413, 1994.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, and Downward J: *Nature* **370:**527–532, 1994.

Rothman P, Kreider B, Azam M, Levy D, Wegenka U, Eilers A, Decker T, Horn F, Kashleva H, Ihle J, and Schindler C: *Immunity* **1:**457–468, 1994.

Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, and Schlossman SF: *Proc. Natl. Acad. Sci. USA* **85:**5190–5194, 1988.

Ruiz-Larrea F, Vicendo P, Yaish P, End P, Panayotou G, Fry MJ, Morgan SJ, Thompson A, Parker PJ, and Waterfield MD: *Biochem. J.* **290:**609–616, 1993.

Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, Goldman AS, Schmalstieg FC, Ihle JN, O'Shea JJ, and Leonard WJ: *Science* **266:**1042–1045, 1994.

Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri RK, Paul WE, and Leonard WJ: *Science* **262:**1880–1883, 1993.

Sabatini DM, Erdjument-Bromage H, Liu M, Tempst P, and Synder SH: *Cell* **78:**35–43, 1994.

Sabers CJ, Martin MM, Brunn GJ, Williams JM, Dumont FJ, Wiederrecht G, and Abraham, RT: *J. Biol. Chem.* **270:**815–822, 1995.

Sadowski HB, Shuai K, Darnell JE Jr, and Gilman MZ: *Science* **261:**1739–1744, 1993.

Saito Y, Tada H, Sabe H, and Honjo T: *J. Biol. Chem.* **266:**22186–22191, 1991.

Saltzman EM, Thom RR, and Casnellie JE: *J. Biol. Chem.* **263:**6956–6959, 1988.

Sato N, Sakamaki K, Terada N, Arai K-i, and Miyajima A: *EMBO J.* **12:**4181–4189, 1993.

Satoh T, Nakafuku M, Miyajima A, and Kaziro Y: *Proc. Natl. Acad. Sci. USA* **88:**3314–3318, 1991.

Satoh T, Minami Y, Kono T, Yamada K, Kawahara A, Taniguchi T, and Kaziro Y: *J. Biol. Chem.* **267:**25423–25427, 1992.

Savitski K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik, R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor MR, Arlett CF, Miki T, Weissman SM, Lovett MF, Collins FS, and Shiloh Y: *Science* **268:**1749–1753, 1995.

Schindler C, Fu X-Y, Improta T, Aebersold R, and Darnell JE Jr: *Proc. Natl. Acad. Sci. USA* **89:**7836–7839, 1992.

Schorle H, Holtschke T, Hunig T, Schimpl A, and Horak I: *Nature* **352:**621–624, 1991.

Schreiber SL: *Science* **251:**283–287, 1991.

Schreiber SL: *Cell* **70:**365–368, 1992.

Schreiber SL, and Crabtree GR: *Immunol. Today* **13:**136–142, 1992.

Serunian LA, Haber MT, Fukui T, Kim JW, Rhee SG, Lowenstein JM, and Cantley LC: *J. Biol. Chem.* **264:**17809–17815, 1989.

Sharon M, Gnarra JR, and Leonard WJ: *J. Immunol.* **143:**2530–2533, 1989.

Sharon M, Klausner RD, Cullen BR, Chizzonite R, and Leonard WJ: *Science* **234:**859–863, 1986.

Shenoy-Scaria AM, Gauen LKT, Kwong J, Shaw AS, and Lublin DM: *Mol. Cell. Biol.* **13:**6385–6392, 1993.

Sherr CJ: *Cell* **73:**1059–1065, 1993.

Sherr CJ: *Cell* **79:**551–555, 1994.

Sherr CJ, and Roberts JM: *Genes Devel.* **9:**1149–1163, 1995.

Shoelson SE, Sivaraja M, Williams KP, Hu P, Schlessinger J, and Weiss MA: *EMBO J.* **12:**795–802, 1993.
Shuai K: *Curr. Op. Cell Biol.* **6:**253–259, 1994.
Shuai K, Horvath CM, Huang LHT, Qureshi SA, Cowburn D, and Darnell JE Jr: *Cell* **76:**821–828, 1994.
Shuai K, Schindler C, Prezioso VR, and Darnell JE Jr: *Science* **259:**1808–1812, 1992.
Shuai K, Stark GR, Kerr IM, and Darnell JE Jr: *Science* **261:**1744–1746, 1993.
Shurtleff SA, Downing JR, Rock CO, Hawkins SA, Roussel MF, and Sherr CJ: *EMBO J.* **9:**2415–2421, 1990.
Sigal CT, Zhou W, Buser CA, McLaughlin S, and Resh MD: *Proc. Natl. Acad. Sci. USA* **91:**12253–12257, 1994.
Sigal NH, and Dumont FJ: *Annu. Rev. Immunol.* **10:**519–560, 1992.
Silvennoinen O, Schindler C, Schlessinger J, and Levy DE: *Science* **261:**1736–1739, 1993.
Silverman L, and Resh MD: *J. Cell. Biol.* **119:**415–425, 1992.
Slingerland JM, Hengst L, Pan C-H, Alexander D, Stampfer MR, and Reed SI: *Mol. Cell. Biol.* **14:**3683–3694, 1994.
Smith KA: *Science* **240:**1169–1178, 1988.
Soler C, Alvarez CV, Beguinot L, and Carpenter G: *Oncogene* **9:**2207–2215, 1994.
Soltoff SP, Rabin SL, Cantley LC, and Kaplan DR: *J. Biol. Chem.* **267:**17472–17477, 1992.
Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN, and Yancopoulos GD: *Science* **263:**92–95, 1994.
Stahl N, Farruggella TJ, Boulton TF, Zhong Z, Darnell JE Jr, and Yancopoulos GD: *Science* **267:**1349–1353, 1995.
Stahl N, and Yancopoulos GD: *Cell* **74:**587–590, 1993.
Stokoe D, Macdonald SG, Cadwallader K, Symons M, and Hancock JF: *Science* **264:**1463–1467, 1994.
Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, and Kishimoto T: *Cell* **58:**573–581, 1989.
Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M, and Sugamura K: *Science* **257:**379–382, 1992.
Taniguchi T: *Science* **268:**251–255, 1995.
Terada N, Lucas JJ, Szepesi A, Franklin RA, Domenico J, and Gelfand EW: *J. Cell. Phys.* **154:**7–15, 1993.
Terada N, Patel HR, Takase K, Kohno K, Nairn AC, and Gelfand EW: *Proc. Natl. Acad. Sci. USA* **91:**11477–11481, 1994.
Teshigawara K, Wang H-M, Kato K, and Smith KA: *J. Exp. Med.* **165:**223–238, 1987.
Toker A, Meyer M, Reddy KK, Falck JR, Aneja R, Aneja A, Parra A, Burns DJ, Ballas LM, and Cantley LC: *J. Biol. Chem.* **269:**32358–32367, 1994.
Torigoe T, Saragovi HU, and Reed JC: *Proc. Natl. Acad. Sci. USA* **89:**2674–2678, 1992.
Treisman R: *Curr. Op. Genet Dev.* **4:**96–101, 1994.
Trouche D, Robin P, Robillard O, Sassone-Corsi P, and Harel-Bellan A: *J. Immunol.* **147:**2398–2403, 1991.
Truitt KE, Mills GB, Turck CW, and Imboden JB: *J. Biol. Chem.* **269:**5937–5943, 1994.
Tsudo M, Karasuyama H, Kitamura F, Tanaka T, Kubo S, Yamamura Y, Tamatani T, Hatakeyama M, Taniguchi T, and Miyasaka M: *J. Immunol.* **145:**599–606, 1990.
Tsudo M, Kozak RW, Goldman CK, and Waldmann TA: *Proc. Natl. Acad. Sci. USA* **83:**9694–9698, 1986.
Turner B, Rapp U, App H, Greene H, Dobashi K, and Reed J: *Proc. Natl. Acad. Sci. USA* **88:**1227–1231, 1991.

Ullman KS, Northrop JP, Verweij CL, and Crabtree GR: *Annu. Rev. Immunol.* **8**:421–452, 1990.
Ullrich A, and Schlessinger J: *Cell* **61**:203–212, 1990.
Valius M, and Kazlauskas A: *Cell* **73**:321–334, 1993.
Veillette A, Bookman MA, Horak EM, and Bolen JB: *Cell* **55**:301–308, 1988.
Velazquez L, Fellous M, Stark GR, and Pellegrini S: *Cell* **70**:313–322, 1992.
Vlahos CJ, Matter WF, Hui KY, and Brown RF: *J. Biol. Chem.* **269**:5241–5248, 1994.
Vogel J, Kres M, Khoury G, and Jay G: *Mol. Cell. Biol.* **6**:3550–3554, 1986.
Wang L-M, Keegan AD, Paul WE, Heidaran MA, Gutkind JS, and Pierce JH: *EMBO J.* **11**:4899–4908, 1992.
Watling D, Guschin D, Muller M, Silvennoinen O, Witthuhn BA, Quelle FW, Rogers NC, Schindler C, Stark GR, Ihle JN, and Kerr IM: *Nature* **366**:166–170, 1993.
Wegenka UM, Lutticken C, Buschmann J, Yuan J, Lottspeich F, Muller-Esterl W, Schindler C, Roeb E, Heinrich PC, and Horn F: *Mol. Cell. Biol.* **14**:3186–3196, 1994.
Weinberg RA: *Cell* **81**:323–330, 1995.
Welham MJ, Duronio V, Leslie KB, Bowtell D, and Schrader JW: *J. Biol. Chem.* **269**:21165–21176, 1994a.
Welham MJ, Duronio V, and Schrader JW: *J. Biol. Chem.* **269**:5865–5873, 1994b.
Weng Q-P, Andrabi K, Kozlowski MT, Grove JR, and Avruch J: *Mol. Cell. Biol.* **15**:2333–2340, 1995.
Williamson P, Merida I, Greene WC, and Gaulton G: *Leukemia* **8**:5186–5189, 1994.
Witthuhn BA, Silvennoinen O, Miura O, Lai KS, Cwik C, Liu ET, and Ihle JN: *Nature* **370**:153–157, 1994.
Xu H, and Littman DR: *Cell* **74**:633–643, 1993.
Yamauchi K, Holt K, and Pessin JE: *J. Biol. Chem.* **268**:14597–14600, 1993.
Yoshimura A, Zimmers T, Neurmann D, Longmore G, Yoshimura Y, and Lodish HF: *J. Biol. Chem.* **267**:11619–11625, 1992.
Zhang X, Blenis J, Li H-C, Schindler C, and Chen-Kiang S: *Science* **267**:1990–1994, 1995.
Zhong Z, Wen Z, and Darnell JE Jr: *Science* **264**:95–98, 1994.
Zhu X, Suen K-L, Barbacid M, Bolen JB, and Fargnoli J: *J. Biol. Chem.* **269**:5518–5522, 1994.

ADVANCES IN IMMUNOLOGY, VOL. 61

Control of the Complement System

M. KATHRYN LISZEWSKI,* TIMOTHY C. FARRIES,† DOUGLAS M. LUBLIN,‡ ISABELLE A. ROONEY,‖ AND JOHN P. ATKINSON*

**Department of Medicine, Division of Rheumatology, and ‡Department of Pathology, Division of Laboratory Medicine, Washington University School of Medicine, St Louis, Missouri 63110; †Molecular Immunopathology Unit, MRC Centre, Cambridge CB2 2QH, United Kingdom; and ‖Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037*

I. Overview of Complement Regulation

More than 600 million years ago primitive components of the alternative pathway of complement likely formed the first humoral immune system (1–3). Subsequent evolution of an adaptive immune response drove the specialization of a second arm of the complement system, the classical pathway, to connect antibody-mediated events to complement-dependent effector mechanisms. A complement system similar to that in mammals has been identified in reptiles, birds, amphibians, and fish. The contemporary human complement system consists of a highly efficient recognition and effector mechanism of humoral immunity designed to destroy infecting microbes. It consists of more than 30 serum or cellular components including activating proteins, receptors, and positive and negative regulators that form an independent immune system.

Because of its potent proinflammatory and destructive capabilities nearly half of complement proteins serve in regulation. In effect, this provides a recognition system to distinguish self from "nonself" (4). That is, foreign surfaces lacking control proteins are "attacked" by complement while host tissue is protected. As evidence for the necessity of regulation, deficiencies of control proteins lead to excessive complement activation and disease. For example, in paroxysmal nocturnal hemoglobinuria, complement-mediated lysis of erythrocytes results from a deficiency of membrane regulators [reviewed in (5)].

The regulatory system is designed to prevent complement activation both in the fluid phase and on "self" tissue (4). Consequently, overlapping activities exist between plasma and membrane inhibitors. Such an arrangement evolved because of the requirement for rapid, unimpeded activation on a microbial target but with strict limitation of activation in both time and space. The *time* of activation must be finite to avoid excessive consumption of complement components for one reaction. Thus, less than 5 min is required to deposit several million copies of C3b on a target and to liberate and equal quantity of C3a into the surrounding milieu upon engagement of either pathway. Restriction of activation in *space* is needed

because the reaction must be focused on the target and not be allowed to spread to the fluid phase or to self tissue. Regulation is accomplished by (i) spontaneous decay of activated proteins and enzyme complexes (i.e., short half-life), (ii) destabilization and inhibition of activation complexes, and (iii) proteolytic cleavage of "activated" components.

Simply put, the activating sequence of complement consists of an initiation or triggering step, an amplification step with a feedback loop, and finally a membrane attack or lytic step. Zymogens become transiently active serine proteases and membrane interactive components acquire the temporary ability to bind to membranes.

The central component of the complement system is C3 (Fig. 1). Generation of C3b by the classical or alternative pathway is crucial for microbial opsonization and lysis. Regulation of complement occurs at the steps preceding as well as following C3b production but the majority are aimed at controlling C3b generation. Table I summarizes selected aspects of the complement regulatory proteins described in this chapter.

The classical pathway is initiated when component C1 via its C1q subcomponent attaches to antibody to form an immune complex. Control of the *initiation* step of the classical pathway is accomplished by C1 inhibitor (C1-Inh). This serpin (*ser*ine *p*rotease *in*hibitor) blocks the active site of C1r and C1s and in the process disassembles them from C1q. C1-Inh does not impede appropriate activation but blocks fluid-phase and chronic or excessive activation on a target.

For the alternative pathway, however, there is no initiating factor equivalent to antibody. Rather, it is in a state of continuous, low-level activation as a result of the spontaneous hydrolysis of a thioester group in native C3. This "tickover" of C3 leads to its binding of nonspecific acceptor molecules in plasma (such as water) or to cell surfaces (6). C3 molecules created in this manner may form C3 convertases and create a feedback loop. Thus,

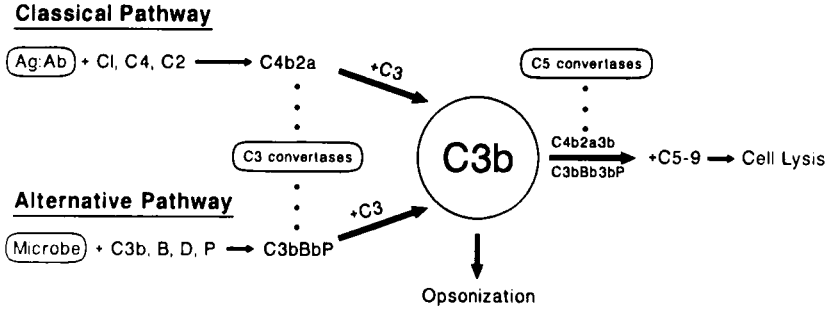


FIG. 1. The central role of C3b in the complement system. C3b is the primary activated component of both the classical and alternative pathways. Its generation promotes opsonization (with subsequent phagocytosis) and, in some cases, cytolysis. From (425).

TABLE I
REGULATORY PROTEINS OF COMPLEMENT

	Molecular Weight (kDa)	Serum Concentration (μg/ml)	Function
Initiation step			
C1 inhibitor	105	120–200	Inactivates C1r and C1s; a serpin
Amplification step			
Factor I	88	35	Cleaves C3b/C4b with cofactor
Membrane cofactor protein	45–70	Membrane	Cofactor for factor I-mediated cleavage of C4b and C3b
Decay-accelerating factor	70	Membrane	Destabilizes C3/C5 convertases
C4-binding protein	560	250	Cofactor for factor I cleavage of C4b; destabilizes CP C3/C5 convertases
Factor H	150	500	Cofactor for factor I cleavage of C3b; destabilizes AP C3/C5 convertases and CP C5 convertase
Complement receptor 1	190–280	Membrane	Receptor for C3b/C4b; inhibitory profile similar to MCP and DAF
Properdin	112–224	25	Stabilizes AP convertases
Membrane attack			
S protein	75–80	250–450	Blocks fluid-phase MAC
Clusterin	80	35–105	Blocks fluid-phase MAC
CD59	18	Membrane	Blocks MAC on host cells
Other			
Anaphylatoxin inactivator	305	35	Inactivates C3a, C4a, C5a (partially)

Note. Abbreviations: CP, classical pathway; AP, alternative pathway; MAC, membrane attack complex.

control of the alternative pathway is focused on regulating convertase assembly (the amplification step and its feedback loop).

The alternative and classical pathway C3 convertases once formed on a target efficiently convert C3 to C3b. This is obviously desirable on a foreign target but must be checked in plasma and on self tissue. In plasma, C4-

binding protein (C4BP) binds C4b, while a related protein, factor H, binds C3b. These two components each possess destabilizing and cofactor activity for their respective ligand individually as well as in convertases. In contrast, on self-tissue, decay-accelerating factor (DAF; CD55) has the dissociative ability for both convertases, while membrane cofactor protein (MCP; CD46) has cofactor activity for both C4b and C3b. Therefore, while the division of labor is different between plasma and self-tissue, the overall functional repertoire is similar.

Regulation of the membrane attack complex (MAC) also occurs in plasma and on host cells. S protein (vitronectin) and clusterin are the fluid-phase inhibitors of the MAC. They attach to the C5b–7 complex, blocking its ability to interact with membranes. MACs that do succeed in depositing on a host cell are inhibited by the glycolipid anchored protein, CD59, which has a binding site for both C8 and C9 to prevent further MAC assembly.

The activation of C3, C4, and C5 leads to the release of a small inflammatory peptide (C3a, C4a, C5a) from the parent molecule. The generation of each "anaphylatoxin" exposes a C-terminal arginine. The plasma regulatory enzyme carboxypeptidase-N inactivates or decreases the activity of the anaphylatoxin by cleaving the arginine residue.

Precise regulation is necessary to protect the host from the proinflammatory and destructive capabilities of complement. The strategy is to permit a rapid, unimpeded but tightly focused attack in time and space against foreign targets while inhibiting the "inappropriate" activation of complement on autologous cells or in plasma (fluid phase).

II. Control of the Initiation Step of the Classical Pathway

A. C1 Inhibitor

1. Introduction

The classical pathway is triggered by the interaction of IgM or IgG with antigen to form an immune complex (IC). The C1q subcomponent of C1 attaches to the Fc portion of antibody that has bound antigen (reviewed in 7,8). Subsequently, the calcium-dependent serine protease tetramer C1s–C1r–C1r–C1s, which is noncovalently bound to C1q, undergoes an as yet uncharacterized internal rearrangement such that C1r cleaves itself and then C1s, converting C1s to an active serine protease. Activated C1s then cleaves C4 to C4b. Regulation of C1r and C1s is provided by C1-Inh, a plasma protein serine protease inhibitor that blocks the active site of C1r and C1s and, in the process, dissociates them from C1q.

2. *Structure*

The isolation and characterization of C1 and its subcomponents also led to the purification and description of C1-Inh as a heat-labile plasma protein that blocked the activity of C1 (reviewed in 9). C1-Inh is a single-chain heavily glycosylated protein of 105 kDa present in plasma at a concentration of 120–200 μg/ml. It consists of 478 amino acids including a 22-residue signal peptide (10,11). The protein contains at least 30% carbohydrate content, making it one of the most heavily glycosylated proteins of plasma (10,11). Much of the oligosaccharide (7 of 13 units) consists of galactosamine-based units O-linked to serine or threonine residues. Additionally, 10 of the 13 units occur within the first 120 residues at the amino terminus.

C1-Inh is a member of the "serpin" superfamily of serine protease inhibitors, sharing homology in the region that extends from residue 120 to the carboxyl terminus. Serpins control susceptible proteases by structurally mimicking the substrate's reactive site in order to bind and trap the protease. C1-Inh shares approximately 25% homology with other serpins. Although seemingly low, this degree of homology is consistent with that observed in other members of the serpin family (9–11).

The gene for C1-Inh is located on chromosome 11 (p11.2q13) and consists of eight exons with introns containing 17 *Alu* repeats for a length of approximately 17 kb (10–12). The C1-Inh gene is unusual relative to the large number of *Alu* repeats that comprise nearly a third of the total intronic sequence.

3. *Function*

C1-Inh reacts with the catalytic site on the β chains of activated C1r and C1s. This covalent binding results in both C1r and C1s rapidly dissociating from the C1 complex, leaving C1q bound to the antibody. The dissociated complex consists of one molecule each of C1r and C1s, and two molecules of C1-Inh (13). The demonstration of the presence of this complex in plasma can be used as a specific indicator of C1 activation (14).

C1-Inh does not prevent or inhibit appropriate complement activation such as the activation of C1 by IgM or IgG on a microbial surface. Its role is to prevent fluid-phase C1 activation and excessive complement activation on a target. Within a few minutes, millions of C3b molecules can be deposited on a bacterium. This quantity provides adequate substrates for forming C5 convertases and clustered ligand for complement receptors. Once this is accomplished, there is no further need for cleavage of C4 and C2. Consequently, when IC are added to plasma, after 3 min there is no further cleavage of C4 and C2 as all of the activated C1 has combined with C1-Inh (15). Moreover, at physiologic concentrations, the rate of

reaction of C1-Inh is very rapid, with the half-life of activated fluid-phase $C1r_2$–$C1s_2$ only 10–20 sec (15).

Thus, C1-Inh surveys plasma for any active C1 complexes which it can immediately inactivate, and more slowly, but eventually with great efficiency, inhibit C1 bound to antibody. Whether this partial protection of C1 in an immune complex represents steric hindrance or involves a more complicated set of events remains to be determined. C1-Inh is the only physiologically important plasma inhibitor of activated C1r and C1s and provides a major portion of the plasma inhibition toward kallikrein and factor XIIa (16). However, C1-Inh probably is not an important inactivator of XIa or plasmin. Thus, the primary role of C1-Inh is to regulate complement and contact (kinin-forming) systems, and possibly the fibrinolytic system (9). There is no widely distributed membrane equivalent of C1-Inh, although on certain cell types there is evidence for a membrane form of the C1-Inh (17).

The critical role performed by C1-Inh in regulating C1r and C1s is exemplified by hereditary angioedema (HAE) (9,16,18). This illness is characterized by recurrent episodes of subcutaneous edema predominantly in the skin but also may manifest in the upper respiratory tract, leading to obstruction and asphyxia, or as abdominal pain due to bowel wall edema. HAE is inherited as an autosomal dominant trait. Patients have low (less than 25% of normal) to undetectable functional activity of C1-Inh. A deficiency of C1-Inh permits C1s to continuously cleave its substrates, C4 and C2, producing a secondary deficiency of these proteins. A fragment of C2 is the likely cause of the angioedema.

III. Control of C3/C5 Convertases on Host Cells

A. Overview of C3/C5 Convertase Control

During the amplification process, C3 convertases cleave C3 to C3b. The goal is to deposit large amounts of clustered C3b on the target. Activities of the C3 convertases are dependent on the association of two components, C4b with C2a in the classical pathway convertase and C3b with Bb in the alternative pathway convertase. For the classical pathway, antibody selects the target and the convertase forms predominantly on the complex. The alternative pathway, however, has no such requirement for antibody, taking more of a "shotgun" approach. Native C3 possesses a labile thioester bond which undergoes continuous, spontaneous, low-level hydrolysis. This so-called *tickover* serves as the trigger to activate C3 allowing it to become part of a convertase with factor B and properdin (6). The resultant C3 convertases generate more C3b, and, thus, more convertases (see Fig. 1). Without regulation, positive feedback continues with more C3 converted

to more C3bBb, amplifying the cycle until the native C3 is consumed. This is perhaps best illustrated in a pathologic setting following the bite of an Asiatic or Egyptian cobra. A C3b-like protein (cobra venom factor, CVF) is produced by cells lining the venom sac (reviewed in 19). After CVF enters the victim's bloodstream, it rapidly binds host factor B and triggers the activation of the alternative pathway. However, CVF is modified such that factor H cannot regulate it. As a result, the venom introduced from a cobra bite may lead to massive uncontrolled fluid-phase C3 activation followed by depletion of C3, generalized anaphylactic effects.

It is critical to ensure that convertases do not form on self tissue or in the fluid phase but are target-directed. Seven proteins are involved in regulating C3/C5 convertases: plasma proteins C4BP, factor H, and factor I, and cell-anchored proteins MCP, DAF, CR1, and CR2. One plasma protein, properdin, serves as a positive regulator that stabilizes alternative pathway convertases.

Six of these regulators (C4BP, factor H, MCP, DAF, CR1, and CR2) belong to a family of genetically, structurally, and functionally related proteins that control complement at this critical C3/C5 convertase step. Termed the Regulators of Complement Activation (RCA) family, they are tightly clustered on the long arm of chromosome 1 at q3.2 (Fig. 2). Each RCA protein is composed largely or entirely of cysteine-rich, repeating modules of approximately 60 amino acids, called complement control protein repeats (CCPRs) or short consensus repeats, that bind C3 and C4 derivatives (20). MCP and DAF possess 4 CCPRs, CR1 has 30, CR2 has 16, factor H has 20, and C4BP has 59 (21,22). CCPRs also occur in other complement proteins including C1r, C1s, C2, factor B, C6, and C7. Additionally, they have been found in many noncomplement proteins (e.g., the interleukin-2 receptor, haptoglobin, clotting factor 13b, and coagulation factor C of the horseshoe crab). Because of its apparent ease of replication and use in multiple gene products, this ancestral motif must possess a useful structure for protein–protein interactions.

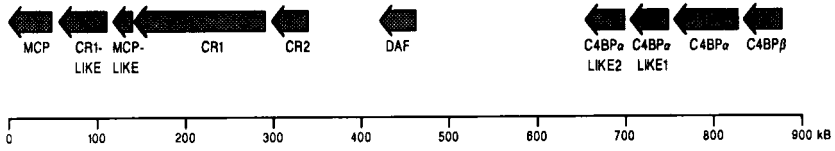


FIG. 2. The regulators of complement activation gene cluster on chromosome 1 at q3.2. Factor H (not shown) lies nearby at an undetermined distance from this complex. Abbreviations: MCP, membrane cofactor protein; CR1, complement receptor type 1; CR2, complement receptor type 2; DAF, decay-accelerating factor; C4BP, C4-binding protein (with α or β chains); "like" refers to a partial duplication of the corresponding gene. From (64).

Each CCPR exhibits a consensus of conserved amino acids including (with approximate positions) Cys2, Pro5, Tyr/Phe29, Cys31, Gly34, Cys45, Trp51, Ala/Pro56, and Cys58 (20,22). The 5th and 16th CCPRs in factor H have been individually examined by two-dimensional ^{1}H-NMR (23,24). The CCPR is folded into a double-loop structure in which Cys2 pairs with Cys45, and Cys31 pairs with Cys58 (i.e., Cys 1 + 3, and 2 + 4) (23,25). Two to 4 CCPRs are required to form a binding site.

To accomplish the goal of convertase control, two primary mechanisms are employed: (a) decay-accelerating activity (DAA), which causes the dissociation of convertase components, and (b) cofactor activity (CA), in which a regulatory protein binds C3b and/or C4b and this, in turn, permits interaction with serine protease factor I to proteolytically degrade C4b and C3b (Fig. 3). Cofactor activity may be mediated by the same protein that serves as a decay accelerator or by a different one (Table II). Interestingly, as previously noted, the plasma inhibitors possess *both* decay-accelerating and cofactor activities for either C4b (i.e., C4BP) or C3b (factor H), while membrane regulatory proteins have *either* cofactor activity for both C4b and C3b (MCP) or decay-accelerating activity for both components (DAF).

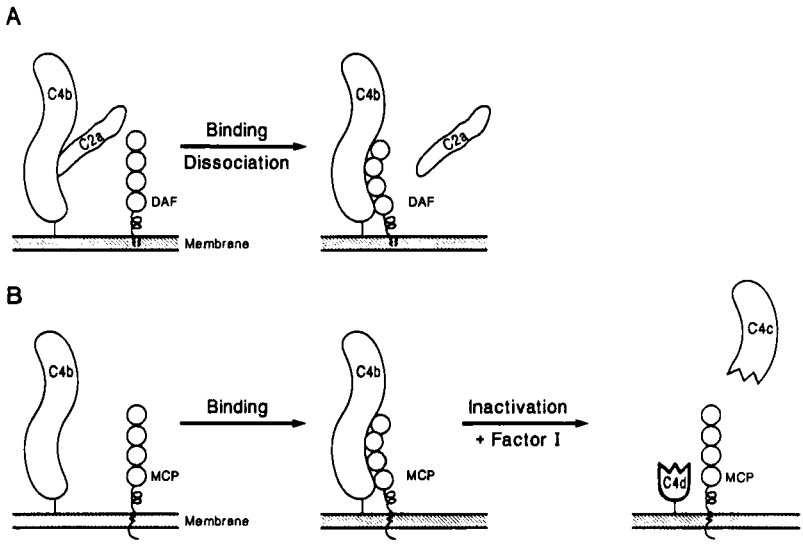


FIG. 3. Regulation of complement by decay-accelerating activity or cofactor activity. This example illustrates regulation on a membrane. Parallel activity occurs in the fluid phase and is mediated by the plasma proteins, factor H and C4BP. Decay of the classical pathway convertase (C4b2a) is mediated by DAF, while proteolytic degradation of C4b alone (or in the convertase) is performed by MCP in the presence of factor I.

TABLE II
COMPARISON OF FLUID PHASE AND MEMBRANE CONVERTASE REGULATORS

	DAA[a]	CA[b]	Substrate[c]
C4BP	+	+	C4b
Factor H	+	+	C3b
DAF	+	−	C3b/C4b
MCP	−	+	C3b/C4b
CR1	+	+	C3b/C4b

[a] DAA is decay-accelerating activity, i.e., the ability to dissociate convertases.

[b] CA is cofactor activity, i.e., the participation of the regulator with factor I to cleave and thereby inactivate C3b/C4b; C4BP and factor H are plasma proteins, while DAF, MCP, and CR1 are membrane-anchored.

[c] For DAA, C4b and C3b are part of a convertase.

The presence of an apparent "equivalent" functional repertoire in the plasma and on cell surfaces for control of C4b and C3b and the convertases containing these proteins implies several points:

1. Neither the plasma nor the membrane group of proteins alone can adequately control complement activation.
2. Control in fluid phase is primarily mediated by the plasma proteins. The concentration of the "membrane"proteins in plasma is so low that it is very unlikely that soluble CR1, DAF, or MCP play a role in the control of fluid-phase complement activation.
3. Control on surfaces is primarily mediated by membrane proteins expressed on the same surface (intrinsic protection) on which the complement activation is taking place. It is possible, though, that there is a role for factor H and C4bp. The polyvalency of C4BP strongly suggests this since such a trait would have no advantage for fluid-phase C4b. Factor H is monomeric but it does have a polyanion binding site (see below). There also are physiochemical features of particles that modulate the affinity of factor H for C3b, for example sialic acid. It should be recognized, however, that the latter studies were performed prior to the recognition of membrane regulators and the principles elucidated for a given particle could not be generalized. It is more likely that factor H and C4BP play a role in preventing excessive activation on a target (possibly analagous to the role of C1-Inh).

1. Nomenclature

The degradation of C3b and C4b follows a specific order. The activation of the parent molecule by hydrolysis or proteolysis is required before

degradation can occur (see Fig. 4). The spontaneous or induced hydrolysis of C3 and C4 produces a species termed C3i or C4i. These have not undergone proteolytic cleavage to C3b or C4b. These derivatives with an inactivated thioester group (C3i and C4i) are degraded in a manner identical to C3b or C4b.

Activation of C3 and C4 by enzymatic cleavage releases a small peptide (C3a or C4a) while the larger form (C3b, C4b) may bind to a target. Cleavage of C3b by factor I and a cofactor (C4BP, MCP, CR1) initially breaks the α chain, releasing a small fragment (C3f) and forming iC3b. Further degradation of this derivative releases the C3c fragment while the C3dg fragment remains attached to the surface. Activation of C4 by enzymatic cleavage produces the C4a peptide and the larger C4b fragment. In contrast to C3b, the initial cleavage of C4b generally releases the C4c fragment, while the C4d fragment remains attached to the target.

B. Factor I

1. Introduction

Factor I is a serine protease that mediates the proteolytic degradation of C3b, iC3b, and C4b (Fig. 5). However, factor I does not act alone. All human factor I-mediated cleavage reactions require a substrate-binding cofactor protein that promotes the binding of the enzyme. Cofactors for C3 and C4 degradation on host cells are MCP and, to a lesser extent, CR1. Cofactor proteins in plasma are C4BP and factor H.

In addition to being demonstrated in mammals, factor I has been demonstrated in both sand bass (26) and frog (27), and the products of C3 degradation indicate that an equivalent activity is present in chickens, trout, and possibly even lamprey (the most divergent species in which a functionally C3-like molecule is known) (28,29).

Factor I is constitutively active and essential for the control of both fluid-phase and cellular complement reactions. Genetic deficiency of factor I produces uncontrolled generation of C3 convertases and consequent chronic consumption of C3, resulting in permanent incapacitation of the complement-dependent effector system (30–32). This observation provided the original indication that there is continuous C3 conversion that, in the resting state, is reduced to a steady tickover by inhibitory proteins (31). Factor I is also required for the processing of surface-bound C3 and C4 derivatives, determining both their specificity for cellular receptors (CR1, CR2, CR3) and their ability to become components of convertases that amplify C3 deposition. Direct modulation of the interaction of factor I with its substrate could in principle be used to up- or down-regulate complement activation. However, the physiological mechanisms that have

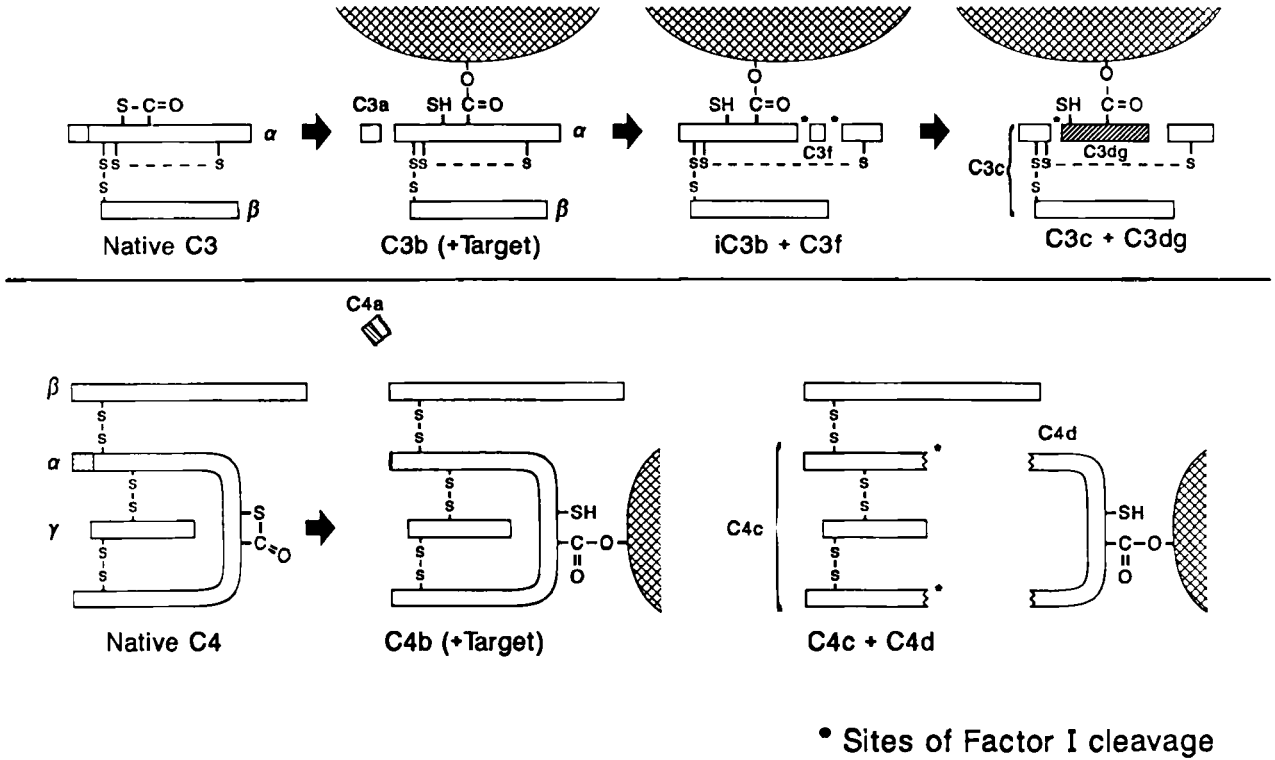


FIG. 4. Specific degradation patterns of C3b and C4b mediated by factor I and a cofactor. The examples show the initial cleavage which removes C3a and C4a to produce C3b and C4b. An identical proteolytic cleavage pattern occurs for C3 and C4 with a hydrolyzed thioester bond (C3i and C4i) but the fragments still possess the C3a and C4a domains. The cofactors, not shown above, are MCP, factor H, or CR1 for C3 and MCP, C4BP, or CR1 for C4.

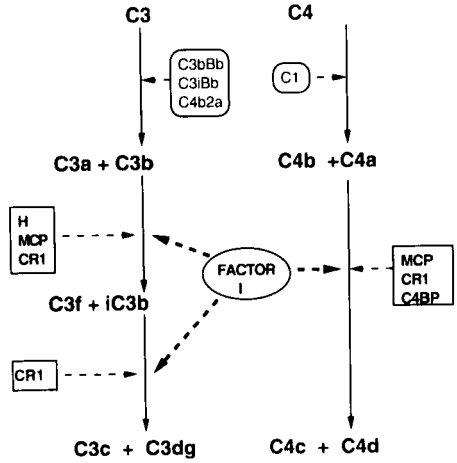


FIG. 5. Factor I-mediated cleavage of C3/C4. Factor I in the presence of a cofactor, as shown in the boxes, proteolytically cleaves C3b and C4b.

so far been described have all concerned variations in the activities or distribution of cofactors and other regulatory molecules. It is not known if any potential target surfaces have specific effects on factor I itself, or on its interaction with C3b or C4b.

2. *Structure*

Factor I is a two-chain disulfide-linked protein synthesized from a single-chain precursor (33,34). The heavy chain of about 50 kDa is derived from the amino-terminal part of the precursor and includes several different structural modules. The light chain is 38 kDa and its sequence indicates that it has the structure of a serine protease. Although factor I cannot be inhibited by conventional serine protease inhibitors, there can be little doubt that this is the enzymatic domain of the molecule. The genomic structure suggests that the relationship to trypsin is closer than that to other complement serine proteases: C2, factor B, C1r, and C1s (35). The activities mediated by the heavy chain, or its individual structural modules, have not been defined, but presumably relate to specific interactions with the substrate and/or cofactor proteins. Neutron and X-ray scattering and electron microscopy show that the complete protein has a globular structure (36,37). The dimensions indicate that the domains cannot be fully extended tandemly in a rod-like manner (36). If the structure is somehow folded back on itself, there could be direct interactions between domains that are not adjacent in primary sequence.

The heavy-chain sequence includes four units of recognizable homology: one "FI/C6/C7" unit, followed by a "CD5" module and two "LDLr" repeats (21,33.34). Finally there are about 40 carboxy-terminal residues of no known homology. There is currently no tertiary structural model for any of these presumed independently folded domains. The FI/C6/C7 unit is a segment of 67 amino acids, including 8 or 10 conserved cysteine residues. There are two tandem units at the carboxy terminus of both C6 and C7 (21). The CD5 module is about 100 amino acids long, and occurs in CD5, CD6 cell surface molecules and in macrophage scavenging receptors. The LDLr repeat of 35–40 amino acids is also found in C6 and C7, plus C8 (α and β) and C9, as well as in the LDL and α2 macroglobulin receptors.

3. Genetics and Biosynthesis

The gene for human factor I is located on chromosome 4, unlinked to the RCA gene cluster on chromosome 1 (33). It comprises 13 exons, including 1 for the FI/C6/C7 module, 1 for each LDLr repeat, 2 for the CD5 module, and 5 for the light-chain serine protease domain (35).

More than 20 cases of genetic deficiency of factor I have now been reported (30–32). They are characterized by ineffective inactivation of fluid-phase C3i and C3b, resulting in excessive C3 convertase activity and overconsumption of C3. As it cannot be degraded, the C3b persists and may interfere with the wide range of C3-dependent physiological processes. For example, there is increased association of C3b with erythrocytes (presumably bound through CR1) (32). In addition, patients exhibit increased susceptibility to bacterial infections, typical of dysfunctional alternative pathway conditions.

Factor I is synthesized by the liver (38), monocytes (39), and human umbilical vein endothelial cells (40). In the latter case, synthesis can be stimulated by interferon-γ.

4. Enzymatic Properties

The only known substrates of factor I are C3b, C4b, and their degradation intermediates. Human C3b is cleaved by factor I at Arg1303–Ser1304 (numbered according to the primary translation product) and Arg-1320–Ser1321 bonds to release the 17-amino-acid peptide, C3f, and form iC3b (41,42). This cleavage uses as cofactor factor H, MCP, or CR1. Acidic conditions (pH <6) favor preferential cleavage at only Arg1303 (43). Further cleavage of iC3b, which is normally dependent on CR1, splits the Arg954–Glu955 bond to produce C3dg (which includes the surface-binding site and is a ligand for CR2) and C3c. Cleavage of C4b to C4d occurs initially at Arg1336–Asn1337 and then at Arg956–Thr957. In all other species so far defined, factor I cleavages similarly occur carboxy-

terminal to arginine residues (29). The activities of factor I against other basic (or nonbasic) residues have not yet been reported, but the narrow substrate range indicates that target-site specificity probably depends on other areas of protein–protein interaction (possibly involving the cofactor), besides the cleavage site itself. The regulatory implications of cleavages, including the circumstances which determine whether these occur efficiently or inefficiently, depend on properties of the cofactor proteins and will be described in more detail in the appropriate subsequent sections.

C. Membrane Cofactor Protein

1. Introduction

Membrane Cofactor Protein (MCP; CD46; measles virus receptor) is a widely expressed regulator of complement activation that serves as a cofactor for the factor I-mediated degradation of C3b and C4b. Discovered a decade ago during an analysis of C3b binding proteins of human peripheral blood mononuclear cells (PBMC) (44) and originally termed gp45-70 to reflect its electrophoretic mobility, it was subsequently shown to possess cofactor activity and renamed to reflect this finding (45). During this same time several groups developed monoclonal antibodies (mAb) to an unknown 60- to 70-kDa protein that subsequently was identified as MCP (46–51).

More recently MCP was demonstrated to be the receptor for measles virus (52–55) and to be involved in the adherence of group A *Streptococcus pyogenes* to epithelial cells (56). Additionally, MCP may play a major role in reproductive biology (reviewed in 57) and, along with related proteins, in xenotransplantation as an inhibitor of complement activation (reviewed in 58).

2. Structure

A distinguishing structural characteristic of MCP on SDS–PAGE is the presence of two heterogeneous protein species of 51–58 kDa ("lower" band) and 59–68 kDa ("upper" band). Typically, PBMC and most tissues demonstrate one of three phenotypic patterns: upper band predominance in 65% of the population, approximately equal distribution in 29%, and lower band predominance in 6% (59). This phenotypic pattern is under autosomal codominant control and is associated with a *Hin*dIII RFLP (59–62). The broadness within each band results primarily from N-linked glycosylation (unpublished observation) while the distinct two-band pattern results from alternative splicing in the region of O-glycosylation (63). The latter and the alternatively spliced cytoplasmic domain produce a family of four regularly expressed MCP isoforms (reviewed in 64 and 65). While most cells in a given individual express the same ratio of all four isoforms

(63), variations have been noted in kidney, salivary gland, and brain (66). Additionally, other rarer isoforms have been noted (63,67,68).

MCP isoforms (see Fig. 6) share the identical amino-terminal portion of the protein that consists of four CCPRs, which contain the binding sites for C3b and C4b. Within the CCPR domains are four invariant cysteines (paired with each other in an arrangement that forms a double-loop structure) and 10–18 other highly conserved residues. Following this is the alternatively spliced "STP" region. Rich in serines, threonines, and prolines, this segment is extensively O-glycosylated. The STP region of MCP consists of 14 or 29 amino acids depending on the splicing in or out of STP exon B (15 amino acids). The presence of STP-B produces the higher-molecular-weight protein isoforms while its absence generates the lower-molecular-weight forms (63). "Upper" forms (BC) are more heavily O-glycosylated than "lower" (C) isoforms (69). The function of such O-glycosylation may be to protect MCP from proteolysis (70,71). Adjacent to the STP region is a juxtamembraneous segment of 12 amino acids of unknown function that is encoded by a separate exon. A transmembrane domain, intracytoplasmic anchor, and one of two distinct cytoplasmic tails (CYT-1 or CYT-2) com-

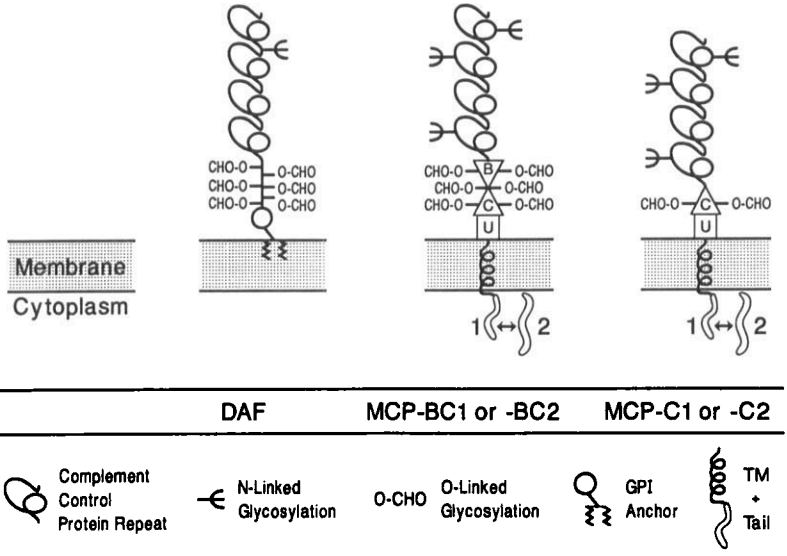


FIG. 6. Structure of DAF and the four regularly expressed isoforms of MCP. DAF and MCP are composed of four CCPRs, some of which are N-glycosylated. This is followed by an STP segment (of variable length in MCP) which is a site for O-glycosylation. In MCP this region is followed by a segment of undefined function, a transmembrane anchor, and one of two alternatively spliced cytoplasmic tails. DAF is tethered to the cell membrane by a glycosylphosphatidyl-inositol (GPI) anchor that is added post-translationally. From (64).

plete the carboxyl-terminus. Designations of the four commonly expressed isoforms are MCP-BC1, MCP-BC2, MCP-C1, and MCP-C2, where B and C refer to the STP region and 1 or 2 to the cytoplasmic tail.

The 43-kb single-copy MCP gene lies within the RCA cluster and consists of 14 exons and 13 introns (60,63,72) (see Fig. 7). Interesting features include a split exon coding for CCPR-2, closely spaced alternatively spliced STP exons, i.e., 7, 8, and 9, a large gap (13 kb) after exon 9, and the alternatively spliced cytoplasmic tails (exons 13 and 14). The promoter of MCP is found within a CG-rich region typical of "housekeeping" genes located in the first few hundred nucleotides upstream from the transcriptional start site (73). The MCP gene does not have characteristic TATA promoter sequences although numerous putative *cis*-acting regulatory elements are present (73). Additionally, an MCP-like element was found which includes sequences homologous to the 5′ end of MCP, i.e., the signal peptide and CCPRs 1–3 (63,73,74). The MCP-like genetic element is 93 and 84% homologous to MCP at the nucleotide and amino acid level, respectively. It is unknown if this partial duplication produces a protein.

3. *Biosynthesis and Tissue Distribution*

The precursors of MCP (Pro-MCP) possess three high-mannose units within the CCPRs, lack O-glycosylation, and demonstrate a M_r of 40–45 kDa (69,75). Pro-MCP is differentially processed depending upon which cytoplasmic tail is present. Precursors bearing cytoplasmic tail one (CYT-1) are processed into their mature forms approximately fourfold faster than those with CYT-2 (69,75).

The widespread expression of MCP is a logical consequence of its critical role in protecting host tissue from complement activation. MCP is expressed on almost every cell examined with the curious exception of the erythrocyte (E) (reviewed in 76). In contrast, most nonhuman primates possess MCP analogs on E (77,78). Additionally, small amounts of soluble forms have been found in plasma, tears, and seminal fluid (79–81). While most cells in a given individual express the same ratio of isoforms (63), selective expression of certain isoforms have been noted in brain, salivary gland, and kidney (66). Additionally, molecular weight variation secondary to glycosylation has been noted for MCP of granulocytes and spermatozoa (82–84).

Tissues may differ in the relative expression and the cellular distribution of MCP. The pattern of MCP expression has been examined in kidney (85–87), skin (88,89), eye (90), thyroid (91,92), and fetal (93) and adult (94) liver, at the feto–maternal interface (95,96), and during spermatogenesis (97). The biological significance of the expression patterns within a given tissue is unknown.

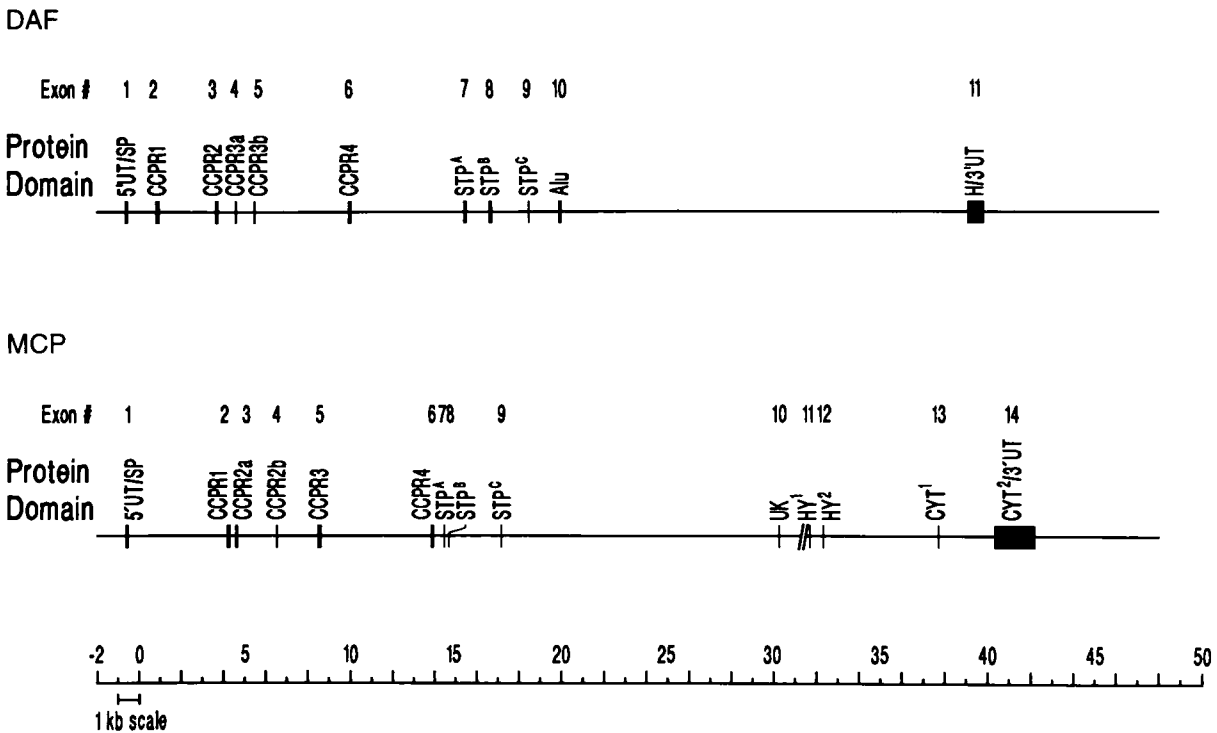


FIG. 7. Organization of MCP and DAF genes. Abbreviations for exons are: 5′UT/SP, 5′-untranslated region/signal peptide; CCPR, complement control protein repeat; STP, serine/threonine/proline-rich domain (alternatively spliced in MCP); Alu, Alu family sequence (in a minor class of mRNA); UK, unknown function; HY, membrane-spanning hydrophobic exons; CYT^1 and CYT^2/3′UT, alternatively spliced cytoplasmic tails of MCP with 3′-untranslated; H/3′UT, hydrophobic carboxy-terminal domain/3′untranslated (in DAF, carboxy-terminal amino acids replaced post-translationally with a GPI anchor). From (64).

No individuals have been found to have an MCP deficiency. Several studies have addressed MCP levels in disease states. MCP expression is increased in certain hematologic malignancies (98). Most tumor cell lines, except those of B-cell lineage, possess 2- to 8-fold more MCP in comparison with their normal counterparts (99). SV40 transformation of fetal fibroblast lines produced a 5- to 10-fold increase in MCP levels and preferential expression of the C isoforms (100). The levels of MCP were increased in glomerular capillary walls and mesangial regions of diseased kidney tissues (85) as well as in astrocytes following cytomegalovirus infection (101). Additionally, MCP levels on endothelial cells are regulated by several cytokines (102).

MCP has been quantified on PBMC, granulocytes, platelets and several cell lines. PBMC and granulocytes expressed ~10,000/cell, hemopoietic cell lines 20,000–50,000/cell, and two human epithelial carcinoma cell lines, HeLa and HEp-2, 100,000 and 250,000/cell, respectively (51,99,103).

4. Function

The wide tissue distribution, affinity for C3b and C4b, and factor I-dependent cofactor activity strongly suggested in early investigations that MCP would be a regulatory protein of the complement system (45). It was speculated and later shown that MCP is an intrinsically acting regulator; i.e., it only inactivates C3b and C4b deposited on the same cell on which MCP is expressed (104).

MCP has a complementary regulatory activity to DAF, i.e., DAF possesses decay-accelerating activity but no cofactor activity (105) (see Section IIID). As a result, MCP and DAF act jointly to inhibit C3b/C4b deposition on self tissue (104,106,107). In one system, a mAb to MCP that abrogates ligand binding and cofactor activity was incubated with DAF-negative T cell lines (107). If then exposed to normal human serum, these lines became coated with C3b via the alternative pathway. If the T cell line also was deficient in CD59, the cells were lysed. In another experimental system, Chinese hamster ovary cells and NIH-3T3 mouse embryo fibroblasts transfected with human MCP cDNA were shown to be protected from lysis by the alternative and classical pathway of human complement (104). A mAb to MCP that blocks its cofactor activity reversed this protective effect.

CCPRs contain binding sites for C3b/C4b. Based on deletion mutants and mAb binding, the third and fourth CCPRs of MCP were found to be necessary for both ligand binding and cofactor activity (103). Additionally a mutant without CCPR-2 bound C3b, but not C4b, and lacked cofactor activity. An MCP mutant deleted of CCPR-1 retained its complement inhibitory profile.

MCP isoforms bear one of two distinct tails of 16 or 23 amino acids. As mentioned, Pro-MCP with CYT-1 processed approximately fourfold faster into their mature, membrane-attached forms than those with CYT-2 (69,75). The significance of this is unknown, although the carboxyl terminus of the CYT-1 appears to dictate this differential processing (75). Additionally, the tails of MCP may be involved in signal transduction since both bear potential signals for phosphorylation (75). However, the only known physiologic role of MCP is that of complement inactivation on a cell surface. A signal need not be sent into cells to accomplish this since MCP only requires a transient interaction with C3b or C4b. Indeed, the tail is not even necessary for C3b/C4b binding and cofactor activity. In one experimental system, transmembrane and glycolipid-linked versions of MCP demonstrated equivalent efficiency in protecting cells from complement-mediated cell damage (108). However, since most cells contain both proteins, the presence of MCP and DAF is likely to have synergistic effects. This question has not been systematically examined, although a GPI-linked hybrid of MCP and DAF exerted a more protective effect than either alone (109).

MCP is most effective against C3b covalently bound to large membrane proteins and in controlling the C5 convertase (110). The C5 convertase of the alternative pathway consists of dimeric C3b (i.e., C3bBbC3b). Degradation of only one of the two C3bs to iC3b by MCP and factor I was sufficient to inactivate the convertase (110). These data demonstrate an interesting parallel between the function of DAF and MCP. DAF prevents the assembly of convertases by binding to C4b or C3b but has an even higher affinity for the convertase as it forms (C4b2a or C3bBb) (111). MCP binds monomeric C4b and C3b but acts more efficiently on a C3b dimer including the C5 convertase (C3bBbC3b) (110) and presumably for a C4b–C3b dimer as well. That MCP can bind C3b or C4b in convertases is also demonstrated by the finding that, in the absence of factor I, MCP stabilizes the alternative and, to a lesser extent, the classical pathway C3 convertases (106).

a. MCP and Reproduction. The role of MCP has expanded recently to the areas of reproductive biology, xenotransplantation, and infectious diseases. During reproduction, two "foreign" elements are introduced into the female: seminal fluid bearing spermatozoa and consequently the developing fetus. These possess foreign antigens and are at risk for rejection by the maternal immune system. The presence of MCP (as well as other regulators such as DAF and CD59) at the maternal–fetal interface and on fetal tissue may protect against maternal complement attack (reviewed in 57). Alterations in MCP function or expression also may have a bearing on habitual abortion or infertility (62). MCP (as well as DAF) is found as

a partially or completely deglycosylated species on the inner acrosomal membrane of spermatozoa (83,84,112). MCP may be part of the egg–sperm attachment mechanism and/or protect against C3b deposition during penetration by sperm (113). Regarding the latter, the protease activated during sperm penetration of the egg may be able to cleave C3. Thus, the sperm head may require MCP to inhibit convertase formation on its surface (57).

b. MCP and Transplantation. In transplant biology, MCP (as well as DAF and CD59) is being engineered to produce transgenic pigs whose organs will be tested in xenotransplantation (reviewed in 58,114). Each year in the United States nearly 43,000 patients with heart failure are denied potentially life-saving transplants due to organ shortage (115). While the transplantation of primate organs has been attempted in a very limited way, the supply, cost, and ethical issues prevent this strategy from being suitable on a large scale. However, use of transgenic pigs whose tissue, especially the endothelium, bears one or more complement regulators could abrogate acute rejection mediated by antibody and complement (116). Additionally, soluble forms of the same regulators and CR1 are being examined for their ability to inhibit complement activation in human autoimmune disease and in reperfusion injury (58,117,118).

c. MCP and Measles Infection. Complement regulatory proteins are involved in the pathogenesis of an increasing number of infectious diseases (reviewed in 119). Their abuse by microorganisms takes two general forms. In the first, the host proteins are used as microbial receptors while in the second the pathogen synthesizes proteins that mimic the regulatory activity of the host protein. Recently, MCP was found to be the receptor for measles virus (MV) (52–55). Despite the introduction of measles vaccines in the 1970s, measles still afflicts an estimated 44 million individuals annually, killing 1.5 million. In developing countries, it is the number one killer of young children (120). Several groups identified MCP as the MV receptor. The receptor was isolated using a monoclonal antibody that blocks MV binding to human cells. Following cloning, the expressed protein bound MV and produced syncytia (52). A second team mapped the receptor to chromosome 1, produced cDNA transfectants permissive to MV, and used blocking polyclonal antibody to confirm MCP as the receptor (53). Transfected rodent cells (nonpermissive for MV) with each of the four primary MCP cDNAs bound MV and produced syncytia (54). Interestingly, cell surface MCP is down-regulated following infection (121). That the tail of MCP is *not* involved in bindng of MV was shown by a glycolipid anchored version of MCP that was permissive to MV infection (122,123). The MV binding site appears to be largely distinct from the C3b/C4b binding sites

(122). Chimeric proteins exchanging the four extracellular CCPRs between MCP and DAF indicated that only molecules with both CCPRs-1 and -2 of MCP allowed a productive MV infection. Monoclonal antibodies against either CCPR-1 or -2 of MCP blocked MV infection (122). Additionally, the binding of MV was abolished after enzymatic release of N-linked (but not O-linked) oligosaccharides (124). Since both CCPR-1 and -2 of MCP have N-glycans, they may be part of the receptor determinant or alter the conformation of the site.

d. MCP and Streptococcus. MCP also serves as a receptor for *Streptococcus pyogenes* (group A streptococcus) (56). This gram-positive bacterium is an important human pathogen that causes a number of serious suppurative and inflammatory infections of the skin (e.g., impetigo, erysipelas, cellulitis, and necrotizing fasciitis) and of the throat (pharyngitis). Additionally, postinfection sequelae include rheumatic fever and acute glomerulonephritis. The M protein of *S. pyogenes* mediates adherence to keratinocytes, the most numerous cell type in the epidermis. *Streptococcus pyogenes* previously had been shown to bind factor H to the bacterial surface where it presumably serves as a cofactor to inactivate C3b and down-regulate complement (125,126). Considering the possibility that a common mechanism underlies factor H binding, a series of C repeat mutants was developed and found to be responsible for recognition of MCP as well as factor H (56). The binding of factor H to the C repeat region of M protein blocked bacterial adherence. Additionally, purified MCP could competitively inhibit the adherence of *S. pyogenes* to keratinocytes. Finally, the M protein was found to directly bind MCP, whereas mutant M proteins lacking the C repeat domain did not bind MCP. A model was proposed that, following adherence of streptococci, the inflammatory response causes influx of plasma factor H which then competes with MCP for bacterial binding. Moreover, factor H-coated bacteria subsequently block activation of the alternative pathway on the bacterial surface. These two processes would promote bacterial adherence and then spreading, yet use only one region within the organism. This could explain the tendency of streptococci to spread in skin (cellulitis) and disseminate into deeper tissues (56).

e. MCP and AIDS. In addition to synthesizing proteins that mimic the inhibitory profile of the host proteins, viruses such as HIV coat themselves with the host's own inhibitors in order to gain protection from complement-mediated attack (127,128). It is likely that viruses have evolved mechanisms to select a host's membrane or intracellular proteins that provide a survival advantage.

D. Decay-Accelerating Factor (DAF, CD55)

1. Initial Identification and Purification

The first identification of membrane factors that could regulate complement activation came from Hoffmann and colleagues in 1969. They made butanol extracts of human erythrocyte stroma and found that a substance remaining in the aqueous phase could inhibit the hemolysis of sheep erythrocytes by antibody and complement (129). Furthermore, this activity could be separated based on high- and low-salt extractions, with one component causing inhibition at the EAC14b2a step (130). It is now known that the human erythrocyte possesses three complement regulatory proteins, namely DAF, CR1, and CD59, and several of these proteins were probably contained in the erythrocyte extracts.

DAF was purified by Nicholson-Weller and colleagues in 1981 using a butanol extraction step as did Hoffmann. They purified DAF from guinea pig (131) and then human (132) erythrocytes by use of sequential chromatography on DEAE-Sephacel, hydroxylapatite, phenyl-Sepharose, and trypan blue–Sepharose, following the material through its activity of accelerating the decay of the classical pathway C3 convertase. The purified DAF was a single-chain glycoprotein with a M_r of 60,000 (guinea pig) or 70,000 (human) on SDS–PAGE.

2. Structure of DAF

a. Peptide Sequence. The structure of DAF has been elucidated by a combination of biochemical studies and by the molecular cloning of cDNA [additional details and references on DAF can be found in two earlier comprehensive reviews (133,134)]. The cDNA for human DAF encodes a 34-amino acid signal peptide followed by a 347-amino acid sequence of the protein (135,136) (see Fig. 6). The amino terminus of the protein consists of four CCPR domains (137). This region is followed by a 67-amino acid stretch composed of 43% serine and threonine residues. This serine/threonine-rich region is heavily modified by O-linked glycosylation (see below). The carboxyl terminus consists of a hydrophobic domain typical of transmembrane domains, although there is no cytoplasmic tail segment. This hydrophobic domain is removed for attachment of a glycosylphosphatidylinositol (GPI) anchor post-translationally (see below).

b. Structure of DAF Gene. The DAF cDNA (mRNA) is transcribed from the human DAF gene spanning 40 kb (138) in the RCA cluster (139) (see Fig. 2). There are 10 exons, with CCPR domains 1, 2, and 4 encoded on single exons and CCPR3 encoded on 2 exons (Figure 7). A minor species of DAF mRNA (136) arises from alternate splicing of an additional

exon located within the last intron (138), but there is no evidence to date demonstrating a corresponding protein species being expressed *in vivo* (133). The promoter region of the DAF gene does not have any typical TATA or CAAT boxes, but there are conserved sequences corresponding to Sp1 binding sites, AP-1 and AP-2 elements, and cAMP-responsive elements (140,141).

c. Glycosylation of DAF. DAF is modified by one N-linked, complex-type oligosaccharide unit (142) which contributes 4000 to the M_r of the protein and is attached to the asparagine residue 61. The protein is extensively modified by O-linked oligosaccharides (142), which are all attached to the serine/threonine-rich region (71). These O-linked oligosaccharide side chains account for an apparent ~26,000 of the M_r of DAF, with two-thirds of this amount due to sialic acid. The N-linked oligosaccharide is added cotranslationally in the endoplasmic reticulum, whereas the O-linked oligosaccharides are attached post-translationally in the Golgi apparatus. Thus, DAF is initially synthesized as a precursor of 46,000 M_r [very brief pulses with radiolabeled amino acids also demonstrate an earlier DAF precursor form of 43,000 M_r, but the exact biochemical structure is not known (142)], which gives rise to the mature DAF on the cell surface with a M_r of ~70,000 to 80,000 due to heterogeneity in glycosylation.

d. GPI Anchor of DAF. DAF is anchored through covalent attachment to a GPI anchor (143,144), a property shared with the complement regulatory protein CD59. The GPI anchor forms a stable membrane attachment, with the fatty acids of the anchor inserted in the outer leaflet of the membrane bilayer (Fig. 3). Investigation of the possible GPI anchoring of DAF was prompted by the observation that purified DAF could reincorporate into cell membranes as a functional membrane protein (145). Studies demonstrated the GPI anchoring of DAF based on the ability to release DAF from the cell surface with the enzyme PI-specific phospholipase C (PI-PLC). Detailed biochemical characterization confirmed the GPI anchor and showed that the anchor contained ethanolamine, glucosamine, mannose, inositol, and saturated and unsaturated fatty acids (144). The GPI anchor is added as an early post-translational modification in the endoplasmic reticulum, with the removal of the 28 carboxyl-terminal amino acids and their replacement with the preformed GPI anchor attached covalently to Ser319 (146).

e. Alternate Forms of DAF. Several alternate forms of DAF of different M_r have been described in tissues, including a high M_r form (147) that represents a crosslinked DAF homodimer (77). In addition, soluble forms

are found in body fluids (148). These soluble forms might arise from membrane forms by the action of a phospholipase or a protease, but there are no data to directly implicate the source of soluble DAF. An earlier suggestion that an alternatively spliced form of DAF (136) might account for soluble DAF has not been supported by subsequent analysis (133).

DAF protein has been purified from human (132), guinea pig (131), mouse (149), and rabbit (150). DAF has been cloned from human (135,136), rhesus monkey (D. M. Lublin, unpublished data), orangutan (77), and mouse (151). Interestingly, in the mouse there are two DAF genes that are closely linked on chromosome 1. They encode a GPI-anchored and a transmembrane form of DAF. The GPI-anchored form is the predominant form expressed in mouse tissues (151).

3. *Function of DAF*

a. Mechanism of Action. DAF was initially purified based on its ability to accelerate the decay of the classical pathway C3 convertase, C4b2a (131). It carries out the same function with respect to the alternative pathway C3 convertase, C3bBb, but does not have any cofactor activity for the factor I-mediated proteolytic degradation of C3b or C4b (152). It was found that purified DAF could be reincorporated into erythrocyte membranes (a property later ascribed to the GPI anchor of DAF) and prevent complement-mediated hemolysis by inhibiting the C3 and C5 convertases (145). Detailed studies following radiolabeled C2 showed that DAF does not inhibit the initial binding of C2 to a cell with deposited C4b, but that DAF leads to the rapid release of C2a from its binding site on C4b, thus dissociating the C3 convertase (111). Similar results held for the release of Bb from the alternative pathway C3 convertase.

The exact binding site of DAF on the convertase is not clear. Experiments using homobifunctional crosslinking reagents demonstrated an endogenous association of DAF with C4b and C3b on the cell surface, not requiring C2 or factor B (153). Indeed, for all other members of the RCA family, the CCPR domains are responsible for binding to C3b or C4b. However, measurements by fluid-phase inhibition indicate that the binding of DAF to the C3 convertase is much greater than that to C3b or C4b alone (154). Since there are a limited number of DAF molecules on the cell surface, it would be advantageous if the binding of DAF to the C3 convertase was lowered following dissociation of C2a or Bb, since then the DAF would be able to bind to and dissociate another intact C3 convertase.

The complement protective function of membrane DAF on cells is only exerted intrinsically, i.e., on C3 convertase assembled on the same cell as DAF (145). Soluble DAF (which is found in body fluids and can be produced *in vitro* by treatment of cells with PIPLC) cannot incorporate

into cell membranes, and it has a much lower efficiency than membrane DAF for inhibiting complement activation on cell surfaces (144).

b. Physiological Role of DAF. The above data demonstrate the basic molecular mechanism for the protective function of DAF. The question is how that function is translated into a physiologically relevant role in the organism. Related to this issue is the fact that there are multiple members of the RCA gene family, all of which can inhibit the complement cascade; thus, the question arises as to what are the relative and possibly overlapping roles of each protein in the organism. There are several approaches to these questions and all indicate that DAF plays a physiologically relevant role in protecting tissues from damage by the complement system. However, the results from different systems leave somewhat unsettled the exact importance of DAF relative to other RCA family members.

DAF is expressed on essentially all hematopoietic cells and on endothelial and epithelial tissues, including the vascular endothelium, gastrointestinal tract, genitourinary tract, central nervous system, and extracellular matrix (148,155). DAF is highly expressed in the placenta at the feto–maternal interface (156) and is also present in plasma and body fluids as a soluble form (148). Overall, this wide tissue distribution of DAF is consistent with its playing an important role in complement protection for the organism.

Blocking of DAF function on human cells and cell lines by polyclonal or monoclonal antibodies confirms that DAF protects the cell from complement-mediated lysis at the C3 convertase step; cells whose DAF function is blocked show increased deposition of C3b and increased cytotoxicity following complement activation (152,157,158). Furthermore, expression of human DAF by transfection in animal cell lines results in decreased C3b deposition and decreased cytotoxicity, thus protecting those cells from complement-mediated damage (108,159). Similar experiments with cells transfected with MCP or CD59 likewise show that these molecules can also block complement-mediated cytotoxicity (108,159–161). Hence, the key physiological question is how DAF fits in with these different RCA proteins to provide separate or overlapping complement protection.

Several lines of evidence suggest that the relative quantitative order of importance is CD59 > DAF > MCP, but it should be kept in mind that all of these studies have limitations that do not allow a complete resolution of the problem. Using cytotoxicity as an end point, antibody blockade of individual RCA proteins demonstrates that CD59 has the greatest influence, with DAF having a lesser effect and MCP the smallest (157,158), although interpretation of these results depends on the expression levels of these RCA proteins on the particular cell being studied. Studies of hemolysis of erythrocytes from PNH cells, which lack both DAF and CD59

as well as other GPI-anchored proteins, also suggest that CD59 exerts the greater quantitative effect (157). However, *in vivo* the C3 and C5 convertases generate soluble fragments C3a and C5a with anaphylatoxic and chemotactic properties, and C3b deposited on the cell surface can lead to binding by phagocytic cells with complement receptors; these effects are not measured by *in vitro* cytotoxicity assays.

Analysis of rare individuals who are genetically deficient in DAF or CD59 due to a specific mutation in that gene (and not due to a lack of GPI anchor synthesis) indicates that only in the CD59-deficient case is there a PNH syndrome including hemolytic anemia (162), whereas the DAF-deficient individuals [who constitute the Inab phenotype of the Cromer blood group carried on the DAF molecule (163)], do not have any apparent hemolytic anemia, although they do have a mild increased complement sensitivity when measured *in vitro* (164). On the other hand, incorporation of purified human DAF or CD59 into animal erythrocytes and testing with human complement shows that DAF has a greater protective effect than CD59 in some cases depending on the animal species being tested (165).

Overall, it is fair to state that DAF can exert an important level of protection from complement-mediated damage, but that the exact interaction of the different RCA proteins in the total scheme of complement protection is not yet clear and might vary in different tissues and species.

c. Mapping of Functional Domains in DAF. Studies of mutant DAF molecules in which each of the CCPR domains has been deleted indicate that CCPR2, 3, and 4 are all required for function, as judged by the ability of the transfected DAF mutant to protect cells from C3b deposition and cytotoxicity (71). Studies of functional blockade by monoclonal antibodies has indicated that only antibodies targeted to SCR3 exert a complete functional block, suggesting that this region is most critical to function (71), although CCPR2 and CCPR4 must also contribute based on the deletion mutant data.

DAF mutants lacking the single N-linked oligosaccharide functioned normally. Analysis of the serine/threonine-rich region of DAF and its extensive O-linked glycosylation was more complex. Deletion of this region abrogated function, but this could be reversed by replacement of this region with a major segment of the HLA-B44 protein (77). This suggests that the serine/threonine-rich region is serving as a nonspecific spacer arm to project the functional SCR2-4 region out from the cell surface. Interestingly, when DAF is expressed in a mutant Chinese hamster ovary cell line that lacks the ability to add O-linked oligosaccharides, the protein is rapidly degraded, leading to very low surface expression (70). The overall

picture of the serine/threonine-rich region is that it forms a rigid rod which projects the functional SCR2-4 domain above the cell surface (past the glycocalyx region), and the O-linked oligosaccharides protect this exposed peptide segment from proteases.

Finally, the role of the GPI anchor of DAF, notably also a feature of CD59, has been investigated by construction of an artificial transmembrane (TM) form of DAF, made by replacing the GPI anchoring signal at the carboxyl terminus with a TM and cytoplasmic domain. Studies of complement protection demonstrated that the GPI and TM forms protected cells equally well (108). This was unexpected, since biophysical measurements had shown that DAF has a very high lateral mobility in the plane of the membrane, a property that would be expected to contribute to its function (166). However, this apparent discrepancy was clarified by measurements of the lateral mobilities of several pairs of proteins expressed in both GPI and TM form, showing that the GPI anchor only contributed marginally to the lateral mobility (167) (however, other cytoplasmic tails that attach the protein to the cytoskeleton can cause a major decrease in lateral mobility).

The GPI anchor was critical to the ability of DAF to cause T cell activation following crosslinking with antibody (168,169). Recent investigations have shown that this arises because the GPI anchor of DAF targets it to plasma membrane domains that are enriched in glycosphingolipids and cholesterol (and resistant to extraction with the nonionic detergent Triton X-100), and that this leads to a noncovalent association with the src family protein tyrosine $p56^{lck}$ and $p59^{fyn}$ (169,170), which appear to be part of the signaling pathway. It is not established whether binding of DAF by C3b, C4b, or C3 convertases can trigger the same activation pathway as antibody crosslinking.

d. DAF as a Microorganism Receptor. DAF also serves in a pathophysiological role. It has been identified as the receptor for certain strains of *Escherichia coli* (171). Binding of the *E. coli* adhesins of the Dr family to DAF could serve as a virulence factor in urinary tract infection. Several enteroviruses, including types of echovirus (172,173) and coxsackie B virus (174), have also been shown to bind to cells through DAF.

e. Conclusions. Biochemical, functional, and molecular biological studies have given a clear picture of the structure and function of DAF. Building on this base, recent investigations have demonstrated the potential for using recombinant DAF as a therapeutic agent to block inflammatory processes. Preliminary studies have shown that purified DAF is able to block inflammatory reactions (118) and to block steps that underlie the process of hyperacute rejection in xenotransplants (116,175). Future work

will focus on extending these exciting initial therapeutic results, and continue to refine our understanding of the precise physiological role of DAF in protection of the individual organism from complement-mediated damage.

E. Complement Receptors as Regulators

1. Introduction

Complement receptors type one (CR1; C3b/C4b receptor; immune adherence receptor; CD35) and type two (CR2; C3d receptor; EBV receptor; CD21) interact with derivatives of C3 (CR1 and CR2) and/or C4 (CR1) in immune complexes (IC) (20). For CR1, such attachment facilitates both complement regulation and immune complex processing. CR2 binds only to C3b-derived ligands that have been degraded by other regulators. Thus, the primary ligands for CR2 are C3dg and, to a lesser extent, iC3b. CR2 probably plays only a minor role in complement regulation but likely a major role in the antigen-dependent activation of B lymphocytes.

2. CR1 Structure/Distribution

CR1 is a polymorphic membrane protein of 190–280 kDa that is present on all peripheral blood cells except platelets (reviewed in 176). The erythrocyte expresses 100 to 400 copies/cell while leukocytes have 10–50,000/cell. However, since there are approximately 1000 times more erythrocytes than other vascular cells, erythrocytes possess more than 85% of total CR1 in blood. The most common form of CR1 consists of 30 CCPRs with three sites for C4b and two sites for C3b binding (177–179). The presence of these distinct ligand recognition sites suggests that each receptor molecule can interact multivalently with complexes containing multiple (clustered) C4b and C3b molecules.

CR1 is composed of 2039 residues including a 41-amino-acid signal peptide, an extracellular domain of 1930 residues, a 25-amino-acid transmembrane region, and a 43-amino-acid cytoplasmic domain. CR1 has an interesting structural arrangement in which contiguous blocks of every seven CCPRs form a larger highly homologous repeating array called a long homologous repeat (LHR). Thus, the extracellular domain of the common form of CR1 is composed of four LHRs (a total of 28 CCPRs) with 2 additional CCPRs. Ligand binding sites are in each of the first three LHRs. It is unlikely that this arrangement has arisen by chance, suggesting that this spacing of 7 SCRs before the next binding site facilitates interactions with clusters of ligand on a target.

3. Function

Clusters of C3b and C4b on target serve as ligands for CR1. Although CR1 possesses both decay-accelerating and cofactor activities, its limited

tissue distribution indicates that its regulatory action is directed at reducing complement activity once C3b/C4b bearing IC have become adherent to cells. This would prevent excessive and unnecessary complement activation on IC especially with cells bearing IC in plasma or at tissue sites. Further, complement regulatory activity of CR1 converts to C3b to iC3b which is a ligand for complement receptor type 3 (CR3; CD11b/18) and to C3dg for uptake by CR2. Additionally, another benefit of the regulatory abilities of CR1 is the release of receptor-bound IC from erythrocytes (180). Regarding the latter, CR1 of erythrocytes serves as a taxi that, following binding of IC, carries the complex to the spleen for processing by immune cells or to the liver for elimination (reviewed in 181,182). The conversion of C3b to iC3b and then to C3dg facilitates the transfer from a CR1-bearing erythrocyte to a hepatic macrophage bearing CR3. Likewise, a mixture of C3b, iC3b, and C3dg on an antigen might be an ideal combination to locate and process immune complexes in the follicular area of a lymphoid follicle since B lymphocytes possess all three complement receptors and monocyte/macrophages possess CR1 and CR3.

As noted, it is unlikely that CR1 has a major role in regulating complement activation in the fluid phase or on host tissue. The limited tissue distribution, especially when compared with DAF, MCP, and CD59, and its very low plasma concentration, 1000- to 10,000-fold less than that of factor H or C4BP, support this conclusion. On the other hand, in contrast to DAF and MCP, CR1 can bind to C3b/C4b-bearing complexes in its environment (i.e., IC *not* attached to the same cell expressing CR1). Therefore, there is the potential for CR1 on an erythrocyte to inhibit complement activation on a nearby cell or tissue (extrinsic protection) whereas MCP and DAF are intrinsic regulators (i.e., they only protect the cell on which they are anchored). At sites of inflammation CR1 could serve as an extrinsic regulator of complement activation on host tissue. The importance of CR1 as an intrinsic regulator is less clear, although if transfected into rodent cells it can be shown to protect against complement activation (183).

Recently, a soluble, recombinant form of CR1 (sCR1) (lacking the transmembrane and cytoplasmic region) was produced and tested for use as a complement inhibitor in complement-dependent animal models of tissue injury (184,185). Infusion of sCR1 blocked several types of inflammatory and vasculitic destructive processes that were complement-dependent as would have been predicted based on similar experiments utilizing complement-deficient animals. Surprisingly, though, complement inhibition by sCR1 also reduced infarct size in two animal model systems of myocardial infarction and in several other models of reperfusion injury. These remarkable results suggest that complement plays a role in removal of dead and dying tissue. This activity was likely designed to promote

healing of cutaneous injury but could be undesirable for partially damaged myocardial tissue.

4. Complement Receptor Type Two (CR2; CD21)

CR2 is a 145-kDa protein that binds to C3dg and iC3b and is expressed on B lymphocytes, follicular-dendritic cells, some epithelial cells, and, in lesser amounts, on peripheral blood T cells (176). It is composed of 16 CCPRs with a ligand-binding site in the first two CCPRs. CR2 serves primarily to localize complement-bearing immune complexes to B-lymphocyte-rich areas of the spleen and lymph nodes in order to promote antigen-driven activation of B cells (186). CR2 is also the receptor for the Epstein–Barr virus (EBV) which possesses a membrane protein with structural similarities to C3dg that binds to an overlapping region of CCPR-1 and -2 (186–188). CR2 may have a minor role in regulating complement since it serves as a relatively weak cofactor for the factor I-mediated cleavage of membrane-bound iC3b to C3dg and C3c (189). The purpose of this activity could be to facilitate localization of an IC in the spleen or lymph nodes.

III. Control of Fluid-Phase C3/C5 Convertases

A. Factor H

1. Introduction

Factor H is a soluble glycoprotein present in normal plasma at concentrations of 0.3–0.5 mg/ml (190,191). Its primary function is to control the fluid-phase tickover of the alternative pathway, as its absence, either *in vitro* (when immunochemically depleted from serum) or *in vivo* [genetic deficiency (192)], results in uncontrolled amplification of C3 conversion until no active C3 remains. Regulation depends on three key activities of factor H: (i) binding to C3b (or C3i), (ii) displacement of Bb from C3bBb (or C3iBb), and (iii) cofactor activity for the factor I-mediated cleavage of C3b (and C3i). These activities occur both in the fluid phase and on surface-bound C3b, but the effectiveness of factor H is reduced on many surfaces. Protection from factor H is a determining factor for triggering alternative pathway activation. In addition to suppressing the alternative pathway-mediated amplification cycle, factor H also inhibits responses to the classical activation pathway by acting on C3b, which is the critical product of both pathways.

2. Structure

a. Basic Structure. Factor H has a single polypeptide chain of molecular weight of 150 kDa. Hydrodynamic data indicate a highly elongated shape,

and electron migrographs reveal an approximately 50 × 3.4-nm chain, bent and twisted in a variety of different ways (37). Most molecules are seen to be folded in the middle, often bringing the two ends close together. X-ray and neutron diffraction studies, performed at high protein concentrations, fit a similar folded rod shape, but imply a larger and apparently dimeric structure (193). However, diffusion and sedimentation coefficients support a monomeric model (37).

The human cDNA-derived amino acid sequence shows that the mature polypeptide of 1213 amino acids consists entirely of 20 contiguous short consensus repeat (CCPR) units (194) (see below). The complete sequence of the equivalent mouse protein is 61% identical to human factor H (195).

b. Functional Sites. Electron micrographs show that C3b binds to one end of the factor H molecule (37). An isolated 38-kDa tryptic fragment encompassing the amino-terminal CCPRs 1–5 possesses some affinity for C3b, as well as cofactor activity (196). Furthermore, the product of a short transcript of the human factor H gene, consisting only of CCPRs 1–7, also displays both C3b-binding and cofactor activities (197). The binding site for C3b is therefore likely to be close to the amino-terminal end of the molecule.

3. *Genetics*

The gene for human factor H is tightly linked to that for the homologous b subunit of coagulation factor XIII and these genes are close to the RCA gene cluster on chromosome 1 (20). The factor H-related genes, described below, are also closely linked (198). The murine chromosome 1 carries a cluster of genes of its own RCA molecules including factor H and coagulation factor XIIIb. Mouse factor H is encoded in 22 exons, including one for each CCPR, except for CCPR 2, whose coding sequence is split between two exons at the same position (Gly34 in the consensus sequence) as the split found in other CCPR-encoding exons of RCA proteins (199).

Genetic deficiency of factor H is rare in humans. The result is uncontrolled complement activation producing depletion of plasma C3 (32,192). This also occurs in a factor H deficiency found in pigs (200). In both species massive renal deposition of complement products is associated with consequent membranoproliferative glomerulonephritis. A number of polymorphisms have been described, but these have not been linked to any functional variation.

4. *Biosynthesis*

Factor H is synthesized by several tissues, including monocytes (39), skin fibroblasts (201), and liver and kidney (202). Synthesis by mouse

L cells can be induced by interferon-γ and dexamethasone (202). Interferon-γ also stimulates secretion of factor H by human umbilical vein endothelial cells, whereas interleukin-1 is suppressive (203). As yet there is no clear picture as to how these *in vitro* effects modulate the physiological functioning of factor H.

5. *Functions*

a. Factor H and Tickover of the Alternative Pathway. Slow spontaneous hydrolysis of the thioester in C3 produces C3i. Combination with factor B and cleavage of the C3iB by factor D can then generate an active C3 convertase, C3iBb. This bimolecular enzyme catalyzes the cleavage of native C3 into C3b which, like C3i, can react with factors B and D to produce more C3 convertases (C3bBb). Without regulation, positive feedback continues, with more C3 converted to more C3bBb, amplifying the cycle until all the native C3 is consumed. This amplification cycle is shown schematically in Fig. 8. Factor H prevents positive feedback in the fluid phase and reduces the cycle to tickover. Some C3 convertases form but are rapidly inactivated. Small amounts of nascent C3b are generated and

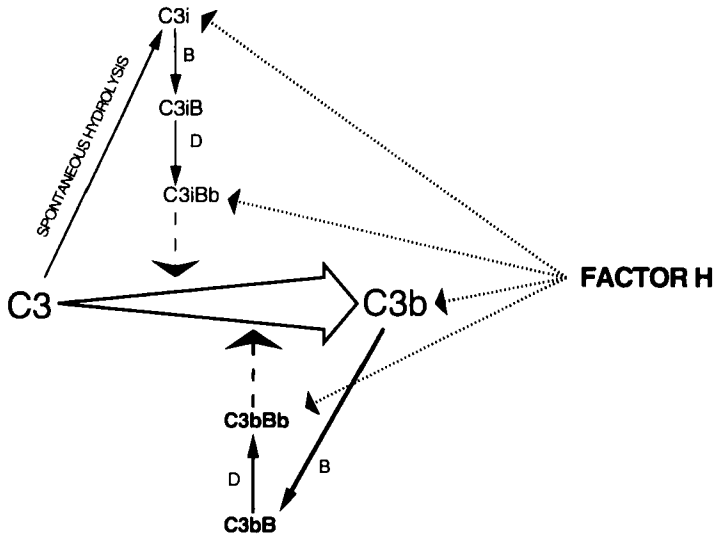


FIG. 8. Control of the alternative pathway C3 tickover by factor H. Serum C3 undergoes spontaneous hydrolysis (upper part of figure) at a low but continual level due to a labile thioester bond in its α chain. This tickover-activated C3 may also form convertases and lead to a feedback amplification (lower part of figure). To prevent inappropriate activation, such as in the fluid phase, factor H (with factor I) binds and degrades C3b and C3i alone or in the convertase.

some deposit randomly onto available surfaces. Regulation by factor H permits active C3 and B to survive in plasma and hence to be able to respond with amplified C3 conversion at an appropriate site, such as a pathogenic cell surface. There are three activities of factor H crucial to this regulatory role:

(i)	Binding:	C3b (or C3i) + H → C3bH (or C3iH)
(ii)	Decay acceleration:	C3bBb (or C3iBb) + H → Bb + C3bH (or C3iH)
(iii)	Cofactor activity:	C3bH (or C3iH) + I → iC3b (or iC3i) + C3f + H + I

b. Binding: C3b (or C3i) + H → C3bH (or C3iH). The affinity of factor H for fluid-phase C3b has been measured at $1.6 \times 10^6 M^{-1}$ at 37°C, but can be considerably less on surface-bound C3b (204). The corresponding K_d of $6 \times 10^{-7} M$ compares with a fluid-phase concentration of approximately $2.5–5 \times 10^{-6} M$, so lower concentrations of C3b will be readily saturated by factor H. The binding of factor H to C3b (i) blocks the binding of factor B and the consequent generation of a C3 convertase enzyme (205); (ii) blocks interaction with CR1 and CR2, preventing undesirable interference of fluid-phase components with the recognition of specifically C3b-coated particles (154,206); and (iii) inhibits the binding of C5 that could lead to the unwanted production of membrane attack complexes (205). Factor H does not inhibit the binding of properdin (205).

Attempts have been made to define the binding site for factor H on C3b using synthetic peptides corresponding to regions of C3 sequence. Two regions on the α' chain have been implicated, covering residues 767–776 and 1209–1271 (numbered according to the primary translation product) (207,208) but supporting evidence, for example by mutagenesis, is presently lacking.

c. Decay Acceleration: C3bBb (or C3iBb) + H → Bb + C3bH (or C3iH). Factor H binds to C3bBb with an affinity similar to that for C3b alone (154). In doing so, it irreversibly displaces the Bb component and thereby destroys the active C3 convertase. Properdin stabilizes C3bBb, but is ineffective at providing protection from factor H action in the fluid phase (209). The membrane proteins DAF and CR1 will similarly dissociate C3bBb on the cell surface. The mechanism either may be mediated by an allosteric effect on the C3b conformation, or could involve a specific interaction between Bb and the displacing molecule (154).

d. Cofactor Activity: C3bH (or C3iH) + I → iC3b (or iC3i) + C3f + H + I. Factor H, MCP, and CR1 are all cofactors for the inactiva-

tion of C3b (and C3i) by the protease factor I. Factor H greatly increases the affinity of factor I for C3b (210), possibly due to a direct contact between the cofactor and factor I, as suggested by evidence of species incompatibilities in this interaction (206). At present, it is unclear from mutagenesis studies on MCP and CR1 whether it may be possible to separate the cofactor activity from the C3b-binding site (103,177). The high affinity accounts for the very low K_m ($0.25–1 \times 10^{-7} M$ at 25°C) of factor I for C3b in the presence of factor H (211). The consequence is that factor I in combination with factor H is very efficient at scavenging and inactivating the low concentrations of C3b and C3i formed when the system is ticking over. However, the low enzymatic turnover rate (<10 min^{-1} at 25°C) means that this regulatory system is unable to stop amplification when fast C3b generation has already started at a site of activation.

The conversion of C3b to iC3b has two important consequences in the fluid phase: (i) it prevents the reformation of C3 convertase enzyme C3bBb, and (ii) it prevents the binding of C5 and consequent generation of membrane attack complexes. When surface bound C3b is converted to iC3b there are additional consequences: (i) iC3b is a ligand for CR3, promoting phagocytosis by macrophages, etc., and (ii) iC3b can be further cleaved by factor I, in the presence of CR1, into C3c, which is released, and C3dg, which remains bound and is a ligand for CR2 on B cells. At low ionic strength factor H binds to iC3b, and can then also act as a cofactor for the further cleavage to C3dg + C3c.

d. Influence of Surfaces on Factor H Interaction with C3b. The nature of a surface to which C3b is bound has important effects on the affinity of the interaction with factor H. When factor H binding is impaired, surface-bound C3bBb complexes have the potential to form more readily, to persist longer, and to generate more C3b around that area such that complement-mediated immune response mechanisms are triggered. The alternative pathway is activated at surfaces on which C3b is well protected from factor H (210,212). Carbohydrate-rich polymers, such as the cell walls of yeast and many bacteria, are often particularly powerful "protected surfaces" enabling the system to react against many such pathogens. Also of physiological importance is the activation of the alternative pathway by IgG–antigen complexes, which may be determined by the relative protection from factor H that is afforded to C3b when it is covalently bound to these antibodies (213).

Much effort has been put into the identification of the physicochemical features of a particle that influences the affinity of bound C3b for factor H. It was expected that the discrimination was dependent on C3b and

that binding to certain substrates either affected the conformation of the C3b or imposed a steric interference on factor H binding. The early observation that removal of sialic acid from the surface of sheep erythrocytes produced a "protected" state that activated the alternative pathway focused attention on the effects of electrostatic charge (210). A polyanion binding site has been found on factor H and occupation of this site appears to increase the affinity for C3b (214,215), but it also has been reported that this does not enhance cofactor or decay-accelerating activities (216).

e. Direct Interaction between Surfaces and Factor H. A number of polyanionic particles bind factor H directly. The consequent sequestration of factor H can be sufficient to deregulate fluid-phase convertases and cause amplified C3b generation (217,218). In the case of streptococci, however, binding of factor H to the M protein correlates with inhibition of alternative pathway activation (219), presumably by increasing the interaction between factor H and bacterium-bound C3b. It is proposed that the main physiological role of the polyanion binding site is to bind factor H to heparin, heparin sulfate, or other polyanionic surfaces of host tissue and, thereby, to protect them from attack by autologous complement (220).

6. Mammalian Factor H-like Proteins

There are several immunologically cross-reacting factor H-like serum proteins and a number of cross-hybridizing mRNA transcripts (221). There appear to be two alternative transcripts expressed from the human factor H gene. One of 4 kb encodes the full-length 150-kDa molecule. A second of 1.8 kb encodes a 38-kDa secreted protein that comprises only the first seven amino-terminal CCPRs and possesses C3b-binding and cofactor activities (197).

There are two other small factor H-like serum proteins (of four and five CCPRs) that are derived from very closely related genes. Another highly homologous transcript is known but the predicted secreted product (five CCPRs) has not yet been found (222). These other genes are linked to the factor H locus (198) and could have arisen by anomalous recombination (221). The products probably lack factor H-like activities because they do not have the equivalents of factor H CCPRs 1–5 that are critical for C3b-binding and cofactor activities. No other potential function is known. In the mouse there are also additional homologous transcripts that would encode factor H-like proteins of 32, 36, 49, and 89 kDa (223).

B. C4b-Binding Protein (C4BP)

1. Introduction

In addition to structural homology, C4 exhibits homologous functional properties with C3 in that: (i) the internal thioester slowly hydrolyzes to

generate C4i; (ii) C4 is cleaved during classical pathway activation, into C4b and C4a; and (iii) some of this C4b deposits covalently and noncovalently onto surfaces such as immune complexes and all membranes. These products, like those of C3, require mechanisms for their inactivation and removal both from the fluid phase and from surfaces. Hence there is a need for C4b-binding protein (C4BP), which has cofactor activity for the proteolytic cleavage of C4b and C4i by factor I, and decay-accelerating activity against C4b2a. These activities apply to both isotypes of C4 (C4A and C4B) and are highly analogous to the cofactor and decay-accelerating activities of factor H against C3b and C3bBb. There are, however, important distinctions in both structure and function.

2. *Structure*

a. Basic Structure. The predominant form of C4b-binding protein (C4BP) in normal human plasma consists of seven identical 75-kDa α chains plus one 45-kDa β chain linked together by disulfide bonds near their carboxy termini (224). The result is a spider-like arrangement, as shown diagrammatically in Fig. 9 and supported by electron micrographic images in which the "tentacles" appear to be 33 nm long and flexible (225). Synchrotron X-ray scattering and hydrodynamic analysis corroborate the basic arrangement, but imply that the arms are more rigid and much less spread out (226).

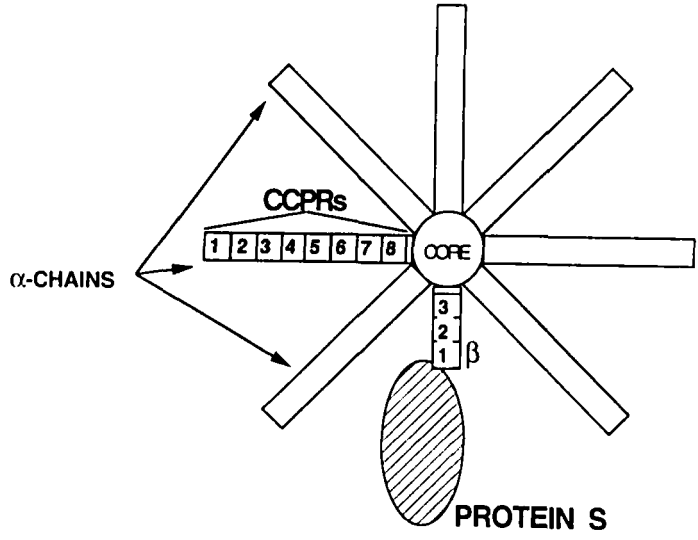


FIG. 9. Diagrammatic representation of the structure of human C4BP. This structure is based on Refs. (224,225,228,232). The predominant $\alpha 7;\beta 1$ form is shown.

In addition to $\alpha 7{:}\beta 1$ (85% of the total in plasma), there are also minor populations of $\alpha 7{:}\beta 0$ and $\alpha 6{:}\beta 1$ molecules as well as free β chains. Protein S, a vitamin K-dependent regulator of the coagulation system, binds to the complex through the β chain (227) and does not bind to $\alpha 7{:}\beta 0$ molecules (228).

b. The α Chains. The α chains are composed of 549 amino acids divided into eight CCPRs (of structure homologous to those found in factor H and other complement regulatory proteins) plus a 58-residue carboxy-terminal non-CCPR segment that is disulfide bonded to other chains in the core region through cysteine 498 and/or 510 (224). The mouse has a similar complex α chains but without interchain disulfide bonds, indicating the additional importance of noncovalent forces (229). In addition, mouse C4BP has no β chain.

A study on proteolytically generated fragments of human C4BP suggested that a region between residues 332 and 395 (in CCPR 6) was important in binding (230). However, the mouse α chain has only six CCPR units and homology indicates that it lacks the equivalents of the human CCPRs 5 and 6 (229). Deletion mutants of mouse C4BP α chain (fusion-protein constructs with membrane-spanning carboxy-termini) have now demonstrated that CCPRs 1–3, on the outward projecting amino-termini, are critical for C4b binding (231). Electron micrographs (using human components) provide supporting evidence that C4b binds to the outer ends of the arms (225). The rest of the chain may be required to allow the extended arms to bind to multiple C4b molecules without steric interference from each other or from the central core.

c. The Beta Chain and Protein S. The human C4BP β chain comprises three CCPRs attached to a C-terminal domain homologous to the core domains of the α chains (232). The attachment to the core includes one or more disulfide bonds and presumably occurs in the same way that the α chains are linked to each other. The β chain mediates the high-affinity ($K_d \approx 6 \times 10^{-10}\ M$ in the presence of Ca^{2+}) binding of protein S (227,233), and is not thought to interact with C4b. There is no complex formed between protein S and C4BP in bovine blood. The bovine C4BP β chain has only two CCPR units and lacks the homolog of the human β chain CCPR 1 (234). The conclusion that CCPR 1 includes at least part of the binding site is corroborated by another study that found that protein S binds to a peptide corresponding to residues 31–45 (within CCPR 1) of the β chain (235).

d. Gene Structure. The human α and β chain genes are closely linked in a head-to-tail arrangement in the RCA cluster on chromosome 1

(236,237). The α chain is encoded by 12 exons including a separate one for each of the 8 CCPRs, except for CCPR 2 which is encoded by two exons split at the same position as that found in other genes of the locus (238,239). There is clearly a close evolutionary relationship with the other structurally and functionally homologous C3b/C4b-binding proteins. The first two CCPRs of the β chain are encoded by separate exons while the third is split between two exons, the last exon also containing part of the C-terminal domain (240). The α and β chain genes probably arose by gene duplication. There also have been described two α-chain-related pseudogenes, C4BPAL1 (241) and C4BPAL2 (242), presumably products of further duplications.

e. Biosynthesis. The normal site of C4BP synthesis is likely to be the liver (243). The human hepatic cell line HepG2 secretes C4BP. The synthesis is stimulated by interleukin-6 and tumor necrosis factor, which is consistent with the elevation of *in vivo* levels of C4BP by as much as 286% (244) in acute phase, the purpose of which is unclear. It could be to provide increased protection of damaged host tissues from complement attack. Alternatively, it may serve to inhibit protein S activity and, thereby, promote coagulation. The expression of the α- and β-chain genes does not appear to be tightly coordinated because excess production of one chain can occur (245). In humans (but not in mice) the chain are covalently linked by disulfide bonds, so the various molecular forms (α7:β1, α7:β0, α6:β1, and free β) may not really interconvert. This implies that the chains are associated before secretion, but that the structure, the core region, and the process of chain assembly are not known.

3. *Function*

a. Role of C4BP in the Control of the Classical Pathway of Complement Activation. C4BP is present in the fluid phase of normal plasma at concentrations of about 0.2 mg/ml (246). Its immunochemical removal does not result in rapid spontaneous consumption of C3, because, unlike the alternative pathway, the classical pathway has an earlier control protein, C1 inhibitor, to prevent spontaneous activation of C1 that would lead to cleavage of C2 and C4. While C4 slowly hydrolyzes to C4i (C4b-like), the formation of the convertase, C4i2a, still requires C1 activity. Addition of excess active C1s to C4BP-depleted serum does promote C3 consumption which can be prevented by exogenously added C4BP (246). Thus, C4BP has a role in the fluid-phase control of C4b2a C3 convertase resembling, in some respects, the role of factor H in controlling fluid-phase tickover of the alternative pathway. There are three crucial reactions whereby C4BP controls the fluid-phase classical pathway: (i) binding to C4b,

(ii) displacement of bound C2a, and (iii) cofactor activity for factor I-mediated cleavage of C4b (246).

b. Binding to C4b. The seven subunits of C4BP impart the possibility of multivalent interaction: one C4BP can simultaneously bind four to six molecules of C4b, depending on the ionic strength (247). The binding sites appear identical ($K_d \approx 10^{-7}$ *M*) but binding of a seventh C4b may be sterically obstructed. The effects are to prevent C2 binding and to allow factor I action, as detailed below. C4BP also binds to C4i and weakly to C4c, but not to native C4 or C4d. In addition it has a weak affinity for, and cofactor activity against, C3b. It is unclear whether there are any physiological circumstances in which it can significantly augment the efficient fluid-phase action of factor H.

There are data to indicate that the binding of C4BP involves residues in the C-terminal α-3 fragment of C4 (248), although it would be surprising if the interaction was markedly different to that between C3b and factor H. Heparin binds to C4BP in competition with the binding of C4b, which points to a possible *in vivo* mechanism of regulating activity (249).

c. Decay Acceleration Activity against C4b2a. Unlike C3bBb, the classical pathway C3 convertase is not stabilized by an additional molecule (equivalent to properdin) but has a greater intrinsic stability. For example, a half-life of 7.5 min was measured at 30°C (in a low-ionic-strength buffer) (246). However, C4BP displaces the C2a in a manner analogous to the decay-accelerating activity of factor H against C3bBb. Once dissociated, C2a cannot rebind, implying that it has undergone an irreversible conformational change.

d. Cofactor Activity for Factor I-Mediated Cleavage of C4b. The factor I-dependent cleavage of C4 in the presence of C4BP is similar to the cleavage of C3b in the presence of H but differs in two respects. First, the conversion of C3b to iC3b involves cleavage at two close sites (C-terminal to arginine residues 1303 and 1320), with concomitant release of C3f, whereas the cleavage of C4b to iC4b only appears to involve one site (at Arg1336, equivalent to Arg1320 in C3) (250). Second, iC4b is only a transient intermediate because it is readily further cleaved by factor I to C4d plus C4c (at Arg956) (246,250), whereas for factor H the equivalent cleavage of iC3b to C3dg plus C3c only occurs at low ionic strength. These phenomena presumably reflect the fact that factor H has only a weak affinity for iC3b and normally dissociates unless the interaction is artificially stabilized, while C4BP apparently retains affinity for its primary product. In this respect the cofactor activity of C4BP is more analogous to that of

CR1, which acts as a cofactor not only for the conversion of C3b to iC3b, but also for additional processing to C3dg and C3c. In addition, it has been found that the iC4b intermediate is still capable of forming an active C3 convertase with C2 (251) and therefore requires the second cleavage for effective inactivation. In contrast, iC3b cannot combine with factor B to form a C3 convertase but is a functional ligand for CR3, which explains why it may be allowed to persist on target surfaces.

e. Regulation of Protein S Activity. Protein S is a cofactor for the inactivation of coagulation factor Va by activated protein C and was recently reported to be a direct inhibitor of factor Xa (252). However, it is *inactive* when bound to C4BP (253), so the concentration of free active S protein is determined by the relative concentration of C4BP. Normally 50% or more of the protein S is complexed in this way. Deficiencies of C4BP are associated with increased activity (254). Elevated levels of C4BP are found in acute-phase and some autoimmune diseases. Such conditions potentially result in down-regulation of protein S activity which might result in increased clotting tendencies comparable to those found in protein S deficiency. However, the increase is often more pronounced in α than β chains, resulting primarily in an accumulation of non-protein S-binding $\alpha 7{:}\beta 0$ molecules (245). The lack of an essential functional role for the interaction is indicated by its absence in other species: the bovine β chain does not bind proteins (234) and there is no β chain component in mouse (255) or rabbit (256). The mouse has a gene that probably once encoded for a β chain, but has now become a pseudogene (255). This may be an evolutionary adaptation to overcome adverse effects of reducing protein S activity. In contrast, in rat plasma C4BP does complex with protein S (257).

f. Possible Role of C4BP on Surfaces. The presence of the β chain with or without bound protein S does not seem to affect C4BP activity on C4 *in vitro* (258). However, through protein S the complex adheres to phospholipid cell membranes. It has been suggested that this provides a means of protecting host cell surfaces from local classical pathway activation (259,260). Nevertheless, the lack of conservation (discussed above) indicates that this interaction cannot be essential.

The polyvalency of C4BP for C4b contrasts with the apparent monovalency of factor H and would impart a great increase in avidity for C4b sites clustered on a surface. In fluid-phase regulation, there is no apparent advantage in a polymeric molecule compared to the alternative of unassociated monovalent α chains. This suggests that C4BP should have a significant role on surfaces, either to dampen-down (or clear-up after) specific activa-

tion on a target, or to protect host cells from complement attack (even though host cells are already protected by the membrane molecules MCP, DAF, and CR1, all of which have activity for C4b as well as for C3b).

C4BP also associates with serum amyloid P component (261). This interaction is independent of protein S but inhibits C4BP activity (262). The physiological implications are not clear.

4. Vaccinia Virus Complement-Control Protein (VCP)

Cells infected with vaccinia virus secrete several virally encoded polypeptides into the medium. One of these is the 35-kDa vaccinia virus complement-control protein (VCP) that consists of 4 CCPRs homologous to complement regulatory proteins (263). The greatest homology is with CCPRs 1–4 of the C4BP α-chain, the segment likely to carry the binding site for C4b. Like C4b-binding protein, VCP binds to C4b, has decay-accelerating activity against C4b2a, and has cofactor activity for the factor I-mediated cleavage of C4b to C4c plus C4d (264). In addition, it has much weaker binding, cofactor, and decay-accelerating activities on C3b and C3bBb. Therefore, despite its smaller size, VCP appears to be a close structural and functional homolog of C4BP. By being secreted, it can protect viral particles released from the cell from antibody-dependent classical-pathway-mediated attack and can, thereby, augment viral virulence (265). In an animal model of vaccinia infection, a mutant virus deficient in its ability to secrete VCP produced smaller pox, but otherwise was similar in its effects. The result of a larger, slower-healing pox would be a longer period of infectivity.

C. Properdin

1. Introduction

Properdin acts against the effects of negative regulators such as factor H by: (i) promoting the association of factor B with C3b, (ii) stabilizing C3bBb against spontaneous and factor H-mediated decay, and (iii) inhibiting the cleavage of C3b by factor I (209,266). Such activities are desirable on foreign surfaces, e.g., bacteria, but clearly undesirable in the fluid phase or on a host cell. The unusual polymeric structure of properdin assures that its activities are highly selective for C3b molecules clustered on a target particle.

2. Structure

a. Basic Structure. The history of properdin dates back to the 1940s when it was first described by Pillemer as a component of the newly discovered alternative pathway (267). However, there has been confusion about its oligomeric structure. The purified protein is composed solely of

identical, noncovalently linked 56-kDa subunits (268). Original measurements of the molecular weight suggested that properdin may be trimeric, but further analyses resolved it into a mixture of dimers (30%), trimers (45%), tetramers (10%), and larger species (16%) (269). They appear in electron micrographs as circular polymers of subunits as shown in Fig. 10, presumably linked head-to-tail. Neutron and X-ray scattering studies performed on the dimer and trimer were consistent with these structures and confirmed a subunit length of about 26 nm (270). The subunits carry an N-linked glycosylation site that is not required for function (271).

The sequence of the human primary precursor consists of 469 residues, including a 27-residue signal sequence (272). Residues 77 to 437 comprise six repeating units of homology rich in cysteine, glycine, proline and serine and conforming to the pattern of the structural element known as the thrombospondin repeat or TSR. [The sixth domain may consist of only part of a TSR that has been genetically fused with another structural element (273).] The cDNA-derived sequence of mouse properdin indicates that it has a similar size and six homologous TSRs (274).

b. Thrombospondin Repeats. In addition to properdin and thrombospondin, TSRs also have been found in malarial parasite circumsporozoite proteins (274). A related, but slightly different, class of domains occur in the terminal complement components C6, C7, C8a, C8b, and C9 (21,274). Each unit consists of approximately 60 amino acids usually with three conserved disulfide bonds. The consensus TSR motif in properdin has been characterized as

WS-WS/GPWSPCSVTCS-G-Q---R-R-C--PAP--G-PCAG-A-----QAC----PCP--G-

(strongly conserved residues are underlined) (270,272). Fourier transform infrared spectroscopy and sequence-derived secondary structure predictions indicate that β-sheet and β-turn structures predominate (275). The dimensions of each TSR are $4 \times 1.7 \times 1.7$ nm (270), but more precise structural definition is presently lacking.

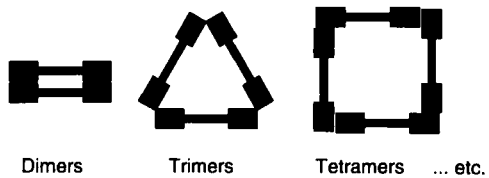


FIG. 10. Schematic representations of the oligomeric structures of properdin.

3. *Genetics*

Properdin deficiency is inherited as an X-linked recessive disorder, and, therefore, is normally observed only in males (276). The human gene lies on the X chromosome within band Xp11.23–Xp21.1 (277). It is organized into 10 exons, including 1 for each of TSRs 1–5 and 2 exons for TSR 6 (273). The mouse gene is also on the X chromosome (278). Deficiency of properdin, like those of C3 and terminal complement components, is associated with susceptibility to meningococcal infections (276). The X linkage explains why it appears to be more common than deficiencies in any other alternative pathway components. In addition, unlike the other deficiencies, the absence of properdin does not completely disable the alternative pathway. Residual properdin-independent activity can be detected against sensitive targets, such as the rabbit erythrocytes that are commonly used to measure alternative pathway activity. Also, cases of partial deficiency, in which the small amounts of properdin present displayed a lower oligomeric molecular weight (279), and of a dysfunctional protein that is present at normal levels (280), have been described.

4. *Biosynthesis*

Another intriguing aspect of properdin is its biosynthesis. Unlike other plasma complement proteins, current evidence suggests that peripheral blood leukocytes are more significant sources than the liver, where mRNA transcripts have been difficult to detect (274). There are several reports in the literature of synthesis of properdin by human peripheral blood monocytes (39,281) and monocyte-related cell lines (271,282). Most unusually, properdin is also synthesized by T lymphocytes of both CD4-positive and CD8-positive phenotypes (283). It is not known how this might relate to the primary immunological functions of T cells.

The process of biosynthesis has been studied in the human promyelocytic cell line HL-60 (271). It was found that the properdin is assembled into functional C3bBb-binding oligomers before secretion and that blocking the N-linked glycosylation with tunicamycin does not interfere with this process.

5. *Function*

a. Binding to C3b and Other Fragments. The interactions of properdin with C3b and other C3 derivatives in the absence of factor B are weak and generally have to be studied at low ionic strength. Where there are clusters of surface-bound ligands, such as C3b molecules deposited around a site of specific activation (alternative or classical pathway mediated), properdin can bind polyvalently. Higher-molecular-weight oligomers, which carry more binding sites (presumably one per subunit), bind with

more avidity and, therefore, exhibit greater activity (269,284,285). This heterogeneity makes it difficult to ascribe overall affinities to the binding reactions. Fluid-phase interactions are effectively monovalent and, therefore, much weaker, but they do allow associations with other C3 derivatives to be studied.

Properdin does not bind to native C3 or C3i, both of which normally occur only in the fluid phase (209). It does bind to C3b, iC3b, and C3c, all with similar affinity. In contrast, on surfaces properdin binds more strongly to C3b than to iC3b. Experiments with C3-derived peptides have identified a putative site of interaction between residues 1424 and 1457 of the C3 α chain (part of C3c) (286). Possibly the conformational change consequent upon factor I-mediated cleavage of surface-fixed C3b to iC3b results in partial occlusion of the properdin binding site. In the fluid phase the binding site is available but the monovalent interactions are much weaker.

The binding of properdin to C3b is not competitive with factor H but it does block the H-dependent binding and action of factor I (209). Prevention of C3b inactivation is another mechanism whereby properdin amplifies complement deposition. Properdin also inhibits the binding of C5 to C3b (205).

b. Effect on C3bB and C3bBb. Properdin binds, in order of affinity, C3bBb > C3bB > C3b (287). Association of properdin with C3b has the reciprocal effect of increasing the affinity for factor B. A very weak interaction with factor B alone can be detected, so that it is likely that properdin stabilizes the complex by making contact with both components. The effect on C3bBb is to dramatically retard the rates of spontaneous and factor H-dependent dissociation (266). It is unclear if there is a direct interaction between properdin and Bb, because only links to the Ba component were detected when P was chemically cross-linked to C3bB (287). For each ligand, the larger properdin oligomers have higher polyvalent avidities.

c. Effects in Fluid Phase. Properdin is a positive regulator of the alternative pathway and as such its activity is not required when the system is just ticking-over in the fluid phase. However, the original isolations of the protein, obtained by elution from zymosan after alternative pathway activation in serum, would produce C3 consumption when added back to properdin-depleted serum. This property is clearly incompatible with the normal stability of the complement levels in intact serum. It was thought that properdin became "activated" as a consequence of interaction with C3bBb (288). It is now known that this is an artifact of aggregation occurring

during isolation and that there is no transformation consequent to binding and dissociation from the convertase (284).

d. Effects on Surfaces. The molecular architecture of properdin gives it a strong selectivity for the surfaces of an activating particle where it is most needed. For example, in one study performed at low ionic strength the average affinity for C3b-coated erythrocytes was measured at $3.1 \times 10^7\ M^{-1}$, compared to an estimated of $2 \times 10^6\ M^{-1}$ in the fluid phase (209). Thus, it is likely that properdin does not participate effectively in the alternative pathway response until after C3b has deposited onto the prospective target. If there are no endogenous regulatory molecules, and the C3b is protected from factor H, then properdin and factor B can associate to form a stabilized C3 convertase. As more C3b is deposited around the initial site, properdin will bind with greater avidity. It will protect convertases from factor H and factor I, contributing to the positive feedback amplification process. *In vitro* this can lead to substantial depletion of properdin from the fluid phase. The higher oligomers are incorporated first and released last (285). When C3b is cleaved by factor I, it loses affinity for both factor B and properdin (209).

e. Direct Interaction with Surfaces. Properdin has been found to bind to sulfatide [Gal (3-SO_4) β1-1Cer], dextran sulfate, and fucoidan (another sulfated polysaccharide) (289). This behavior, characteristic of thrombospondin-like proteins, has been attributed to a region in the TSRs containing the sequence CSVTCG (and close variants) that is thought to mediate the cell-adhesive activities of thrombospondin (290). It has been suggested that sulfated carbohydrates and lipids on cell surfaces also may promote properdin binding, increasing its effect on C3b deposited on these surfaces (289). Properdin enhances the interaction between C3b and C4b (291), possibly binding to the sulfate groups on C4b (289). However, there is no unequivocal evidence of a direct interaction between properdin and C4b (291).

D. Nephritic Factors

Nephritic factors (NeFs) are pathologically associated immunoglobulin molecules that bind to and stabilize either of the C3 convertases, C3bBb (by "C3NeF") or C4b2a (by "C4NeF"), resulting in abnormal, chronic C3 cleavage (292,293). They are found in some cases of membranoproliferative glomerulonephritis (MPGN) and partial lipodystrophy, where they are associated with depleted levels of plasma C3 and renal glomerular deposition of C3 fragments (294) (although the actual relationship to the disease pathology is not certain).

The molecules have been purified from serum on the basis of their affinity for the convertase (e.g., on erythrocyte-C3bBb) (293–295), as well as from the supernatants of cell lines derived by transformation of patients' B lymphocytes with Epstein–Barr virus (296,297). Most are IgGs (apparently unrestricted in subclass) that have unusually large amounts of carbohydrate (298). This may be significant for the activity which has been shown to reside in the Fab portion (294) and is commonly thought to involve an interaction between the antigen binding site and a neoepitope on the convertase. Stabilization could then be achieved by a binding site that bridges the two proteins of the convertase. Studies of the activities on convertases containing heterologous sources of C3b and B have shown that while some NeFs interact with both components, others interact primarily with the Bb (299). It is evident that Bb bound to C3b must be conformationally distinct both from intact B, because this does not cleave C3, and from dissociated Bb ("Bbi"), because this cannot rebind to C3b. It is feasible that the major site of interaction could be with this subcomponent and that the antibody acts by stabilizing the bound conformation. Indeed, NeF-like stabilizing activity has been described in Bb-reactive monoclonal antibodies raised against purified factor B (300).

In addition to the C3 convertase, nephritic factors also bind to a 21-kDa molecule on the surface of erythrocytes (301). However, it is still not clear what stimulates production of these autoantibodies: is it the convertase itself or perhaps an unidentified cross-reacting antigen from an infectious agent? There also may be a contribution from genetic factors whose mechanism and importance remain to be clarified (302,303).

C3 NeF acts like properdin in retarding the dissociation of Bb from C3bBb. Unlike C3bBbP, however, C3bBbNeF complexes are generally resistant to accelerated dissociation mediated by factor H (191) and hence also resistant to factor I-dependent proteolytic inactivation. Furthermore, whereas properdin is primarily effective on a C3b-coated surface, nephritic factors have a sufficient monovalent affinity to work on fluid-phase convertases. It is these properties that allow NeFs to bypass normal homeostatic mechanisms, promoting excessive C3 conversion and ultimate C3 depletion from the circulation. The NeF-stabilized convertases also display resistance to the decay-accelerating activity of CR1 (304,305) and some resistance to that of DAF (306).

It is important to realize that nephritic factors represent a heterogeneous collection of molecules with various levels of activity. There are some C3NeFs that stabilize C3bBb *in vitro* but do not protect against factor H and do not cause C3 depletion *in vivo* (307). In some other cases the activity is dependent on properdin (308). It is possible that these C3NeFs act by enhancing the properdin interaction and indirectly stabilizing

C3bBb. There are also weak C3NeFs which are ineffective in the fluid phase but stabilize surface-bound convertases causing more depletion of C5 than of C3 (309). In addition there are the C4NeFs which retard the spontaneous dissociation of C4b2a and provide some resistance to C4BP (and hence also to factor I), CR1, and DAF (305,306,310). The effect is again uncontrolled C3 consumption and C3 fragment deposition.

V. Control of Fluid-Phase MAC

A. S Protein/Vitronectin

1. Introduction

S protein (site-specific protein) is a 75- to 80-kDa glycoprotein originally described by Kolb and Muller-Eberhard (311) as a component of the nonlytic terminal complement complex (TCC) which is generated during formation of the membrane attack complex (MAC) on cells. Comparison of cDNA-derived amino acid sequences (312,313) later showed that this protein was identical with vitronectin or "serum spreading factor" (314).

2. Structure/Genetics/Biosynthesis

S protein is present in human plasma at 0.25–0.45 mg/ml and is also present in amniotic fluid and in urine (315,316). Plasma S protein is derived mainly from liver; however, the protein is also synthesized by platelets, megakaryocytes, macrophages, and umbilical vein endothelial cells (317–321). The S-protein gene recently has been mapped to the centromeric region of chromosome 17q (322). It consists of eight exons and seven introns and has a single mRNA transcript of 1700 bp (323). In hepatocytes S protein is initially synthesized as a single-chain polypeptide, including a 19-amino-acid signal sequence which is cleaved to yield a mature polypeptide of 459 amino acids (323,324). Thereafter the protein is processed differently in different individuals. In some plasma samples all S protein exists in single-chain form, whereas plasma from other individuals contains a two-chain form, which results from proteolytic cleavage of the original translation product, or a mixture of the one-chain and two-chain forms (325). These different forms are due to allelic variation involving a single amino acid change (from methionine to threonine at position 381) which results in a difference in susceptibility of the protein to proteolysis (326).

The sequence and organization of the S-protein molecule has been well described (323). The amino-terminal signal peptide is followed by a Somatomedin B domain—an independently folded structural module containing eight interlinked cysteines which is of unknown functional significance. This is followed by a linear sequence, the connecting segment,

which contains the RGD sequence responsible for binding of S protein to integrins, a highly acidic sequence (residues 53–64), a putative crosslinking site, and a collagen binding site. Two domains of S protein which have homology with hemopexin, a human heme–plasma binding protein, are present between amino acids 132–268 and 269–459, suggesting incomplete duplication of a primordial four-repeat hemopexin domain during evolution (323). At the carboxy terminus of the protein is located a heparin binding domain, represented by a 40-amino-acid segment rich in basic residues, which is responsible for binding of S protein to sulfated polysaccharide and related compounds (327) and which is partly responsible for the MAC regulatory activity of this protein.

3. *Functions*

a. Inhibition of MAC Formation. S protein binds to the metastable binding site of the nascent C5b-7 complex, preventing insertion of this complex into cell membranes (328). Nascent MACs have the potential to insert into any cell membrane and are not restricted to the membrane on which the complement cascade is activated. The MAC inhibitory function of S protein in plasma is, therefore, important for the protection of "self cells" from "bystander" MAC attack at sites of complement activation. Although the resulting SC5b-7 complex can incorporate C8 and C9 molecules, forming soluble SC5b-8 and SC5b-9 complexes, these complexes are lytically inactive (329). In addition to its effect on the C5b-7 complex, S protein has been shown to bind C8 and C9 and to inhibit polymerization within the forming TCC (330–332). Further, S protein inhibits polymerization of the T-cell-derived pore-forming toxin perforin (331). In a recent study, using separate assays, Milis *et al.* (333) compared the effect of S protein on MAC formation at the C5b-7 stage with its effect on polymerization of C9 within the TCC. In these experiments, S protein has a greater inhibitory effect on MAC formation at the C5b-7 stage than at the stage of C9 polymerization. Further, the authors examined the effect on these reactions of two synthetic 15-mer peptides which span the heparin binding site of S protein. The peptides inhibited C9 polymerization but had no effect on insertion of C5b-7 into membranes. Thus, it appears that S protein acts via the heparin binding site to inhibit C9 polymerization within forming TCCs but has a greater effect on insertion of C5b-7 into cell membranes, which operates by an unknown mechanism.

b. Other Functions. The structural properties of S protein allow its interaction in diverse biological systems. There is considerable evidence that, via its several ligand binding sites, this protein is important in cell–matrix interactions. Further, it may be involved in bacterial adhesion to

host cells, facilitating both infection and phagocytosis. S protein also has important effects on the coagulation system. It forms stable complexes with thrombin and antithrombin III during thrombin inactivation, resulting in net protection of thrombin. Further, it binds to heparin-like molecules and stabilizes plasminogen activator type I in the subendothelial matrix at sites of tissue injury, resulting in a net procoagulatory effect. It is not possible to describe the various functions of S protein in detail here; for a comprehensive review see Ref. (334)

B. Clusterin [SP-40,40, Cytolysis Inhibitor (CLI)]

1. Introduction

Clusterin, which also has been named SP-40,40 (335), cytolysis inhibitor [CLI (336)], sulfated glycoprotein 2 [SGP2 (336)], and apolipoprotein J (337), is an 80-kDa heterodimeric glycoprotein which was originally identified as a previously unrecognized component of the SC5b-9 complex in glomerular immune deposits (335). Cloning and sequencing studies established that the human protein has strong sequence homology with a major sertoli cell product of rat and ram (336,338–340) and the same protein has also been characterized as a novel, high-density HDL-associated component, apo J, in plasma (337,341,342). These findings resulted in the proliferation of names listed above (clusterin is now preferred) and to the realization that this protein has multiple functions (see below).

2. Structure/Genetics/Biosynthesis

Clusterin is composed of two nonidentical subunits, α and β, of approximately equal size (35–40 kDa) (336,338). Southern blotting and cross-hybridization studies using the cDNA of different mammalian species have shown that clusterin is the translation product of a single-copy gene (CLI) on chromosome 8 (343–345) at position 8p21 (346) proximal to the lipoprotein lipase (LPL) gene. The single-chain precursor has 445 amino acids of which the first 21 represent a typical signal peptide. The two subunits are generated by proteolytic cleavage between Arg205 and Ser206. Each subunit has three N-linked glycosylation sites and 5 cysteines, one of which is involved in the intrachain disulfide link (336). The amino-terminus sequence indicates an α helical terminus followed by a short segment (positions 77 to 98) which has homology to the cysteine-rich thrombospondin type 1 modules in terminal complement proteins (336). The molecule is transported along the constitutive secretory pathway to the apical surface in several cell types (reviewed in 323).

Clusterin has been identified in a wide variety of cells, tissues, and species (337) and is found in all human tissues examined except for T lymphocytes. The protein is present in human serum at 35–105 μg/ml

(335), where it associates with lipoproteins, forming the Apo A1–clusterin–lipoprotein complex (341,342).

3. *Functions*

a. Complement Inhibition. Like S protein, clusterin inhibits the terminal complement pathway after formation of the C5b-7 complex (336,347,348). However, the relative importance of S protein versus clusterin in control of the fluid-phase MAC remains to be determined. Clusterin also incorporates into nascent TCCs, preventing insertion of the C5b-7 complex into cell membranes and resulting in complexes which are soluble and lytically inactive (347–349). This protein is therefore important in protecting self cells from MAC attack during complement activation. Clusterin's MAC inhibitory function is dependent on the thrombospondin-like aminoterminal region mentioned above which is suitable for interaction with hydrophobic regions of the terminal complement proteins and which allows clusterin to bind with high affinity to C7, to the β chain of C8, and to the b domain of C9 (350).

In addition to *in vitro* demonstrations of clusterin's MAC inhibitory activity, much evidence indicates the importance of clusterin as a regulator of MAC formation *in vivo*. Clusterin has been demonstrated in association with the MAC in a number of disease states in which complement activation is implicated, including glomerulonephritis (335), myocardial infarction (351), and Alzheimer's disease (352). Further, the protein has been shown to colocalize with the MAC in glomerular capillary walls in the isolated perfused kidney rat Heymann nephritis model of human membranous glomerulonephritis (353). In recent experiments using the same model, kidneys perfused with clusterin-depleted plasma developed significantly greater proteinuria than control kidneys, providing direct evidence for the importance of clusterin in controlling complement-mediated pathology.

b. Clusterin in the Male Reproductive Tract. Clusterin is synthesized by testis, seminal vesicle, and epididymis (354) and is present at high concentration in human seminal plasma [published values are from 250 μg/ml (336) to 15 mg/ml (314)]. This protein may therefore be important for immune protection of sperm, which are highly susceptible to MAC-mediated damage and which are at risk of MAC attack in the female reproductive tract (reviewed in 355). A minority of spermatozoa (51) have a coat of clusterin on their surface (356). These cells are grossly abnormal and agglutinated, suggesting that clusterin may play a role in agglutination of abnormal sperm. Recently, it has been reported that the spermatozoal acrosome (an enzyme-filled vacuole whose contents facilitate penetration of the glycoprotein coat surrounding the ovum) contains a protein which

reacts with antibodies to the clusterin α subunit but not with antibodies to the heterodimer [β-chain specific antibodies are not yet available (356)]. The precise nature of this protein and its functional significance are unknown.

c. Other Functions. Clusterin has been implicated in a number of biological processes in addition to its role in regulation of complement activation. Clusterin expression has been found to be up-regulated at sites of tissue injury, including neural tissues in Alzheimer's disease (357) and in tissues which have undergone ischemic injury (358). This upregulation might be in response to secondary MAC attack or, alternatively, clusterin might be involved in tissue remodeling. Clusterin up-regulation has also been noted in tissues undergoing programmed cell death, which has led to suggestions that this protein might be a mediator of apoptosis (359,360). Recently, however, it has been shown that clusterin gene expression is confined to surviving cells in populations undergoing programmed cell death (361). The physiological significance of this finding is not yet understood. Further, the association of clusterin with HDL particles and the observation that the clusterin–HDL complex copurifies with cholesterol ester transport protein activity in human plasma have led to the suggestion that the protein may play a role in lipid transport (335). Finally, the protein's abundance and location in the male reproductive tract indicate a possible role for the protein in sperm maturation.

VI. Control of Deposited MAC

A. CD59

1. Introduction

CD59 is an 18- to 20-kDa membrane-bound inhibitor of MAC formation which was originally described on circulatory cells. The protein was identified independently by several groups and consequently is known by a variety of names; P18 (362), homologous restriction factor 20 [HRF20 (363)], MAC inhibitory factor [MACIF (364)], membrane inhibitor of reactive lysis [MIRL (365)], CD59 antigen (366), and protectin (367).

2. Structure/Genetics/Biosynthesis

CD59 is a single-chain glycoprotein which, like DAF, is GPI-anchored (366). The gene is located on chromosome 11 at position 11p13 (368,369). This gene spans over 20 kb of DNA and contains four exons (370,371). The precursor protein contains 128 amino acids of which 25 represent an amino-terminal signal peptide, while a hydrophobic sequence of 26 amino

acids at the carboxy terminus provides the signal for GPI-anchor attachment (372). As in the case of DAF, this stretch of amino acids is removed in the endoplasmic reticulum as an early post-translation event and replaced with the glycosylphosphatidylinositol moiety.

The mature protein has a backbone of 77 amino acids with the GPI anchor attached to Asn77. There are 10 cysteine residues, resulting in extensive intrachain disulfide bonding and a single N-linked glycosylation site at position Asn18. The sugar is heterogeneous and mainly of biantennary or triantennary complex type (373). The amino acid sequence and gene structure show considerable homology to the murine lymphocyte antigen Ly 6 (366,374) and CD59 also has 48% amino acid identity with a viral membrane protein of Herpes Saimiri (VSV-15) (375). The structure of CD59 in solution has recently been determined by nuclear magnetic resonance spectroscopy (376,377) and shows a folding pattern similar to several snake venom toxins; the protein is folded into a disc-shaped domain with the disulfide bonds clustered in the hydrophobic center and with four looped extensions protruding from the disc.

CD59 is very widely distributed in human cells and tissues (378). It is present on all circulating blood cells (366), endothelial cells (379,380), epithelial cells (378), cardiomyocytes (381), spermatozoa (382), the fetal membranes amnion (383), trophoblast (95), and on fetal tissues from early in development (93). Its absence from certain cells, for example oligodendrocytes (384), may explain their extreme sensitivity to complement-mediated lysis. The protein is also present in most body fluids. In urine, CD59 is soluble, having lost the GPI anchor (366); however, in other body fluids a GPI-anchored form of the protein exists in association with lipid which may protect the hydrophobic anchor moiety from damage (385–387).

a. Control of CD59 Synthesis. CD59 expression by cells is upregulated in response to a number of stimulants including cytokines, phorbol myristate acetate, and calcium ionophore (102,388–390). Levels of CD59, together with MCP and DAF, are increased in joints affected by inflammatory arthritis, possibly in response to attack by activated complement components (391). There is evidence that CD59 is upregulated at the level of transcription (390) and also that, in some cells, an intracellular pool of the protein exists that can be rapidly mobilized (389).

b. Functions. The only function of CD59 which has been convincingly demonstrated so far is inhibition of formation of the membrane attack complex on cells. Abundant evidence for this function has accrued from many investigators using several experimental systems:

1. Antibody blocking experiments. Blocking of CD59 activity with monoclonal antibodies has been shown to render numerous cell types, including erythrocytes (366), epithelial cells (383,392), endothelial cells (380), and spermatozoa (382), significantly more susceptible to MAC-mediated killing.

2. Protective effect of exogenous CD59 on heterologous cells. Purified CD59 can incorporate into cell membranes via the hydrophobic GPI anchor. Exogenous CD59 has been shown to increase the resistance of heterologous erythrocytes (365) and endothelial cells (393) to MAC attack and the conferred resistance can be partially reversed by antibody blocking of CD59. Further, many cell types—including rat endothelial cells (394), porcine endothelial cells (395), mouse fibroblasts (396), rat T cells (397), and Chinese hamster ovary cells (160) (transfected with human CD59)—have acquired increased resistance to MAC attack which can be reversed by treatment with anti-CD59.

3. Susceptibility to MAC attack of human cells lacking CD59: paroxysmal nocturnal hemoglobinuria. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disease characterized by increased susceptibility of blood cells to complement attack; the clinical features include a chronic hemolytic anemia, pancytopenia, and venous thrombosis (398). The disorder is caused by a somatic mutation at the level of bone marrow stem cells which results in inability of cells to synthesize GPI anchors. Consequently, PNH cells are deficient in both CD59 and DAF. Incorporation of exogenous CD59 into PNH cells has been shown to increase the resistance of these cells to MAC-mediated lysis (365) and recently a CD59-deficient B-cell line isolated from a PNH patient has been transfected with CD59 with a resultant increase in the cell's resistance to MAC attack (399).

4. *In vivo* experimentation. The rat (400) and sheep (401) analogs of CD59 have recently been isolated and will allow direct analysis of the role of CD59 in control of complement activation *in vivo*. Already, important progress has been made in this area: functional inhibition of the rat analog of CD59 using monoclonal antibodies increased the severity of renal damage in rats with experimental glomerulonephritis, providing direct evidence that CD59 is important for prevention of complement-related renal pathology.

c. Mechanism of CD59 Function. CD59 inhibits MAC formation after the stage of C5b-7 insertion into cell membranes (366). The protein incorporates into forming MACs, inhibiting both incorporation of the first C9 and the subsequent polymerization of C9 within the complex (367,402). CD59 has binding sites for both the α chain of C8 and the b domain of C9 (403); the precise regions of contact between the molecules are currently being investigated. Two groups recently have sought to identify the

region of C9 which is recognized by CD59 by examining the CD59 binding activity of synthetic peptides which correspond to different regions of C9. Tomlinson *et al.* (404) reported that a peptide from the "hinge" region of C9 (residues 247–261), which is thought to unfold during C9 insertion into the MAC, has CD59 binding activity; further, this peptide enhanced MAC-mediated lysis of human erythrocytes in their experiments. This finding is, however, controversial: Chang *et al.* (405) failed to demonstrate CD59 binding activity of hinge region-derived peptides but reported that peptides spanning amino acids 359–411 of C9 were able to bind the protein.

The regions of CD59 involved in C8 and C9 binding have not been identified; however, the Asn-linked sugar of the protein is of some functional importance. Ninoyima *et al.* (406) reported that, although removal of this sugar did not affect ability of CD59 to bind C8 or C9, the MAC inhibitory activity of the protein was reduced by this modification.

d. Species Selectivity of CD59. CD59 was originally described as a "homologous restriction factor," implying that its inhibitory activity is restricted to human complement. It is now clear that CD59 inhibits complement from other species and that this inhibition is species-selective rather than species-specific (402). This finding is supported by observations that the rat, sheep, and porcine analogs of CD59 are effective against complement from other species (407).

e. Other Functions of CD59. Antibody crosslinking of CD59 on the surface of T cells and neutrophils has been shown to result in their activation (408,409). This phenomenon also has been observed when a variety of other GPI-linked proteins are crosslinked on cells. Morgan *et al.* have shown recently that the effect is mediated through tyrosine kinases (409). The possible association of GPI-anchored proteins with tyrosine kinases in specific cell membrane domains may partly explain this effect (410). However the precise mechanism by which it occurs and its physiological relevance *in vivo* have yet to be elucidated.

f. CD59 as a Ligand for CD2. CD2 is a T cell surface protein that functions in cell adhesion and activation. Its principal ligand is CD58. It has been reported that CD59 may function as a second ligand for CD2 (411,412) and also that CD59 functions in CD58-dependent costimulation of T cells (413). Recent studies, however, have failed to demonstrate any interaction between CD59 and CD2 and the physiological relevance of such an interaction remains doubtful.

B. Homologous Restriction Factor

Homologous restriction factor (HRF) is a 60-kDa glycoprotein which was described independently by three groups and appears in the literature

as HRF (414), C8 binding protein [C8bp (415)], and MAC inhibitory protein [MIP (416)]. Like CD59 and DAF it is GPI-anchored (417). Purified HRF has been shown to incorporate into PNH erythrocytes and to render them more resistant to MAC attack (418). Depletion of this protein from serum has been reported to render the serum more lytically active (416). The MAC inhibitory activity of HRF is ~1% that of CD59, raising the possibility that trace contamination of HRF preparations with CD59 may have been responsible for some of these results. Also, CD59 has a tendency to form dimers and trimers during purification and storage. The trimeric form could comigrate with HRF. A 1% contamination by CD59 would account for the functional activity reported for HRF. The protein has not been cloned or sequenced and assessment of its importance as a MAC inhibitor *in vivo* is, therefore, difficult. [For a recent review see Ref. (419).]

VII. Control of Anaphylatoxins

During activation of the complement system, peptides of 74–77 amino acids are released by the splitting of a single Arg–X bond at the amino terminus of the α chains of C3, C4, and C5 (420). Although the anaphylatoxins (C3a, C4a, and C5a) are similar structurally, they differ vastly in their relative potencies (421–423). C5a is by far the most powerful anaphylatoxin followed by C3a and remotely by C4a. Additionally, C5a is a potent chemotactic factor.

The spasmogenic activities of the anaphylatoxins are rapidly abrogated by a plasma enzyme that acts as an anaphylatoxin inactivator, serum carboxypeptidase N (420). This exopeptidase removes the carboxyl-terminal arginyl residue from each anaphylatoxin converting them into their des-Arg forms. While completely inactivating C3a and C4a, the activity of C5a des Arg is approximately 10% that of C5a. Thus, C5a differs from C3a and C4a in several important ways: (a) it is a less efficient substrate for serum carboxypeptidase N and is formed more slowly than C3a des Arg, (b) some C5a may be resistant to degradation, allowing its involvement in systemic inflammation, and (c) C5a-des Arg retains about 10% of its original biological activity (424).

VIII. Summary and Conclusions

The complement system has developed a remarkably simple but elegant manner of regulating itself. It has faced and successfully dealt with how to facilitate activation on a microbe while preventing the same on host tissue. It solved this problem primarily by creating a series of secreted and

membrane-regulatory proteins that prevent two highly undesirable events: activation in the fluid phase (no target) and on host tissue (inappropriate target). Also, if not checked, even on an appropriate target, the system would go to exhaustion and have nothing left for the next microbe. Therefore, the complement enzymes have an intrinsic instability and the fluid-phase control proteins play a major role in limiting activation in time. The symmetry of the regulatory process between fluid phase and membrane inhibitors at the C4/C3 step of amplification and convertase formation as well as at the MAC steps are particularly striking features of the self/nonself discrimination system. The use of glycolipid anchored proteins on membranes to decay enzymes and block membrane insertion events is unlikely to be by chance. Finally, it is economical for the cofactor regulatory activity to produce derivatives of C3b that now specifically engage additional receptors. Likewise, C1-Inh leads to C1q remaining on the immune complex to interact with the C1q receptor. Thus the complement system is designed to allow rapid, efficient, unimpeded activation on an appropriate foreign target while regulatory proteins intervene to prevent three undesirable consequences of complement activation: excessive activation on a single target, fluid phase activation, and activation on self.

A. Some Remaining Questions

A number of issues concerning complement regulation have been generated in this review. Some of the more intriguing questions are summarized below.

1. How much of a role does C1-Inh play in regulating complement activation on a cell surface? Is there a need for a membrane equivalent or is it unnecessary since C1 on a target is always directed by and bound to antibody?
2. Aside from C1 regulation, does C1-Inh dissociate C1r and C1s from C1q in order that C1q can interact with its receptor? Is this analagous to cofactor activity in which C3b is converted to iC3b or C3dg and these cleavage products serve as ligands for CR2 and CR3?
3. Do plasma convertase regulators factor H and C4BP have a role in membrane regulation?
4. Are C3/C5 convertases regulated synergistically by DAF and MCP? What is the relative role of decay-accelerating activity versus cofactor activity in controlling complement activation on a cell surface? For example, if C3b or C4b deposits on a host cell, does MCP or DAF get there first? What is the optimal ratio of DAF to MCP on a host cell?
5. Given the very restricted tissue distribution of CR1, but its potent decay-accelerating and cofactor activities for convertases of both the alter-

native and classical pathways, what other regulatory roles might CR1 play? Is this regulatory profile primarily designed for processing immune complexes?

6. What is the relative role of S protein versus clusterin in the control of fluid-phase MAC?

7. Is HRF a second inhibitor, in addition to CD59 of the MAC, or is its activity accounted for by contamination of the HRF preparations with CD59?

8. What is the biologic advantage for having DAF and CD59 attached to cells by a GPI anchor?

9. What is the biologic advantage for having the tightly linked genes for the RCA proteins?

10. What are the variations on these regulatory themes employed by other species to control their complement systems?

Hopefully, answers will soon be forthcoming to these and many other questions related to how the complement system is regulated.

Finally, the complement system has undergone a renaissance over the past decade. Much of this revival relates to the discovery and characterization of the membrane regulatory proteins whose roles have broadened to include other areas of biology. For example, the regulatory proteins may assist fertilization—they are highly expressed at the maternal–fetal interface and on spermatozoa. Additionally, membrane regulatory proteins are targets for microbes who use them as receptors for adherence and cellular entry. Pathogens also may possess proteins that mimic the inhibitory activity of complement regulators or may "capture" regulators during their reproduction in an infected cell. The cloning of regulators has allowed their genetic manipulation. By liberating them from their tethering, regulators are being tested for use as soluble inhibitors of complement activation. Such engineering has led to the discovery of complement's role in mediating reperfusion injury. Transgenic animals, especially pigs, expressing human complement membrane inhibitors have been developed for use in xenotransplantation. These are now available for trials in the hope that the acute xenograft rejection phenomenon, mediated in part by complement, can be abrogated. Such therapy could positively impact thousands of patients who currently are denied potentially life-saving transplants due to organ shortage. These exciting developments and others yet to be anticipated are an outcome of our improved understanding of the regulation of the complement system.

References

1. Lachmann, P. J. 1979. An evolutionary view of the complement system. *Behring Inst. Mitt.* **63**:25–37.

2. Farries, T. C., and J. P. Atkinson. 1991. Evolution of the complement system. *Immunol. Today* **12:**295–300.
3. Dodds, A. W., and A. J. Day. 1993. The phylogeny and evolution of the complement system. *In* Complement in Health and Disease (K. Whaley, M. Loos, and J. M. Weiler, Eds.), pp. 39–88. Kluwer Academic, Boston.
4. Farries, T. C., and J. P. Atkinson. 1987. Separation of self from non-self in the complement system. *Immunol. Today* **8:**212–215.
5. Rosse, W. F. 1992. Paroxysmal nocturnal hemoglobinuria. *Curr. Top. Microbiol. Immunol.* **178:**163–173.
6. Lachmann, P. J., and P. A. E. Nicol. 1973. Reaction mechanism of the alternative pathway of complement fixation. *Lancet* **i:**465–467.
7. Cooper, N. R. 1985. The classical complement pathway. Activation and regulation of the first complement component. *Adv. Immunol.* **37:**151–216.
8. Sim, R. B., and K. B. M. Reid. 1991. C1: Molecular interactions with activating systems. *Immunol. Today* **12:**307–311.
9. Davis, III, A. E. 1988. C1 inhibitor and hereditary angioneurotic edema. *Annu. Rev. Immunol.* **6:**595–628.
10. Davis, III, A. E., A. S. Whitehead, R. A. Harrison, A. Dauphinais, G. A. P. Bruns, M. Cicardi, and F. S. Rosen. 1986. Human inhibitor of the first component of complement, C1: Characterization of cDNA clones and localization of the gene to chromosome II. *Proc. Natl. Acad. Sci. USA* **83:**3161–3165.
11. Bock, S. C., K. Skriver, E. Nielsen, M. C. Thogersen, B. Wiman, V. H. Donaldson, R. L. Eddy, J. Marrinan, E. Radziejewska, R. Huber, T. Shows, and S. Magnussen. 1986. Human C1 inhibitor: Primary structure, cDNA cloning, and chromosomal localization. *Biochemistry* **25:**4292–4301.
12. Carter, P. E., C. Duponchel, M. Tosi, and J. E. Fothergill. 1991. Complete nucleotide sequence of the gene for human C1 inhibitor with an unusually high density of Alu elements. *Eur. J. Biochem.* **197:**301–308.
13. Perkins, J. J., K. F. Smith, S. Amatayakul, D. Ashford, T. W. Rademacher, R. A. Dwek, P. J. Lachmann, and R. A. Harrison. 1990. Two-domain structure of the native and reactive centre cleaved forms of C1 inhibitor of human complement by neutron scattering. *J. Mol. Biol.* **214:**751–763.
14. Laurell, A. B., U. Martensson, and A. G. Sjohdin. 1979. Quantitation of C1r-Cls-C1 inactivator complexes by electroimmunoassay. *Acta Pathol. Microbiol. Scand. Sec. C* **87:**79–85.
15. Ziccardi, R. J. 1981. Activation of the early components of the classical complement pathway under physiologic conditions. *J. Immunol.* **126:**1769–1773.
16. Davis, III, A. E., K. S. Aulak, K. Zahedi, J. J. Bissler, and R. A. Harrison. 1993. C1 Inhibitor. *Methods Enzymol.* **223:**97–120.
17. Kaul, M., and M. Loos. 1993. C1q, the collagen-like subcomponent of the first component of complement C1, is a membrane protein of guinea pig macrophages. *Eur. J. Immunol.* **23:**2166–2174.
18. Oltvai, Z. N., E. C. C. Wong, J. P. Atkinson, and K. S. K. Tung, 1991. C1 inhibitor deficiency: Molecular and immunologic basis of hereditary and acquired angioedema. *Lab. Invest.* **65:**381–388.
19. Vogel, C.-W. 1991. Handbook of Natural Toxins, Reptile and Amphibian Venoms, Vol. 5. Dekker, New York.
20. Hourcade, D., V. M. Holers, and J. P. Atkinson. 1989. The regulators of complement activation (RCA) gene cluster. *Adv. Immunol.* **45:**381–416.

21. Reid, K. B. M., and A. J. Day. 1989. Structure-function relationships of the complement components. *Immunol. Today* **10:**177–180.
22. Reid, K. B. M., D. R. Bentley, R. D. Campbell, L P. Chung, R. B. Sim, T. Kristensen, and B. F. Tack. 1986. Complement system proteins which interact with C3b or C4b. *Immunol. Today* **7:**230–234.
23. Norman, D. G., P. N. Barlow, M. Baron, A. J. Day, R. B. Sim, and I. D. Campbell. 1991. Three-dimensional structure of a complement control protein module in solution. *J. Mol. Biol.* **219:**717–725.
24. Barlow, P. N., D. G. Norman, A. Steinkasserer, T. J. Horne, J. Pearce, P. C. Driscoll, R. B. Sim, and I. D. Campbell. 1992. Solution structure of the fifth repeat of factor H: A second example of the complement control protein module. *Biochemistry* **31:**3626–3634.
25. Janatova, J., K. B. M. Reid, and A. C. Willis. 1989. Disulfide bonds are localized within the short consensus repeat units of complement regulatory proteins: C4b-binding protein. *Biochemistry* **28:**4754–4761.
26. Kaidoh, T., and I. Gigli. 1989. Phylogeny of regulatory proteins of the complement system. Isolation of a C4b/C3b inhibitor and a cofactor from sand bass plasma. *J. Immunol.* **142:**1605–1613.
27. Kunnath-Muglia, L. M., G. H. Chang, R. B. Sim, A. J. Day, and R. A. Ezekowitz. 1993. Characterization of Xenopus laevis complement factor I structure-conservation of modular structure except for an unusual insert not present in human factor I. *Mol. Immunol.* **30:**1249–1256.
28. Nonaka, M., T. Fujii, T. Kaidoh, S. Natsuume-Sakai, M. Nohaka, N. Yamaguchi, and M. Takahashi. 1984. Purification of a lamprey complement protein homologous to the third component of the mammalian complement system. *J. Immunol.* **133:**3242–3249.
29. Mavroidis, M., J. O. Sunyer, and J. D. Lambris. 1995. Isolation, primary structure, and evolution of the third component of chicken complement and evidence for a new member of the alpha2-macroglobulin family. *J. Immunol.* **154:**2164–2174.
30. Abramson, N., C. A. Alper, P. J. Lachmann, F. S. Rosen, and J. H. Jandl. 1971. Deficiency of C3 Inactivator in man. *J. Immunol.* **107:**19–27.
31. Nicol, P. A. E., and P. J. Lachmann. 1973. The alternative pathway of complement activation. The role of C3 and its inactivator (KAF). *Immunology* **24:**259–275.
32. Vyse, T. J., P. J. Spath, K. A. Davies, B. J. Morley, P. Philippe, P. Athanassiou, C. M. Giles, and M. J. Walport. 1994. Hereditary complement factor I deficiency. *Q. J. Med.* **87:**385–401.
33. Goldberger, G., G. A. Bruns, M. Rits, M. D. Edge, and D. J. Kwiatkowski. 1987. Human complement factor I: Analysis of cDNA-derived primary structure and assignment of its gene to chromosome 4. *J. Biol. Chem.* **262:**10065–10071.
34. Catterall, C. F., A. Lyons, R. B. Sim, A. J. Day, and T. J. R. Harris. 1987. Characterization of the primary amino acid sequence of human complement control protein Factor I from an analysis of cDNA clones. *Biochem. J.* **242:**849–856.
35. Vyse, T. J., G. P. Bates, M. J. Walport, and B. J. Morley. 1994. The organization of the human complement factor I gene (IF): A member of the serine protease gene family. *Genomics* **24:**90–98.
36. Perkins, S. J., K. F. Smith, and R. B. Sim. 1993. Molecular modelling of the domain structure of factor I of human complement by X-ray and neutron solution scattering. *Biochem. J.* **295:**101–108.
37. DiScipio, R. G. 1992. Ultrastructures and interactions of complement factors H and I. *J. Immunol.* **149:**2592–2599.

38. Goldberger, G., M. A. Arnaout, D. Aden, R. Kay, M. Rits, and H. R. Colten. 1984. Biosynthesis and postsynthetic processing of human C3b/C4b inactivator (factor I) in three hepatoma cell lines. *J. Biol. Chem.* **259:**6492–6497.
39. Whaley, K. 1980. Biosynthesis of the complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *J. Exp. Med.* **151:**501–516.
40. Julen, H., H. Dauchel, C. Lemercier, R. B. Sim, M. Fontaine, and J. Ripoche. 1992. In vitro biosynthesis of complement factor I by human endothelial cells. *Eur. J. Immunol.* **22:**213–217.
41. Harrison, R. A., and P. J. Lachmann. 1980. The physiological breakdown of the third component of human complement. *Mol. Immunol.* **17:**9–20.
42. Harrison, R. A., T. C. Farries, F. D. Northrop, P. J. Lachmann, and A. E. Davis. 1988. Structure of C3f, a small peptide specifically released during inactivation of the third component of complement. *Complement* **5:**27–32.
43. Sim, E., A. B. Wood, L. M. Hsiung, and R. B. Sim. 1981. Pattern of degradation of human complement fragment, C3b. *FEBS Lett.* **132:**55–60.
44. Cole, J., G. A. Housley, T. R. Dykman, R. P. MacDermott, and J. P. Atkinson. 1985. Identification of an additional class of C3-binding membrane proteins of human peripheral blood leukocytes and cell lines. *Proc. Natl. Acad. Sci. USA* **82:**859–863.
45. Seya, T., J. R. Turner, and J. P. Atkinson. 1986. Purification and characterization of a membrane protein (gp45–70) that is a cofactor for cleavage of C3b and C4b. *J. Exp. Med.* **163:**837–855.
46. Johnson, P. M., H. M. Cheng, C. M. Molloy. C. M. M. Stern, and M. B. Slade. 1981. Human trophoblast-specific surface antigens identified using monoclonal antibodies. *Am. J. Reprod. Immunol.* **1:**246–254.
47. Hsi, B. L., C-J. G. Yeh, P. Fenichel, M. Samson, and C. Grivaux. 1988. Monoclonal antibody GB24 recognizes a trophoblast-lymphocyte cross-reactive antigen. *Am. J. Reprod. Immunol. Microbiol.* **18:**21–27.
48. Sparrow, R. L., and I. F. C. McKenzie. 1983. HuLy-m5: A unique antigen physically associated with HLA molecules. *Hum. Immunol.* **7:**1–15.
49. Andrews, P. W., B. B. Knowles, M. Parkar, B. Pym, K. Stanley, and P. N. Goodfellow. 1985. A human cell-surface antigen defined by a monoclonal antibody and controlled by a gene on human chromosome 1. *Ann. Hum. Genet.* **49:**31–39.
50. Purcell, D. F. J., I. F. C. McKenzie, D. M. Lublin, P. M. Johnson, and J. P. Atkinson. 1990. The human cell-surface glycoproteins HuLy-m5, membrane cofactor protein (MCP) of the complement system, and trophoblast-leukocyte common (TLX) antigen are CD46. *Immunology* **70:**155–161.
51. Cho, S. W., T. J. Oglesby, B. L. Hsi, E. M. Adams, and J. P. Atkinson. 1991. Characterization of three monoclonal antibodies to membrane cofactor protein (MCP) of the complement system and quantitation of MCP by radioassay. *Clin. Exp. Immunol.* **83:**257–261.
52. Naniche, D., G. Varior-Krishnan, F. Cervoni, T. F. Wild, B. Rossi, C. Rabourdin-Combe, and D. Gerlier. 1993. Human Membrane Cofactor Protein (CD46) acts as a cellular receptor for measles virus. *J. Virol.* **67:**6025–6032.
53. Dorig, R. E., A. Marcil, A. Chopra, and C. D. Richardson. 1993. The human CD46 molecule is a receptor for measles virus (Edmonton strain). *Cell* **75:**295–305.
54. Manchester, M., M. K. Liszewski, J. P. Atkinson, and M. B. A. Oldstone. 1994. Multiple isoforms of CD46 (membrane cofactor protein) serve as receptors for measles virus. *Proc. Natl. Acad. Sci. USA* **91:**2161–2165.

55. Dorig, R. E., A. Marcil, and C. D. Richardson. 1994. CD46, a primate-specific receptor for measles virus. *Trends Microbiol.* **2**:312–318.
56. Okada, N., M. K. Liszewski, J. P. Atkinson, and M. Caparon. 1995. Membrane Cofactor Protein (MCP; CD46) is a keratinocyte receptor for the M protein of group A streptococcus. *Proc. Natl. Acad. Sci. USA* **92**:2489–2493.
57. Rooney, I. A., T. J. Oglesby, and J. P. Atkinson. 1993. Complement in human reproduction: Activation and control. *Immunol. Res.* **12**:276–294.
58. Rooney, I. A., M. K. Liszewski, and J. P. Atkinson. 1993. Using membrane-bound complement regulatory proteins to inhibit rejection. *Xeno* **1**:29–35.
59. Ballard, L., T. Seya, J. Teckman, D. M. Lublin, and J. P. Atkinson. 1987. A polymorphism of the complement regulatory protein MCP (membrane cofactor protein or gp45-70). *J. Immunol.* **138**:3850–3855.
60. Bora, N. S., T. W. Post, and J. P. Atkinson. 1991. Membrane cofactor protein (MCP) of the complement system: A Hind III RFLP that correlates with expression polymorphism. *J. Immunol.* **146**:2821–2825.
61. Wilton, A. N., R. W. Johnstone, I. F. C. McKenzie, and D. F. J. Purcell. 1992. Strong associations between RFLP and protein polymorphisms for CD46. *Immunogenetics* **36**:79–85.
62. Risk, J. M., B. F. Flanagan, and P. M. Johnson. 1991. Polymorphism of the human CD46 gene in normal individuals and in recurrent spontaneous abortion. *Hum. Immunol.* **30**:162–167.
63. Post, T. W., M. K. Liszewski, E. M. Adams, I. Tedja, E. A. Miller, and J. P. Atkinson. 1991. Membrane cofactor protein of the complement system: Alternative splicing of serine/threonine/proline-rich exons and cytoplasmic tails produces multiple isoforms which correlate with protein phenotype. *J. Exp. Med.* **174**:93–102.
64. Liszewski, M. K., and J. P. Atkinson. 1996. Membrane cofactor protein (CD46) and decay accelerating factor (CD55). *In* The Complement System (M. Hansch, G. Till, and K. Rother, Eds.) Springer Verlag, New York.
65. Liszewski, M. K., T. W. Post, and J. P. Atkinson. 1991. Membrane cofactor protein (MCP or CD46): Newest member of the regulators of complement activation gene cluster. *Annu. Rev. Immunol.* **9**:431–455.
66. Johnstone, R. W., S. M. Russell, B. E. Loveland, and I. F. C. McKenzie. 1993. Polymorphic expression of CD46 protein isoforms due to tissue-specific RNA splicing. *Mol. Immunol.* **30**:1231–1241.
67. Purcell, D. F. J., S. M. Russell, N. J. Deacon, M. A. Brown, D. J. Hooker, and I. F. C. McKenzie. 1991. Alternatively spliced RNAs encode several isoforms of CD46 (MCP), a regulator of complement activation. *Immunogenetics* **33**:335–344.
68. Xing, P.-X., S. Russell, J. Prenzoska, and I. F. C. McKenzie. 1994. Discrimination between alternatively spliced STP-A and -B isoforms of CD46. *Immunology* **83**:122–127.
69. Ballard, L. L., N. S. Bora, G. H. Yu, and J. P. Atkinson. 1988. Biochemical characterization of membrane cofactor protein of the complement system. *J. Immunol.* **141**:3923–3939.
70. Reddy, P., I. Caras, and M. Kreiger. 1989. Effects of O-linked glycosylation on the cell surface expression and stability of decay-accelerating factor, a glycophospholipid-anchored membrane protein. *J. Biol. Chem.* **264**:17329–17336.
71. Coyne, K. E., S. E. Hall, S. Thompson, M. A. Arce, T. Kinoshita, T. Fujita, D. J. Anstee, W. Rosse, and D. M. Lublin. 1992. Mapping of epitopes, glycosylation sites and complement regulatory domains in human decay accelerating factor. *J. Immunol.* **149**:2906–2913.

72. Bora, N. S., D. M. Lublin, B. V. Kumar, R. D. Hockett, V. M. Holers, and J. P. Atkinson. 1989. Structural gene for human membrane cofactor protein (MCP) of complement maps to within 100 kb of the 3′ end of the C3b/C4b receptor gene. *J. Exp. Med.* **169:**597–602.
73. Cui, W., D. Hourcade, T. W. Post, A. C. Greenlund, J. P. Atkinson, and V. Kumar. 1993. Characterization of the promoter region of the membrane cofactor protein (CD46) gene of the human complement system and comparison to a membrane cofactor protein-like genetic element. *J. Immunol.* **151:**4137–4146.
74. Hourcade, D., A. D. Garcia, T. W. Post, P. Taillon-Miller, V. M. Holers, L. M. Wagner, N. S. Bora, and J. P. Atkinson. 1992. Analysis of the human regulators of complement activation (RCA) gene cluster with yeast artificial chromosomes (YACs). *Genomics* **12:**289–300.
75. Liszewski, M. K., I. Tedja, and J. P. Atkinson. 1994. Membrane Cofactor Protein (CD46) of complement: Processing differences related to alternatively spliced cytoplasmic domains. *J. Biol. Chem.* **269:**10776–10779.
76. Liszewski, M. K., and J. P. Atkinson. 1992. Membrane cofactor protein. *Curr. Top. Microbiol. Immunol.* **178:**45–60.
77. Nickells, M. W., J. I. Alvarez, D. M. Lublin, and J. P. Atkinson. 1994. Characterization of DAF-2, a high molecular weight form of decay-accelerating factor (DAF;CD55), as a covalently cross-linked dimer of DAF-1. *J. Immunol.* **152:**676–685.
78. Nickells, M. W., and J. P. Atkinson. 1990. Characterization of CR1 and membrane cofactor protein-like proteins of two primates. *J. Immunol.* **144:**4262–4268.
79. Purcell, D. F. J., G. J. Clark, M. A. Brown, I. F. C. McKenzie, M. S. Sandrin, and J. J. Deacon. 1990. HuLy-m5, an antigen sharing epitopes with envelope gp70 molecules of primate retroviruses, and a structural relationship with complement regulatory molecules. *In* White Cell Differentiation Antigens (W. Knapp, Ed.) pp. 653–656. Oxford University Press, Oxford.
80. Hara, T., S. Kuriyama, H. Kiyohara, Y. Nagase, M. Matsumoto, and T. Seya. 1992. Soluble forms of membrane cofactor protein (CD46,MCP) are present in plasma, tears, and seminal fluid in normal subjects. *Clin. Exp. Immunol.* **89:**490–494.
81. Seya, T., T. Hara, K. Iwata, S-I. Kuriyama, T. Hasegawa, Y. Nagase, S. Miyagawa, M. Matsumoto, M. Hatanaka, J. P. Atkinson, and S. Nagasawa. 1995. Purification and functional properties of soluble forms of membrane cofactor protein (CD46) of complement: Identification of forms increased in cancer patients' sera. *Int. Immunol.* **7:**727–736.
82. Seya, T., L. L. Ballard, N. S. Bora, V. Kumar, W. Cui, and J. P. Atkinson. 1988. Distribution of membrane cofactor protein (MCP) of complement on human peripheral blood cells. An altered form is found on granulocytes. *Eur. J. Immunol.* **18:**1289–1294.
83. Cervoni, F., T. J. Oglesby, M. Nickells, C. Milesi-Fluet, P. Fenichel, J. P. Atkinson, and B. L. Hsi. 1992. Identification and characterization of membrane cofactor protein (MCP) on human spermatozoa. *J. Immunol.* **148:**1431–1437.
84. Seya, T., T. Hara, M. Matsumoto, H. Kiyohara, I. Nakanishi, T. Kinouchi, M. Okabe, A. Shimizu, and H. Akedo. 1993. Membrane cofactor protein (MCP,CD46) in seminal plasma and on spermatozoa in normal and "sterile" subjects. *Eur. J. Immunol.* **23:**1322–1327.
85. Endoh, M., Yamashina, M., H. Ohi, K. Funashashi, T. Ikuno, T. Yasugi, J. P. Atkinson, and H. Okada. 1993. Immunohistochemical demonstration of membrane cofactor protein (MCP) of complement in normal and diseased kidney tissues. *Clin. Exp. Immunol.* **94:**182–188.

86. Nakanishi, I., A. Moutabarrik, T. Hara, M. Hatanaka, T. Hayashi, T. Syouji, N. Okada, E. Kitamura, Y. Tsubakihara, and M. Matsumoto. 1994. Identification and characterization of membrane cofactor protein (CD46) in the human kidneys. *Eur. J. Immunol.* **24**:1529–1535.
87. Ichida, S., Y. Yuzawa, H. Okada, K. Yoshioka, and S. Matsuo. 1994. Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int.* **46**:89–96.
88. Sayama, K., S. Shiraishi, Y. Shirakata, Y. Kobayashi, T. Seya, and Y. Miki, 1991. Expression and characterization of membrane cofactor protein (MCP) in human skin. *J. Invest. Dermatol.* **97**:722–724.
89. Sayama, K., S. Shiraishi, and Y. Miki. 1992. Distribution of complement regulators (CD46,CD55, and CD59) in skin appendages, and in benign and malignant skin neoplasms. *Br. J. Dermatol.* **127**:1–4.
90. Bora, N. S., C. L. Gobleman, J. P. Atkinson, J. S. Pepose, and H. J. Kaplan. 1993. Differential expression of the complement regulatory proteins in the human eye. *Invest. Ophthalmol. Vis. Sci.* **34**:3579–3584.
91. Yamakawa, M., K. Yamada, T. Tsuge, H. Ohrui, T. Ogata, M. Dobashi, and Y. Imai. 1994. Protection of thyroid cancer cells by complement-regulatory factors. *Cancer* **73**:2808–2817.
92. Tandon, N., S. L. Yan, B. P. Morgan, and A. P. Weetman. 1994. Expression and function of multiple regulators of complement activation in autoimmune thyroid disease. *Immunology* **81**:643–647.
93. Simpson, K. L., M. M. Houlihan, and C. H. Holmes. 1993. Complement regulatory proteins in early human fetal life: CD59, membrane cofactor protein (MCP) and decay-accelerating factor (DAF) are differentially expressed in the developing liver. *Immunology* **80**:183–190.
94. Scoazec, J. Y., D. Delautier, A. Moreau, F. Durand, C. Degott, J. P. Benhamou, J. Belghiti, and G. Feldmann. 1994. Expression of complement-regulatory proteins in normal and UW-preserved human liver. *Gastroenterology* **107**:505–516.
95. Holmes, C. H., K. L. Simpson, H. Okada, N. Okada, S. D. Wainwright, D. F. J. Purcell, and J. M. Houlihan. 1992. Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). *Eur. J. Immunol.* **22**:1579–1585.
96. Hsi, B. L., J. S. Hunt, and J. P. Atkinson. 1991. Differential expression of complement regulatory proteins on subpopulations of human trophoblast cells. *J. Reprod. Immunol.* **19**:209–223.
97. Simpson, K. L., and C. H. Holmes. 1994. Differential expression of complement regulatory proteins decay-accelerating factor (CD55), membrane cofactor protein (CD46) and CD59 during human spermatogenesis. *Immunology* **81**:452–461.
98. Hara, T., A. Kojrima, H. Fukuda, T. Masaoka, Y. Fukumori, M. Matsumoto, and T. Seya. 1992. Levels of complement regulatory proteins, CD3 (CR1), CD46 (MCP) and CD55 (DAF) in human haematological malignancies. *Br. J. Haematol.* **82**:368–373.
99. Seya, T., T. Hara, M. Matsumoto, and H. Akedo. 1990. Quantitative analysis of membrane cofactor protein (MCP) of complement. *J. Immunol.* **145**:238–245.
100. McNearney, T., L. Ballard, T. Seya, and J. P. Atkinson. 1989. Membrane cofactor protein of complement is present on human fibroblast, epithelial and endothelial cells. *J. Clin. Invest.* **84**:538–545.
101. Gordon, D. L., T. A. Sadlon, S. L. Wesselingh, S. M. Russell, R. W. Johnstone, and D. F. J. Purcell. 1992. Human astrocytes express membrane cofactor protein (CD46), a regulator of complement activation. *J. Neuroimmunol.* **36**:199–208.

102. Moutabarrik, A., I. Nakanishi, M. Namiki, T. Hara, M. Matsumoto, M. Ishibashi, A. Okuyama, D. Zaid, and T. Seya. 1993. Cytokine-mediated regulation of the surface expression of complement regulatory proteins CD46 (MCP), CD55 (DAF) and CD59 on human vascular endothelial cells. *Lymphokine Cytokine Res.* **12:**167–172.

103. Adams, E. M., M. C. Brown, M. Nunge, M. Krych, and J. P. Atkinson. 1991. Contribution of the repeating domains of membrane cofactor protein (MCP;CD46) of the complement system to ligand binding and cofactor activity. *J. Immunol.* **147:**3005–3011.

104. Oglesby, T. J., C. J. Allen, M. K. Liszewski, D. J. G. White, and J. P. Atkinson. 1992. Membrane cofactor protein (MCP;CD46) protects cells from complement-mediated attack by an intrinsic mechanism. *J. Exp. Med.* **175:**1547–1551.

105. Lublin, D. M., and J. P. Atkinson. 1989. Decay-accelerating factor and membrane cofactor protein. *Curr. Top. Microbiol. Immunol.* **153:**123–145.

106. Seya, T., and J. P. Atkinson. 1989. Functional properties of membrane cofactor protein of complement. *Biochem. J.* **64:**581–588.

107. Seya, T., T. Hara, M. Matsumoto, Y. Sugita, and H. Akedo. 1990. Complement-mediated tumor cell damage induced by antibodies against membrane cofactor protein (MCP,CD46). *J. Exp. Med.* **172:**1673–1677.

108. Lublin, D. M., and K. E. Coyne. 1991. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. *J. Exp. Med.* **174:**35–44.

109. Iwata, K., T. Seya, H. Ariga, and S. Nagasawa. 1994. Expression of a hybrid complement regulatory protein, membrane cofactor protein decay accelerating factor on Chinese hamster ovary. *J. Immunol.* **152:**3436–3444.

110. Seya, T., M. Okada, M. Matsumoto, K. Hong, T. Kinoshita, and J. P. Atkinson. 1991. Preferential inactivation of the C5 convertase of the alternative complement pathway by factor I and membrane cofactor protein (MCP). *Mol. Immunol.* **28:**1137–1147.

111. Fujita, T., T. Inoue, K. Ogawa, K. Iida, and N. Tamura. 1987. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J. Exp. Med.* **166:**1221–1228.

112. Anderson, D. J., J. S. Michaelson, and P. M. Johnson. 1989. Trophoblast/leukocyte-common antigen is expressed by human testicular germ cells and appears on the surface of acrosome-reacted sperm. *Biol. Reprod.* **41:**285–293.

113. Anderson, D. J., A. F. Abbott, and R. M. Jack. 1993. The role of complement component C3b and its receptors in sperm-oocyte interaction. *Proc. Natl. Acad. Sci. USA* **90:**10051–10055.

114. Platt, J. L., G. M. Vercellotti, A. P. Dalmasso, A. J. Matas, R. M. Bolman, J. S. Najarian, and F. H. Bach. 1990. Transplantation of discordant xenografts: A review of progress. *Immunol. Today* **12:**450–456.

115. Nowak, R. 1994. Xenotransplants set to resume. *Science* **266:**1148–1151.

116. McCurry, K. R., D. L. Kooyman, C. G. Alvarado, A. H. Cotterell, M. J. Martin, J. S. Logan, and J. L. Platt. 1995. Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nature Med.* **1:**423–427.

117. Moore, F. D., Jr. 1994. Therapeutic regulation of the complement system in acute injury states. *Adv. Immunol.* **56:**267–299.

118. Moran, P., H. Beasley, A. Gorrell, E. Martin, P. Gribling, H. Fuchs, N. Gillett, L. E. Burton, and I. W. Caras. 1992. Human recombinant soluble decay accelerating factor inhibits complement activation in vitro and in vivo. *J. Immunol.* **149:**1736–1743.

119. Cooper, N. R. 1991. Complement evasion strategies of microorganisms. *Immunol. Today* **12:**327–331.
120. Weiss, R. 1992. Measles battle loses potent weapon. *Science* **258:**546–547.
121. Gerlier, D., B. Loveland, G. Varior-Krishnan, B. Thorley, I. F. C. McKenzie, and C. Rabourdin-Combe. 1994. Measles virus receptor properties are shared by several CD46 isoforms differing in extracellular regions and cytoplasmic tails. *J. Gen. Virol.* **75:**2163–2171.
122. Manchester, M., A. Valsamakis, R. Kaufman, M. K. Liszewski, J. Alvarez, J. P. Atkinson, D. M. Lublin, and M. B. A. Oldstone. 1995. Measles virus and C3 binding sites are distinct on membrane cofactor protein (CD46). *Proc. Natl. Acad. Sci. USA* **92:**2303–2307.
123. Varior-Krishnan, G., M. C. Trescol-Biemont, D. Naniche, C. Rabourdin-Combe, and D. Gerlier. 1994. Glycosyl-phosphatidylinositol-anchored and transmembrane forms of CD46 display similar measles virus receptor properties: virus binding, fusion, and replication; down-regulation by hemagglutinin; and virus uptake and endocytosis for antigen presentation by major histocompatibility complex class II molecules. *J. Virol.* **68:**7891–7899.
124. Maisner, A., J. Schneider-Schaulies, M. K. Liszewski, J. P. Atkinson, and G. Herrler. 1994. Binding of measles virus to membrane cofactor protein (CD46): Importance of disulfide bonds and N-glycans for the receptor function. *J. Virol.* **68:**6299–6304.
125. Weis, J. J., S. K. Law, R. P. Levine, and P. P. Cleary. 1985. Resistance to phagocytosis by group A streptococci: Failure of deposited complement opsonins to interact with cellular receptors. *J. Immunol.* **134:**500–505.
126. Horstmann, R. D., H. J. Sievertsen, J. Kbobloch, and V. A. Fischetti. 1988. Antiphagocytic activity of streptococcal M protein: Selective binding of complement control protein factor H. *Proc. Natl. Acad. Sci. USA* **85:**1657–1661.
127. Montefiori, D. C., R. J. Cornell, J. Y. Zhou, J. T. Zhou, V. M. Hirsch, and P. R. Johnson. 1994. Complement control proteins, CD45, CD55, and CD59, as common surface constituents of human and simian immunodeficiency viruses and possible targets for vaccine protection. *Virology* **205:**82–92.
128. Saifuddin, M., C. J. Parker, M. E. Peeples, M. K. Gorny, S. Zolla-Pazner, M. Ghassemi, I. A. Rooney, J. P. Atkinson, and G. T. Spear. 1995. Role of virion-associated glycosylphosphatidylinositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. *J. Exp. Med.* **182:**501–509.
129. Hoffmann, E. M. 1969. Inhibition of complement by a substance isolated from human erythrocytes. I. Extraction from human erythrocyte stromata. *Immunochemistry* **6:**391–403.
130. Hoffmann, E. M. 1969. Inhibition of complement by a substance isolated from human erythrocytes. II. Studies on the site and mechanism of action. *Immunochemistry* **6:**405–419.
131. Nicholson-Weller, A., J. Burge, and K. F. Austen. 1981. Purification from guinea pig erythrocyte stroma of a decay-accelerating factor for the classical C3 convertase, C4b,2a. *J. Immunol.* **127:**2035–2039.
132. Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J. Immunol.* **129:**184–189.
133. Lublin, D. M., and J. P. Atkinson. 1989. Decay-accelerating factor: Biochemistry, molecular biology, and function. *Annu. Rev. Immunol.* **7:**35–58.
134. Nicholson-Weller, A. 1992. Decay accelerating factor (CD55). *Curr. Topics Microbiol. Immunol.* **178:**7–30.

135. Medof, M. E., D. M. Lublin, V. M. Holers, D. J. Ayers, R. R. Getty, J. F. Leykam, J. P. Atkinson, and M. L. Tykocinski. 1987. Cloning and characterization of cDNAs encoding the complete sequence of decay-accelerating factor of human complement. *Proc. Natl. Acad. Sci. USA* **84:**2007–2011.

136. Caras, I. W., M. A. Davitz, L. Rhee, G. Weddell, D. W. Martin, Jr., and V. Nussenzweig. 1987. Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. *Nature* **325:**545–549.

137. Nakano, Y., K. Sumida, N. Kikuta, N. H. Muira, T. Tobe, and M. Tomita. 1992. Complete determination of disulfide bonds localized within the short consensus repeat units of decay accelerating factor (CD55 antigen). *Biochem. Biophys. Acta* **1116:**235–240.

138. Post, T. W., M. A. Arce, M. K. Liszewski, E. S. Thompson, J. P. Atkinson, and D. M. Lublin. 1990. Structure of the gene for human complement protein decay accelerating factor. *J. Immunol.* **144:**740–744.

139. Lublin, D. M., R. S. Lemons, M. M. LeBeau, V. M. Holers, M. L. Tykocinski, M. E. Medof, and J. P. Atkinson. 1987. The gene encoding decay-accelerating factor (DAF) is located in the complement-regulatory locus on the long arm of chromosome 1. *J. Exp. Med.* **165:**1731–1736.

140. Ewulonu, U. K., L. Ravi, and M. E. Medof. 1991. Characterization of the decay-accelerating factor gene promoter region. *Proc. Natl. Acad. Sci. USA* **88:**4675–4679.

141. Thomas, D. J., and D. M. Lublin. 1993. Identification of 5′ flanking regions affecting the expression of the human decay accelerating factor gene and their role in tissue-specific expression. *J. Immunol.* **150:**151–160.

142. Lublin, D. M., J. Krsek-Staples, M. K. Pangburn, and J. P. Atkinson. 1986. Biosynthesis and glycosylation of the human complement regulatory protein decay-accelerating factor. *J. Immunol.* **137:**1629–1635.

143. Davitz, M. A., M. G. Low, and V. Nussenzweig. 1986. Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC). Selective modification of a complement regulatory protein. *J. Exp. Med.* **163:**1150–1161.

144. Medof, M. E., E. I. Walter, W. L. Roberts, R. Haas, and T. L. Rosenberry. 1986. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. *Biochemistry* **25:**6740–6747.

145. Medof, M. E., T. Kinoshita, and V. Nussenzweig. 1984. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. *J. Exp. Med.* **160:**1558–1578.

146. Moran, P., H. Raab, W. J. Kohr, and I. W. Caras. 1991. Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site. *J. Biol. Chem.* **266:**1250–1257.

147. Kinoshita, T., S. I. Rosenfield, and V. Nussenzweig. 1987. A high MW form of decay-accelerating factor (DAF-2) exhibits size abnormalities in paroxysmal nocturnal hemoglobinuria erythrocytes. *J. Immunol.* **138:**2994–2998.

148. Medof, M. E., E. I. Walter, J. L. Rutgers, D. M. Knowles, and V. Nussenzweig. 1987. Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J. Exp. Med.* **165:**848–864.

149. Kameyoshi, Y., M. Matsushita, and H. Okada. 1989. Murine membrane inhibitor of complement which accelerates decay of human C3 convertase. *Immunology* **68:**439–444.

150. Sugita, Y., M. Uzawa, and M. Tomita. 1987. Isolation of decay-accelerating factor (DAF) from rabbit erythrocyte membranes. *J. Immunol. Methods* **104:**123–130.

151. Spicer, A. P., M. F. Seldin, and S. J. Gendler. 1995. Molecular cloning and chromosomal localization of the mouse decay-accelerating factor (DAF) genes: Duplicated genes: encode GPI-anchored and transmembrane forms. *J. Immunol.* **155:**3079–3091.
152. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1983. Deficiency of an erythrocyte membrane protein with complement regulatory activity in paroxysmal nocturnal hemoglobinuria. *Proc. Natl. Acad. Sci. USA* **80:**5430–5434.
153. Kinoshita, T., M. E. Medof, and V. Nussenzweig. 1986. Endogenous association of decay-accelerating factor (DAF) with C4b and C3b on cell membranes. *J. Immunol.* **136:**3390–3395.
154. Pangburn, M. K. 1986. Differences between the binding sites of the complement regulatory proteins DAF, CR1, and factor H on C3 convertases. *J. Immunol.* **136:**2216–2221.
155. Asch, A. S., T. Kinoshita, E. A. Jaffe, and V. Nussenzweig. 1986. Decay-accelerating factor is present on cultured human umbilical vein endothelial cells. *J. Exp. Med.* **163:**221–226.
156. Holmes, C. H., K. L. Simpson, S. D. Wainwright, C. G. Tate, J. M. Houlihan, I. H. Sawyer, I. P. Rogers, F. A. Spring, D. J. Anstee, and M. J. Tanner. 1990; Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy. *J. Immunol.* **144:**3099–3105.
157. Wilcox, L. A., J. L. Ezzell, N. J. Bernshaw, and C. J. Parker. 1991. Molecular basis of the enhanced susceptibility of the erythrocytes of paroxysmal nocturnal hemoglobinuria to hemolysis in acidified serum. *Blood* **78:**820–829.
158. Brooimans, R. A., P. A. M. van Wieringer, L. A. van Es, and M. R. Daha. 1992. Relative roles of decay-accelerating factor, membrane cofactor protein, and CD59 in the protection of human endothelial cells against complement-mediated lysis. *Eur. J. Immunol.* **22:**3135–3140.
159. White, D. J. G., T. Oglesby, M. K. Liszewski, I. Tedja, D. Hourcade, M.-W. Wang, L. Wright, J. Wallwork, and J. P. Atkinson. 1992. Expression of human decay accelerating factor or membrane cofactor protein genes on mouse cells inhibits lysis by human complement. *Transplant Proc.* **24:**474–476.
160. Zhao, J., S. A. Rollins, S. E. Maher, A. L. M. Bothwell, and P. J. Sims. 1991. Amplified gene expression in CD59-transfected Chinese hamster ovary cells confers protection against the membrane attack complex of human complement. *J. Biol. Chem.* **266:**13418–13422.
161. Walsh, L. A., M. Tone, and H. Waldmann. 1991. Transfection of CD59 complementary DNA into rat cells confers resistance to human complement. *Eur. J. Immunol.* **21:**847–850.
162. Yamashina, M., E. Ueda, T. Kinoshita, T. Takami, A. Ojima, H. Ono, H. Tanaka, N. Kondo, T. Orii, and N. Oakda. 1990. Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. *N. Eng. J. Med.* **323:**1184–1189.
163. Telen, M. J., S. E. Hall, A. M. Green, J. J. Moulds, and W. F. Rosse. 1988. Identification of human erythrocyte blood group antigens on decay-accelerating factor (DAF) and an erythrocyte phenotype negative for DAF. *J. Exp. Med.* **167:**1993–1998.
164. Telen, M. J., and A. M. Green. 1989. The Inab phenotype: Characterization of the membrane protein and complement regulatory defect. *Blood* **74:**437–441.
165. Miyagawa, S., R. Shirakura, G. Matsumiya, S. Nakata, M. Hatanaka, M. Matsumoto, H. Kitamura, and T. Seya. 1993. Test for ability of decay-accelerating factor (DAF,CD55) and CD59 to alleviate complement-mediated damage of xeno-erythrocytes. *Scand. J. Immunol.* **38:**37–44.

166. Thomas, J., W. Webb, M. A. Davitz, and V. Nussenzweig. 1987. Decay accelerating factor diffuses rapidly on $HeLa_{AE}$ cell surfaces. *Biophys. J.* **51**:522a.

167. Zhang, F., B. Crise, B. Su, Y. Hou, J. K. Rose, A. Bothwell, and K. Jacobson. 1991. Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol-linked proteins: Toward establishing rules governing the lateral mobility of membrane proteins. *J. Cell Biol.* **115**:75–84.

168. Davis, L. S., S. S. Patel, J. P. Atkinson, and P. E. Lipsky. 1988. Decay-accelerating factor functions as a signal transducing molecule for human T cells. *J. Immunol.* **141**:2246–2252.

169. Shenoy-Scaria, A. M., J. Kwong, T. Fujita, M. W. Olszowy, A. S. Shaw, and D. M. Lublin. 1992. Signal transduction through decay accelerating factor: Interaction of GPI anchor and protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. *J. Immunol.* **149**:3535–3541.

170. Shenoy-Scaria, A. M., D. J. Dietzen, J. Kwong, D. C. Link, and D. M. Lublin. 1994. $Cysteine^3$ of src family protein tyrosine kinase determines palmitoylation and localization in caveolae. *J. Cell Biol.* **126**:353–363.

171. Nowicki, B., A. Hart, K. E. Coyne, D. M. Lublin, and S. Nowicki. 1993. Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by Escherichia coli recombinat Dr adhesion in a model of a cell-cell interaction. *J. Exp. Med.* **178**:2115–2121.

172. Bergelson, J. M., M. Chan, K. Solomon, N. F. St. John, H. Lin, and R. W. Finberg. 1994. Decay-accelerating factor, a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proc. Natl. Acad. Sci. USA* **91**:6245–6248.

173. Ward, T., P. A. Pipkin, N. A. Clarkson, D. M. Stone, P. D. Minor, and J. W. Almond. 1994. Decay accelerating factor (CD55) identified as a receptor for echovirus 7 using CELICS, a rapid immuno-focal cloning method. *EMBO J.* **13**:5070–5074.

174. Bergelson, J. M., J. G. Mohanty, R. L. Crowell, N. F. St.John, D. M. Lublin, and R. W. Finberg. 1995. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). *J. Virol.* **69**:1903–1906.

175. Dalmasso, A. P., G. M. Vercellotti, J. L. Platt, and F. H. Bach. 1991. Inhibiton of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. Potential for prevention of xenograft hyperacute rejection. *Transplantation* **52**:530–533.

176. Ahearn, J. M., and D. T. Fearon. 1989. Structure and function of the complement receptors. CR1 (CD35) and CR2 (CD21). *Adv. Immunol.* **46**:183–219.

177. Krych, M., L. Clemenza, D. Howdeshell, R. Hauhart, D. Hourcade, and J. P. Atkinson. 1994. Analysis of the functional domains of complement receptor type 1 (C3b/C4b receptor; CD35) by substitution mutagenesis. *J. Biol. Chem.* **269**:13273–13278.

178. Klickstein, L. B., T. J. Bartow, V. Miletic, L. D. Rabson, J. A. Smith, and D. T. Fearon. 1988. Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. *J. Exp. Med.* **168**:1699–1717.

179. Krych, M., D. Hourcade, and J. P. Atkinson. 1991. Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. *Proc. Natl. Acad. Sci. USA* **88**:4353–4357.

180. Medof, M. E., G. M. Prince, and C. Mold. 1982. Release of soluble immune complexes from immune adherence receptors on human erythrocytes is mediated by C3b inactivator independently of B1H and is accompanied by generation of C3c. *Proc. Natl. Acad. Sci. USA* **79**:5047–5051.

181. Walport, J. J., and P. J. Lachmann. 1988. Erythrocyte complement receptor type 1, immune complexes, and the rheumatic diseases. *Arthritis Rheum.* **31**:153–158.

182. Liszewski, M. K., and J. P. Atkinson. 1991. The role of complement in autoimmunity. *In* Systemic Autoimmunity (M. R. Reichlin and P. Bigazzi, Eds.), pp. 13–37. Marcel Dekker, New York.
183. Kim, S-Y., P. Braverman, and D. Fearon. 1993. Relative complement regulatory functions of membrane CR1, decay-accelerating factor (DAF) and membrane cofactor protein (MCP). *Mol. Immunol.* **30**(Suppl. 1): 23. [Abstract]
184. Weisman, H. F., T. Bartow, M. K. Leppo, Jr., H. C. Marsh, G. R. Carson, M. F. Concino, M. P. Boyle, K. H. Roux, M. L. Weisfeldt, and D. T. Fearon. 1990. Soluble human complement receptor type 1: In vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* **249:**146–151.
185. Moore, Jr., F. D. 1994. Therapeutic regulation of the complement system in acute injury states. *Adv. Immunol.* **56:**267–299.
186. Erdei, A., G. Fust, and J. Gergely. 1991. The role of C3 in the immune response. *Immunol. Today* **12:**332–337.
187. Molina, H., C. Brenner, S. Jacobi, J. Gorka, J. C. Carel, T. Kinoshita, and V. M. Holers. 1991. Analysis of Epstein-Barr virus-binding sites on complement receptor 2 (CR2/CD21) using human-mouse chimeras and peptides. *J. Biol. Chem.* **266:**12173–12179.
188. Nemerow, G. R., R. A. Houghten, M. D. Moore, and N. R. Cooper. 1989. Identification of an epitope in the major envelope protein of Esptein-Barr virus that mediates viral binding to the B lymphocyte EBV receptor (CR2). *Cell* **56:**369–377.
189. Mitomo, K., T. Fujita, and K. Iida. 1987. Functional and antigenic properties of complement receptor type 2, CR2. *J. Exp. Med.* **165:**1424.
190. Whaley, K., and S. Ruddy. 1976. Modulation of C3b hemolytic activity by a plasma protein distinct from C3b inactivator. *Science* **193:**1011–1013.
191. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein beta 1H. *Proc. Natl. Acad. Sci. USA* **73:**3268–3272.
192. Thompson, R. A., and M. H. Winterborn. 1981. Hypocomplementaemia due to a genetic deficiency of beta 1H globulin. *Clin. Exp. Immunol.* **27:**23–29.
193. Perkins, S. J., A. S. Nealis, and R. B. Sim. 1991. Oligomeric domain structure of human complement factor H by X-ray and neutron solution scattering. *Biochemistry* **30:**2847–2857.
194. Ripoche, J., A. J. Day, T. J. R. Harris, and R. B. Sim. 1988. The complete amino acid sequence of human complement factor H. *Biochem. J.* **249:**593–602.
195. Kristensen, T., and B. F. Tack. 1986. Murine protein H is comprised of 20 repeating units, 61 amino acids in length. *Proc. Natl. Acad. Sci. USA* **83:**3963–3967.
196. Alsenz, J., J. D. Lambris, T. F. Schulz, and M. P. Dierich. 1984. Localization of the complement-component-C3b-binding site and the cofactor activity for factor I in the 38 kDa tryptic fragment of factor H. *Biochem. J.* **224:**389–398.
197. Misasi, R., H. P. Huemer, W. Schwaeble, E. Solder, C. Larcher, and M. P. Dierich. 1989. Human complement factor H: An additional gene product of 43 kDa isolated from human plasma shows cofactor activity for the cleavage of the third component of complement. *Eur. J. Immunol.* **19:**1765–1768.
198. Weiss, E. H., W. Schwaeble, A. Steinkasserer, and R. B. Sim. 1993. Genomic organization, evolution and sequence analysis of the human factor H gene family. *Mol. Immunol.* **30**(suppl. 1):62.
199. Vik, D. P., J. B. Keeney, P. Munoz-Canoves, D. D. Chaplin, and B. F. Tack. 1988. Structure of the murine complement factor H gene. *J. Biol. Chem.* **263:**16720–16724.
200. Hogasen, K., J. H. Jansen, T. E. Mollnes, J. Hovdenes, and M. Harboe. 1995. Hereditary porcine membranoproliferative glomerulonephritis type II is caused by factor H deficiency. *J. Clin. Invest.* **95:**1054–1061.

201. Katz, Y., and R. C. Strunk. 1988. Synthesis and regulation of complement protein factor H in human skin fibroblasts. *J. Immunol.* **141**:559–563.

202. Munoz-Canaves, P., B. F. Tack, and D. P. Vik. 1989. Analysis of complement factor H mRNA expression: Dexamethasone and IFN-gamma increase the level of H in L cells. *Biochemistry* **28**:9891–9897.

203. Brooimans, R. A., A. A. van der Ark, W. A. Buurman, L. A. van Es, and M. R. Daha. 1990. Differential regulation of complement factor H and C3 production in human umbilical vein endothelial cells by IFN-gamma and IL-1. *J. Immunol.* **144**:3835–3840.

204. Pangburn, M. K., and H. J. Muller-Eberhard. 1983. Kinetic and thermodynamic analysis of the control of C3b by the complement regulatory proteins factors H and I. *Biochemistry* **22**:178–185.

205. DiScipio, R. G. 1981. The binding of human complement proteins C5, factor B, beta 1H and properdin to complement fragment C3b on zymosan. *Biochem. J.* **199**:485–496.

206. Farries, T. C., T. Seya, R. A. Harrison, and J. P. Atkinson. 1990. Competition for binding sites on C3b by CR1, CR2, MPC, factor B and factor H. *Compl. Inflamm.* **7**:30–41.

207. Fishelson, Z. 1991. Complement C3: A molecular mosaic of binding sites. *Mol. Immunol.* **28**:545–552.

208. Lambris, J. D., D. Avila, J. D. Becherer, and H. J. Muller-Eberhard. 1988. A discontinuous factor H binding site in the third component of complement as delineated by synthetic peptides. *J. Biol. Chem.* **263**:12147–12150.

209. Farries, T. C., P. J. Lachmann, and R. A. Harrison. 1988. Analysis of the interactions between properdin, the third component of complement (C3), and its physiological activation products. *Biochem. J.* **252**:47–54.

210. Pangburn, M. K., and H. J. Muller-Eberhard. 1978. Complement C3 convertase: Cell surface restriction of beta 1H control and generation of restriction on neuraminidase treated cells. *Proc. Natl. Acad. Sci. USA* **75**:2416–2420.

211. Isenman, D. E. 1983. Conformational changes accompanying proteolytic cleavage of human complement protein C3b by the regulatory enzyme factor I and its cofactor H. Spectroscopic and enzymological studies. *J. Biol. Chem.* **258**:4238–4244.

212. Fearon, D. T., and K. F. Austen. 1977. Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Proc. Natl. Acad. Sci. USA* **74**:1683–1687.

213. Fries, L. F., T. A. Gaither, C. H. Hammer, and M. M. Frank. 1984. C3b covalently bound to IgG demonstrates a reduced rate of inactivation by factors H and I. *J. Exp. Med.* **160**:1640–1655.

214. Carreno, M. P., D. Labarre, F. Maillet, M. Jozenfowicz, and M. D. Kazatchkine. 1989. Regulation of the human alternative complement pathway: Formation of a ternary complex between factor H, surface-bound C3b and chemical groups on nonactivating surfaces. *Eur. J. Immunol* **19**:2145–2150.

215. Meri, S., and M. K. Pangburn. 1990. Discrimination between activators and nonactivators of the alternative pathway of complement: Regulation via a sialic acid/polyanion binding site on factor H. *Proc. Natl. Acad. Sci. USA* **87**:3982–3986.

216. Koistinen, V. 1993. Effects of sulphated polyanions on functions of complement factor H. *Mol. Immunol.* **30**:113–118.

217. Bitter-Suermann, D., R. Burger, and U. Hadding. 1981. Activation of the alternative pathway of complement: Efficient fluid-phase amplification by blockade of the regulatory complement protein beta 1H through sulfated polyanions. *Eur. J. Immunol.* **11**:291–295.

218. Winsnes, R., and P. J. Lachmann. 1982. Intestinal glycoprotein activates the alternative complement pathway by reacting with factor H. *Scand. J. Immunol.* **15**:371–378.

219. Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti. 1988. Antiphagocytic activity of streptococcal M protein: Selective binding of complement control protein factor H. *Proc. Natl. Acad. Sci. USA* **85:**1657–1661.
220. Meri, S., and M. K. Pangburn. 1994. Regulation of alternative pathway complement activation by glycosaminoglycans: Specificity of the polyanion binding site on factor H. *Biochem. Biophys. Res. Commun.* **198:**52–59.
221. Zipfel, P. F., and C. Skerka. 1994. Complement factor H and related proteins: An expanding family of complement-regulatory proteins? *Immunol. Today* **15:**121–126.
222. Skerka, C., S. Kuhn, K. Gunther, K. Lingelbach, and P. F. Zipfel. 1993. A novel short consensus repeat-containing molecule is related to human complement factor H. *J. Biol. Chem.* **268:**2904–2908.
223. Vik, D. P., P. Munoz-Canoves, H. Kozono, L. G. Martin, B. F. Tack, and D. D. Chaplin. 1990. Identification and sequence analysis of four complement factor H-related transcripts in mouse liver. *J. Biol. Chem.* **265:**3193–3201.
224. Chung, L. P., D. R. Bentley, and K. B. M. Reid. 1985. Molecular cloning and characterization of the cDNA coding for C4b-binding protein, a regulatory protein of the classical pathway of the human complement system. *Biochem. J.* **230:**133–141.
225. Dahlback, B., C. A. Smith, and H. J. Muller-Eberhard. 1983. Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. *Proc. Natl. Acad. Sci. USA* **80:**3461–3465.
226. Perkins, S. J., L. P. Chung, and K. B. M. Reid. 1986. Unusual ultrastructure of complement-component-C4b-binding protein of human complement by synchrotron X-ray scattering and hydrodynamic analysis. *Biochem. J* **233:**799–807.
227. Hillarp, A., and B. Dahlback. 1988. Novel subunit in C4b-binding protein required for protein S binding. *J. Biol Chem* **263:**12759–12764.
228. Hillarp, A., M. Hessing, and B. Dahlback. 1989. Protein S Binding in relation to the subunit composition of human C4b-binding protein. *FEBS. Lett.* **259:**53–56.
229. Kristensen, T., R. T. Ogata, L. P. Chung, K. B. M. Reid, and B. F. Tack. 1987. cDNA structure of murine C4b-binding protein, a regulatory component of the serum complement system. *Biochemistry* **26:**4668–4674.
230. Chung, L. P., and K. B. M. Reid. 1985. Structural and functional studies on C4b-binding protein, a regulatory component of the human complement system. *Biosci. Rep.* **5:**855–865.
231. Ogata, R. T., P. Mathias, B. M. Bradt, and N. R. Cooper. 1993. Murine C4b-binding protein. Mapping of the ligand binding site and the N-terminus of the pre-protein. *J. Immunol.* **150:**2273–2280.
232. Hillarp, A., and B. Dahlback. 1990. Cloning of cDNA coding for the beta chain of human complement component C4b-binding protein: Sequence homology with the alpha chain. *Proc. Natl. Acad. Sci. USA* **87:**1183–1187.
233. Nelson, R. M., and G. L. Long. 1991. Solution-phase equilibrium binding interaction of human protein S with C4b-binding protein. *Biochemistry* **30:**2384–2390.
234. Hillarp, A., A. Thern, and B. Dahlback. 1994. Bovine C4b binding protein. Molecular cloning of the alpha- and beta-chains provides structural background for lack of complex formation with protein S. *J. Immunol.* **153:**4109–4199.
235. Fernandez, J. A., and J. H. Griffin. 1994. A protein S binding site on C4b-binding protein involves beta chain residues 31-45. *J. Biol. Chem.* **269:**2535–2540.
236. Pardo-Manuel, F., J. Rey-Campos, A. Hillarp, B. Dahlback, and S. Rodriguez-de-Cordoba. 1990. Human genes for the alpha and beta chains of complement C4b-binding protein are closely linked in a head-to-tail arrangement. *Proc. Natl. Acad. Sci. USA* **87:**4529–4532.

237. Andersson, A., B. Dahlback, C. Hanson, A. Hillarp, G. Levan, J. Szpirer, and C. Szpirer. 1990. Genes for C4b-binding protein alpha- and beta-chains (C4BPA and C4BPB) are located on chromosome 1, band 1q32, in humans and on chromosome 13 in rats. *Somat. Cell. Mol. Genet.* **16:**493–500.
238. Aso, T., S. Okamura, T. Matsuguchi, N. Sakamoto, T. Sata, and Y. Niho. 1991. Genomic organization of the alpha chain of the human C4b-binding protein gene. *Biochem. Biophys. Res. Commun.* **174:**222–227.
239. Rodriguez-de-Cordoba, S., P. Sanchez-Corral, and J. Rey-Campos. 1991. Structure of the gene coding for the alpha polypeptide chain of the human complement component C4b-binding protein. *J. Exp. Med.* **173:**1073–1082.
240. Hillarp, A., F. Pardo-Manuel, R. R. Ruiz, S. Rodriguez-de-Cordoba, and B. Dahlback. 1993. The human C4b-binding protein beta-chain gene. *J. Biol. Chem.* **268:**15017–15023.
241. Sanchez-Corral, P., F. Pardo-Manuel-de-Villena, J. Rey-Campos, and S. Rodriguez-de-Cordoba. 1993. C4BPAL1, a member of the human regulator of complement activation (RCA) gene cluster that resulted from the duplication of the gene coding for the alpha-chain of C4b-binding protein. *Genomics* **17:**185–193.
242. Devillena, F. P. M., and S. Rodriguez-de-Cordoba. 1995. C4BPAL2: A second duplication of the C4BPA gene in the human RCA gene cluster. *Immunogenetics* **41:**139–143.
243. Saeki, T., S. Hirose, M. Nukatsuka, Y. Kusunoki, and S. Nagasawa. 1989. Evidence that C4b-binding protein is an acute-phase protein. *Biochem. Biophys. Res. Commun.* **164:**1446–1451.
244. Barnum, S. R., and B. Dahlback. 1990. C4b-binding protein, a regulatory component of the classical pathway of complement, is an acute-phase protein and is elevated in systemic lupus erythematosus. *Compl. Inflamm.* **7:**71–77.
245. Garcia-de-Frutos, P., R. I. Alim, Y. Hardig, B. Zoller, and B. Dahlback. 1994. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* **84:**815–822.
246. Gigli, I., T. Fukita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins, C4 binding protein and C3b inactivator. *Proc. Natl. Acad. Sci. USA* **76:**6596–6600.
247. Ziccardi, R. J., B. Dahlback, and H. J. Muller-Eberhard. 1984. Characterization of the interaction of human C4b-binding protein with physiological ligands. *J. Biol. Chem.* **259:**13674–13679.
248. Hessing, M., C. van-'t-Veer, T. M. Hackeng, B. N. Bouma, and S. Iwanaga. 1990. Importance of the alpha 3 fragment of complement C4 for the binding with C4b-binding protein. *FEBS. Lett.* **271:**131–136.
249. Hessing, M., R. A. Vlooswijk, T. M. Hackeng, D. Kanters, and B. N. Bouma. 1990. The localization of heparin-binding fragments on human C4b-binding protein. *J. Immunol.* **144:**204–208.
250. Press, E. M., and J. Gagnon. 1981. Human complement component C4. Structural studies on the fragments derived from C4b by cleavage with C3b inactivator. *Biochem. J.* **199:**351–357.
251. Yamazaki, M., C. Ichihara, and S. Nagasawa. 1986. Evidence that a nicked C4b, C4b′, is a functionally active C4b derivative. *FEBS. Lett.* **208:**147–150.
252. Heeb, M. J., J. Rosing, H. M. Bakker, J. A. Fernandez, G. Tans, and J. H. Griffin. 1994. Protein-S binds to and inhibits Factor-Xa. *Proc. Natl. Acad. Sci. USA* **91:**2728–2732.
253. Nishioka, J., and K. Suzuki. 1990. Inhibition of cofactor activity of protein S by a complex of protein S and C4b-binding protein. Evidence for inactive ternary complex

formation between protein S, C4b-binding protein. Evidence for inactive ternary complex formation between protein S, C4b-binding protein, and activated protein C. *J. Biol. Chem.* **265:**9072–9076.

254. Comp, P. C., J. Forristall, C. D. West, and R. G. Trapp. 1990. Free protein S levels are elevated in familial C4b-binding protein deficiency. *Blood* **76:**2527–2529.
255. Rodriguez-de-Cordoba, S., M. Perez-Blas, R. Ramos-Ruiz, P. Sanchez-Corral, F. Pardo-Manuel-de-Villena, and J. Rey-Campos. 1994. The gene coding for the beta-chain of C4b-binding protein (C4BPB) has become a pseudogene in the mouse. *Genomics* **21:**501–509.
256. He, X., and B. Dahlback. 1994. Rabbit plasma, unlike its human counterpart, contains no complex between protein S and C4b-binding protein. *Thromb. Haemost.* **71:**446–451.
257. Yasuda, F., T. Hayashi, K. Tanitame, J. Nishioka, and K. Suzuki. 1995. Molecular cloning and functional characterization of rat plasma protein S. *J. Biochem.* **117:**374–383.
258. Dahlback, B., and B. Hildebrand. 1983. Degradation of human complement component C4b in the presence of the C4b-binding protein-protein S complex. *Biochem. J.* **209:**857–863.
259. Schwalbe, R., B. Dahlback, A. Hillarp, and G. Nelsestuen. 1990. Assembly of protein S and C4b-binding protein on membranes. *J. Biol. Chem.* **265:**16074–16081.
260. Furmaniak-Kazmierczak, E., C. Y. Hu, and C. T. Esmon. 1993. Protein S. enhances C4b binding protein interaction with neutrophils. *Blood* **81:**405–411.
261. Schwalbe, R. A., B. Dahlback, and G. L. Nelsestuen. 1990. Independent association of serum amyloid P component, protein S. and complement C4b with complement C4b-binding protein and subsequent association of the complex with membranes. *J. Biol. Chem.* **265:**21749–21757.
262. Garcia-de-Frutos, P., and B. Dahlback. 1994. Interaction between serum amyloid P component and C4b-binding protein associated with inhibition of factor I-mediated C4b degradation. *J. Immunol.* **152:**2430–2437.
263. Kotwal, G. J., and B. Moss. 1988. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* **335:**176–178.
264. McKenzie, R., G. J. Kotwal, B. Moss, C. H. Hammer, and M. M. Frank. 1992. Regulation of complement activity by vaccinia virus complement-control protein. *J. Infect. Dis.* **166:**1245–1250.
265. Isaacs, S. N., G. J. Kotwal, and B. Moss. 1992. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Proc. Natl. Acad. Sci. USA* **89:**628–632.
266. Fearon, D. T., and K. F. Austen. 1975. Properdin: Binding to C3b and stabilisation of the C3b-dependent C3 convertase. *J. Exp. Med.* **142:**856–863.
267. Pillemer, L., L. Blum, I. H. Lepow, O. A. Ross, E. W. Todd, and A. C. Wardlaw. 1954. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science* **120:**279–285.
268. Penksy, J., C. F. Hinz, E. W. Todd, R. J. Wedgwood, J. T. Boyer, and I. H. Lepow. 1968. Properties of highly purified human properdin. *J. Immunol.* **100:**142–158.
269. Smith, C. A., M. K. Pangburn, C. W. Vogel, and H. J. Muller-Eberhard. 1984. Molecular architecture of human properdin, a positive regulator of the alternative pathway of complement. *J. Biol. Chem.* **259:**4582–4588.
270. Smith, K. F., K. F., Nolan, K. B. M. Reid, and S. J. Perkins. 1991. Neutron and X-ray scattering studies on the human complement protein properdin provide an analysis of the thrombospondin repeat. *Biochemistry* **30:**8000–8008.

271. Farries, T. C., and J. P. Atkinson. 1989. Biosynthesis of properdin. *J. Immunol.* **142:**842–847.
272. Nolan, K. F., W. Schwaeble, S. Kaluz, M. P. Dierich, and K. B. M. Reid. 1991. Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. *Eur. J. Immunol.* **21:**771–776.
273. Nolan, K. F., S. Kaluz, J. M. Higgins, D. Goundis, and K. B. M. Reid. 1992. Characterization of the human properdin gene. *Biochem. J.* **287:**291–297.
274. Goundis, D., and K. B. M. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature* **335:**82–85.
275. Perkins, S. J., A. S. Nealis, P. I. Haris, D. Chapman, Goundis, D., and K. B. M. Reid. 1989. Secondary structure in properdin of the complement cascade and related proteins: A study by fourier transform infrared spectroscopy. *Biochemistry* **28:**7176–7182.
276. Densen, P., J. M. Weiler, J. M. Griffiss, and L. G. Hoffmann. 1987. Familial properdin deficiency and fatal meningococcemia. Correction of the bactericidal defect by vaccination. *N. Engl. J. Med.* **316:**922–926.
277. Goundis, D., S. M. Holt, Y. Boyd, and K. B. M. Reid. 1989. Localization of properdin structural locus to Xp11.23-Xp21.1. *Genomics* **5:**56–60.
278. Evans, E. P., M. D. Burtenshaw, S. H. Laval, D. Goundis, K. B. M. Reid, and Y. Boyd. 1990. Localization of the properdin factor complement locus Pfc to band A3 on the mouse X chromosome. *Genet. Res.* **56:**153–155.
279. Sjoholm, A. G., C. Soderstrom, and L. A. Nilsson. 1988. A second variant of properdin deficiency: The detection of properdin at low concentrations in affected males. *Complement* **5:**130–140.
280. Sjoholm, A. G., E. J. Kuijper, C. C. Tijssen, A. Jansz, P. Bol, L. Spanjaard, and H. C. Zanen. 1988. Dysfunctional properdin in a dutch family with meningococcal disease. *N. Engl. J. Med.* **319:**33–37.
281. Schwaeble, W., H. P. Huemer, J. Most, M. P. Dierich, M. Strobel, C. Claus, K. B. M. Reid, and H. W. L. Zieglerheitbrock. 1994. Expression of properdin in human monocytes. *Eur. J. Biochem.* **219:**759–764.
282. Minta, J. O. 1988. Biosynthesis of complement factor P (properdin) by the human pre-monocyte cell line (U-937). *Mol. Immunol.* **25:**1363–1370.
283. Schwaeble, W., W. G. Dippold, M. K. Schafer, H. Pohla, D. Jonas, B. Luttig, E. Weihe, H. P. Huemer, M. P. Dierich, and K. B. M. Reid. 1993. Properdin, a positive regulator of complement activation, is expressed in human T cell lines and peripheral blood T cells. *J. Immunol.* **151:**2521–2528.
284. Farries, T. C., J. T. Finch, P. J. Lachmann, and R. A. Harrison. 1987. Resolution and analysis of 'native' and 'activated' properdin. *Biochem. J.* **243:**507–517.
285. Pangburn, M. K. 1989. Analysis of the natural polymeric forms of human properdin and their functions in complement activation. *J. Immunol.* **142:**202–207.
286. Daoudaki, M. E., J. D. Becherer, and J. D. Lambris. 1988. A 34-amino acid peptide of the third component of complement mediates properdin binding. *J. Immunol.* **140:**1577–1580.
287. Farries, T. C., P. J. Lachmann, and R. A. Harrison. 1988. Analysis of the interaction between properdin and factor B, components of the alternative-pathway C3 convertase of complement. *Biochem. J.* **252:**667–675.
288. Medicus, R. G., O. Gotze, and H. J. Muller-Eberhard. 1976. Alternative pathway of complement: Recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway. *J. Exp. Med.* **144:**1076–1093.

289. Holt, G. D., M. K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide [Gal(3-SO4)beta 1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J. Biol. Chem.* **265**:2852–2855.

290. Tuszynski, G. P., V. L. Rothman, A. H. Deutch, B. K. Hamilton, and J. Eyal. 1992. Biological activities of peptides and peptide analogues derived from common sequences present in thrombospondin, properdin, and malarial proteins. *J. Cell. Biol.* **116**:209–217.

291. Farries, T. C., K. L. Knutzen-Steuer, and J. P. Atkinson. 1990. The mechanism of activation of the alternative pathway of complement by cell-bound C4b. *Mol. Immunol.* **27**:1155–1161.

292. Spitzer, R. E., E. H. Vallota, J. Forristal, R. Sudora, A. Stitzel, N. C. Davis, and C. A. West. 1969. Serum C'3 lytic system in patients with glomerulonephritis. *Science* **164**:436–437.

293. Daha, M. R., K. F. Austen, and D. T. Fearon. 1977. The incorporation of C3 nephritic factor (C3Nef) into a stabilized C3 convertase, C3bBb(C3Nef), and its release after decay of convertase function. *J. Immunol.* **119**:812–817.

294. Scott, D. M., N. Amos, J. G. P. Sissons, P. J. Lachmann, and D. K. Peters. 1978. The immunoglobulin nature of nephritic factor (NeF). *Clin. Exp. Immunol.* **32**:12–24.

295. Schreiber, R. D., O. Gotze, and H. J. Muller-Eberhard. 1976. Nephritic factor: Its structure and function and its relationship to the initiating factor of the alternative pathway. *Scand. J. Immunol.* **5**:705–713.

296. Hiramatsu, M., J. E. Balow, and G. C. Tsokos. 1986. Production of nephritic factor of the alternative complement pathway by Epstein Barr virus-transformed B cell lines derived from a patient with membranoproliferative glomerulonephritis. *J. Immunol.* **136**:4451–4455.

297. Hiramatsu, M., and G. C. Tsokos. 1988. Epstein-Barr virus transformed B cell lines derived from patients with systemic lupus erythematosus produce a nephritic factor of the classical complement pathway. *Clin. Immunol. Immunopathol.* **46**:91–99.

298. Scott, D. M., N. Amos, and S. R. Bartolotti. 1981. The role of carbohydrate in the structure and function of nephritic factor. *Clin. Exp. Immunol.* **46**:120–129.

299. Daha, M. R., and L. A. van Es. 1981. Stabilization of homologous and heterologous cell-bound amplification convertases, C3bBb, by C3 nephritic factor. *Immunology* **43**:33–38.

300. Daha, M. R., A. M. Deelder, and L. A. van Es. 1984. Stabilization of the amplification convertasae of complement by monoclonal antibodies directed against human factor B. *J. Immunol.* **132**:2538–2542.

301. Marin, M. A., G. Fontan, and M. Lopez-Trascasa. 1991. Interaction of C3 nephritic factor (NEF) with erythrocyte membranes complement-independent binding to sheep and patients erythrocytes. *Mol. Immunol.* **28**:133–140.

302. Power, D. A., Y. C. Ng, and J. G. Simpson. 1990. Familial incidence of C3 nephritic factor, partial lipodystrophy and membranoproliferative glomerulonephritis. *Q. J. Med.* **75**:387–398.

303. Lopez-Trascasa, M., J. M. Martin-Villa, J. L. Vicario, M. A. Marin, J. Martinez-Ara, C. Garcia-Messeguer, A. Arnaiz-Villena, and G. Fontan. 1991. Familial incidence of C3 nephritic factor. *Nephron* **59**:261–265.

304. Mold, C., and M. E. Medof. 1985. C3 nephritic factor protects bound C3bBb from cleavage by factor I and human erythrocytes. *Mol. Immunol.* **22**:507–512.

305. Fischer, E., M. D. Kazatchkine, and L. Mercarelli-Halbwachs. 1984. Protection of the classical and alternative complement pathway C3 convertases, stabilized by nephritic factors, from decay by the human C3b receptor. *Eur. J. Immunol.* **14**:1111–1114.

306. Ito, S., N. Tamura, and T. Fujita. 1989. Effect of decay-accelerating factor on the assembly of the classical and alternative pathway C3 convertases in the presence of C4 or C3 nephritic factor. *Immunology* **68:**449–452.

307. Ohi, H., S. Watanabe, T. Fujita, and T. Yasugi. 1992. Significance of C3 nephritic factor (C3NeF) in non-hypocomplementaemic serum with membranoproliferative glomerulonephritis (MPGN). *Clin. Exp. Immunol* **89:**479–484.

308. Tanuma, Y., H. Ohi, and M. Hatano. 1990. Two types of C3 nephritic factor: properdin-dependent C3NeF and properdin-independent C3NeF. *Clin. Immunol. Immunopathol.* **56:**226–238.

309. Ng, Y. C., and D. K. Peters. 1986. C3 nephritic factor (C3NeF): Dissociation of cell-bound and fluid phase stabilization of alternative pathway C3 convertase. *Clin. Exp. Immunol.* **65:**450–457.

310. Gigli, I., J. Sorvillo, L. Mecarelli-Halbwachs, and J. Leibowitch. 1981. Mechanism of action of the C4 nephritic factor. Deregulation of the classical pathway of C3 convertase. *J. Exp. Med.* **154:**1–12.

311. Kolb, W. P., and H. J. Muller-Eberhard. 1975. The membrane attack mechanism of complement. Isolation and subunit composition of the C5b-9 complex. *J. Exp. Med.* **141:**724–735.

312. Jenne, D., and K. K. Stanley. 1985. Molecular cloning of S protein, a link between complement, coagulation and cell-substrate adhesion. *EMBO J.* **4:**3153–3157.

313. Suzuki, S., A. Oldberg, E. G. Hayman, M. D. Piersbacher, and E. Ruoslahti. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment site in vitronectin and fibronectin. *EMBO J.* **4:**2519–2524.

314. Hayman, E. G., M. D. Piersbacher, Y. Ohgren, and E. Ruoslahti. 1983. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc. Natl. Acad. Sci. USA* **80:**4003–4007.

315. Shaffer, M. C., T. P. Foley, and D. W. Barnes. 1984. Quantitation of spreading factor in human biologic fluids. *J. Lab. Clin. Med.* **103:**783–791.

316. Preissner, K. T., R. Wassmuth, and G. Muller-Berghaus. 1985. Physicochemical characterization of human S-protein and its function in the blood coagulation system. *Biochem. J.* **231:**349–355.

317. Preissner, K. L., S. Holzhuter, C. Justus, and G. Muller-Berghaus. 1989. Identification and partial characterization of platelet vitronectin. Evidence for complex formation with platelet-derived plasminogen activator inhibitor-I. *Blood* **74:**1989–1996.

318. Parker, C. J., O. L. Stone, V. F. White, and N. J. Bernshaw. 1989. Vitronectin (S-protein) is associated with platelets. *Br. J. Haematol.* **71:**245–252.

319. Kanz, L., G. W. Lohr, and K. T. Preissner. 1988. Identification of human megakaryocyte vitronectin/S-protein. *Blood* **72:**(Suppl.1):327a.

320. Hetland, G., H. B. Pettersen, T. E. Mollnes, and F. Johnson. 1989. S-protein is synthesized by human monocytes and macrophages in vitro. *Scand. J. Immunol.* **29:**15–21.

321. Berge, V., E. Johnson, K. Hogasen, and G. Hetland. 1992. Human umbilical vein endothelial cells synthesize S-protein (vitronectin) in vitro. *Scand. J. Immunol.* **36:**119–123.

322. Fink, T. M., D. E. Jenne, and P. Lichter. 1992. The human vitronectin (complement S-protein) gene maps to the centromeric region of 17q. *Human Genet.* **88:**569–572.

323. Jenne, D., and K. K. Stanley. 1987. Nucleotide sequence and organization of the human S-protein gene: Repeating peptide motifs in the "pexin" family and a model for their evolution. *Biochemistry* **26:**6735–6742.

324. Barnes, D. W., and J. Reing. 1985. Human spreading factor: Synthesis and response by Hep G2 cells in culture. *J. Cell Physiol.* **125:**207–214.

325. Dahlback, B., and E. R. Podack. 1985. Characterization of human S-protein, an inhibitor of the membrane attack complex of complement. Demonstration of a free reactive thiol group. *Biochemistry* **24:**2368–2374.

326. Kubota, K., M. Hayashi, N. Oishi, and Y. Sakaki. 1990. Polymorphism of the human vitronectin gene causes vitronectin blood type. *Biochem. Biophys. Res. Commun.* **167:**1355–1360.

327. Preissner, K. T., and G. Muller-Berghaus. 1987. Neutralization and binding of heparin by S-protein/vitronectin in the inhibition of factor Xa by antithrombin III. Involvement of an inducible heparin binding domain of S-protein/vitronectin. *J. Biol. Chem.* **262:**12247–12253.

328. Podack, E. R., W. P. Kolb, and H. J. Muller-Eberhard. 1977. The SC5b-7 complex: Formation, isolation, properties and subunit composition. *J. Immunol.* **119:**2024–2029.

329. Bhakdi, S., and J. Tranum-Jensen. 1983. Membrane damage by complement. *Biochim. Biophys. Acta* **737:**343–372.

330. Podack, E. R., K. T. Preissner, and H. J. Muller-Eberhard. 1984. Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein. *Acta Pathol. Microbiol. Immunol. Scand. Suppl.* **284:**89–96.

331. Peitsch, M., and K. T. Preissner. 1988. The heparin binding domain of S-protein/vitronectin binds to complement components C7, C8, and C9 and perforin from cytolytic T cells and inhibits their lytic activities. *Biochemistry* **27:**4103–4109.

332. Johnson, E., V. Berge, and K. Hogsen. 1994. Formation of the terminal complex on agarose beads: Further evidence that vitronectin (complement S-protein) inhibits C9 polymerization. *Scand. J. Immunol.* **39:**281–285.

333. Milis, L., C. A. Morris, M. C. Sheehan, J. H. Charlesworth, and B. A. Pussel. 1993. Vitronectin-mediated inhibition of complement: Evidence for different binding sites for C5b-7 and C9. *Clin. Exp. Immunol.* **92:**114–119.

334. Felding-Habermann, B., and D. A. Cheresh. 1993. Vitronectin and its receptors. *Curr. Opin. Cell. Biol.* **5:**864–868.

335. Murphy, B. B., L. Kirszbaum, I. D. Walker, and A. J. d'Apice. 1988. A newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J. Clin. Invest.* **81:**1858–1864.

336. Jenne, D., and J. Tschopp. 1989. Molecular structure and functional characteristics of a human complement cytolysis inhibitor found in blood and seminal plasma: Identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc. Natl. Acad. Sci. USA* **86:**7123–7127.

337. deSilva, H. V., J. A. Harmony, W. D. Stuart, C. M. Gill, and J. Robbins. 1990. Apolipoprotein J: Structure and tissue distribution. *Biochemistry* **29:**5380–5389.

338. Kirzbaum, L. J., B. F. Sharpe, J. F. d-Apice, B. Classon, P. Hudson, and I. D. Walker. 1989. Molecular cloning and characterization of the novel complement associated protein SP-40,40: A link between the complement and reproductive systems. *EMBO J.* **8:**711–718.

339. Sylvester, S. R., M. K. Skinner, and M. D. Griswold. 1984. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol. Reprod.* **31:**1081–1101.

340. Fritz, I. B., K. Burdzy, B. Setchell, and O. Blaschnik. 1983. Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. *Biol. Reprod.* **28:**1173–1188.

341. deSilva, H. V., W. D. Stuart, C. R. Duvic, J. R. Wetterau, M. J. Ray, D. E. Ferguson, H. W. Albers, W. R. Smith, and J. A. Harmony. 1990. A 70 kDa lipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J. Biol. Chem.* **265:**13240–13247.

342. Jenne, D. E., B. Lowin, M. C. Peitsch, A. Bottcher, G. Schmidt, and J. Tschopp. 1991. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-1 in human plasma. *J. Biol. Chem.* **226:**11030–11036.

343. Slawin, K., I. S. Sawszuk, C. A. Olsson, and R. Buttyan. 1990. Chromosomal assignment of the human homologue encoding SGP2. *Biochem. Biophys. Res. Commun.* **172:**160–164.

344. Purello, M., S. Bettuzzi, C. DiPietro, E. Mirabelli, M. DiBlasi, R. Rimini, K. H. Grezchik, C. Ingletti, A. Corti, and G. Sichel. 1991. The gene for SP-40,40 human homolog of rat sulfated glycoprotein 2, rat clusterin and rat testosterone repressed message 2 maps to chromosome 8. *Genomics* **10:**151–161.

345. Tobe, T., S. Minoshima, S. Yamase, N. H. Choi, M. Tomita, and N. Shimizu. 1991. Assignment of a human serum glycoprotein SP-40,40 gene (CLI) to chromosome 8. *Cytogenet. Cell Genet.* **57:**193.

346. Fink, T. M., M. Zimmer, J. Tschopp, J. Etienne, D. E. Jenne, and P. Lichter. 1993. Human clusterin (CLI) maps to 8p21 in proximity to the lipoprotein lipase (LPL) gene. *Genomics* **16:**526–528.

347. Choi, N. H., T. Mazda, and M. Tomita. 1989. A serum protein SP-40,40 modulates the formation of a membrane attack complex of complement on erythrocytes. *Mol. Immunol.* **26:**835–840.

348. Murphy, B. F., J. R. Saunders, M. K. O'Bryan, L. Kirszbaum, I. D. Walker, and A. J. d'Apice. 1989. SP-40,40 is an inhibitor of C5b-6 initiated hemolysis. *Int. Immunol.* **1:**551–554.

349. Choi, N. H., Y. Nakano, T. Tobe, T. Mazda, and M. Tomita. 1990. Incorporation of SP-40,40 into the soluble membrane attack complex (SMAC, SC5b-9) of complement. *Int. Immunol.* **2:**413–417.

350. Tschopp, J., A. Chonn, S. Hertig, and L. E. French. 1993. Clusterin, the human apolipoprotein and complement inhibitor, binds to complement C7, C8 beta and the b domain of C9. *J. Immunol.* **151:**2159–2165.

351. Vakeva, A., P. Laurila, and S. Meri. 1994. Codeposition of clusterin with the complement membrane attack complex in myocardial infarction. *Immunology* **80:**177–182.

352. McGeer, P. L., and J. Rogers. 1992. Anti-inflammatory agents as a therapeutic approach to Alzheimer's disease. *Neurology* **42:**447–449.

353. Eddy, A. A., and I. B. Fritz. 1991. Localization of clusterin in the epimembranous deposits of passive Heymann nephritis. *Kidney Int.* **39:**247–252.

354. O'Bryan, M. K., H. W. G. Baker, J. R. Saunders, L. Kirszbaum, I. D. Walker, P. Hudson, D. Y. Liu, M. D. Glew, and A. J. d'Apice. 1990. Human seminal clusterin (SP-40,40) isolation and characterization. *J. Clin. Invest.* **85:**1477–1486.

355. Rooney, I. A. 1994. New aspects of complement structure and function. *In* Complement, Complement Inhibitors and Fertilization (A. Erdei, Ed.), pp. 117–131. R. G. Landes, Co., Austin, Texas.

356. O'Bryan, M. K., B. F. Murphy, D. Y Liu, G. N. Clark, and H. W. G. Baker. 1994. The use of anticlusterin monoclonal antibodies for the combined assessment of human sperm morphology and acrosome integrity. *Hum. Reprod.* **9:**1490–1496.

357. Duguid, J. R., C. W. Bohmont, N. G. Liu, and W. W. Tourtellotte. 1989. Changes in brain gene expression shared by scrapie and Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **86:**7260–7264.

358. Rosenberg, M. E., and M. S. Paller. 1991. Differential gene expression in the recovery from ischemic renal injury. *Kidney Int.* **39:**1156–1161.

359. Buttyan, R., C. A. Olsson, J. Pinter, C. Chang, M. Bandyk, P. Y. Ng, and I. S. Sawszuk. 1989. Identification of the TRPM-2 gene in cells undergoing programmed death. *Mol. Cell Biol.* **9:**3473–3481.

360. Bettuzzi, S., L. Troiano, P. Davalli, F. Tropea, M. C. Ingletti, E., Granilli, D. Monti, A. Corti, and C. Franceschi. 1991. In vitro accumulation of sulfated glycoprotein 2 mRNA in rat thymocytes upon dexamethasone induced cell death. *Biochem. Biophys. Res. Commun.* **175:**810–815.

361. French, L. E., A. Wohlwent, A. P. Sappino, J. Tschopp, and J. A. Schifferli. 1994. Human clusterin gene expression is confined to surviving cells during in vitro programmed cell death. *J. Clin. Invest.* **93:**877–884.

362. Sugita, Y., Y. Nakano, and M. Tomita. 1988. Isolation from human erythrocytes of a new membrane protein which inhibits the formation of complement transmembrane channels. *J. Biochem. (Tokyo)* **104:**633–737.

363. Okada, N., R. Harada, T. Fujita, and H. Okada. 1989. Monoclonal antibodies capable of causing hemolysis of neuraminidase treated erythrocytes by homologous complement. *J. Immunol.* **143:**2262–2266.

364. Sugita, Y., T. Tobe, E. Oda, M. Tomita, K. Yasukawa, N. Yami, T. Takemoto, K. Furuichi, M. Takayama, and S. Yano. 1989. Molecular cloning and characterization of MACIF, an inhibitor of membrane channel formation of complement. *J. Biochem. (Tokyo)* **106:**555–557.

365. Holguin, M. H., L. R. Fredrick. N. J. Bernshaw, L. A. Wilcox, and C. J. Parker. 1989. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J. Clin. Invest.* **84:**7–17.

366. Avies, A., D. L. Simmons, G. Hale, R. A. Harrison, H. Tighe, P. J. Lachmann, and H. Waldmann. 1989. CD59, and LY6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J. Exp. Med.* **170:**637–654.

367. Meri, S., B. P. Morgan, A. Davies, R. H. Daniels, M. G. Olavesen, H. Waldmann, P. J. Lachmann. 1990. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology* **71:**1–9.

368. Philbrick, W. M., R. G. Palfree, S. E. Maher, M. M. Bridgett, S. Sirlin, and A. L. Bothwell. 1990. The CD59 antigen is a structural homologue of murine Ly6 antigens but lacks interferon inducibilty. *Eur. J. Immunol.* **20:**87–92.

369. Hekhl-Ostreicher, B., S. Ragg, M. Drechsler, H. Scherthan, and B. Royer-Pokora. 1993. Localization of the human CD59 gene by fluorescence in situ hybridization and pulsed-field gel electrophoresis. *Cytogenet. Cell Genet.* **63:**144–146.

370. Tone, M., L. A. Walsh, and H. Waldmann. 1992. Gene structure of human CD59 and demonstration that discrete mRNAs are generated by alternative polyadenylation. *J. Mol. Biol.* **227:**971–976.

371. Petranka, J. G., D. E. Fleischer, K. Sykes, R. E. Kaufman, and W. F. Rosse. 1992. Structure of the CD59 encoding gene: Further evidence of a relationship to murine lymphocyte antigen Ly6 protein. *Proc. Natl. Acad. Sci. USA* **89:**7876–7879.

372. Sugita, Y., Y. Nakano, E. Oda, K. Noda, T. Tobe, N. H. Miura, and M. Tomita. 1993. Determination of carboxyterminal residue and disulfide bonds of MACIF (CD59) a glycosylphosphatidylinositol anchored membrane protein. *J. Biochem.* **114:**473–477.

373. Nakano, Y., K. Noda, T. Endo, A. Kobata, and M. Tomita. 1993. Structural study on the glycosylphosphatidylinositol anchor and the asparagine-linked sugar chain of a soluble form of CD59 in human urine. *Arch. Biochem. Biophys.* **311**:117–126.

374. Tone, M., L. A. Walsh, and H. Waldmann. 1992. Gene structure of human CD59 encoding gene: Further evidence of a relationship to murine lymphocytic antigen Ly6 protein. *Proc. Natl. Acad. Sci. USA* **89**:7876–7879.

375. Albrecht, J. C., J. Nicholas, K. R. Cameron, C. Newman, B. Fleckenstein, and R. W. Honess. 1992. Herpes virus Saimiri has a gene specifying a homologue of the cellular membrane glycoprotein CD59. *Virology* **190**:527–530.

376. Fletcher, C. M., R. A. Harrison, P. J. Lachmann, and D. Neuhaus. 1994. Structure of a soluble, glycosylated form of the complement regulatory protein CD59. *Structure* **2**:185–199.

377. Kieffer, B., P. C. Driscoll, I. D. Campbell, A. C. Willis, P. A. van der Merwe, and S. J. Davis. 1994. Three dimensional solution structure of the extracellular region of the complement regulatory protein CD59, a new cell-surface protein domain related to snake venom neurotoxins. *Biochemistry* **33**:4471–4482.

378. Meri, S., H. Waldmann, and P. J. Lachmann. 1991. Distribution of protectin (CD59) a complement membrane attack inhibitor in normal human tissues. *Lab. Invest.* **65**:532–537.

379. Nose, M., M. Katoh, N. Okada, M. Kyoguku, and H. Okada. 1990. Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement and its predominant expression on endothelial cells in vivo. *Immunology* **70**:145–149.

380. Brooimans, R. A., A. A. J. van der Ark, M. Tomita, L. A. van Es, and M. R. Daha. 1992. CD59 expressed by human endothelial cells functions as a protective molecule against complement-mediated lysis. *Eur. J. Immunol.* **22**:791–797.

381. Vakeva, A., P. Laurila, and S. Meri. 1992. Loss of expression of protectin (CD59) is associated with the complement membrane attack complex deposition in myocardial infarction. *Lab. Invest.* **67**:608–616.

382. Rooney, I. A., A. Davies, and B. P. Morgan. 1992. Membrane attack complex (MAC)-mediated damage to spermatozoa: Protection of the cells by the presence on their membranes of MAC inhibitory proteins. *Immunology* **75**:499–506.

383. Rooney, I. A., and B. P. Morgan. 1990. Protection of human amniotic epithelial cells (HAEC) from complement-mediated lysis: Expression on the cells of three complement inhibitory membrane proteins. *Immunology* **71**:308–311.

384. Wing, M. G., J. Zajicek, D. J. Seilly, D. A. Compston, and P. J. Lachmann. 1992. Oligodendrocytes lack glycolipid anchored proteins which protect them against complement lysis: Restoration of resistance to lysis by incorporation of CD59. *Immunology* **76**:140–145.

385. Rooney, I. A., J. P. Atkinson, E. S. Krul, G. Schonfeld, K. Polakoski, J. E. Saffitz, and B. P. Morgan. 1993. Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes and inhibits complement mediated lysis. *J. Exp. Med.* **177**:1409–1420.

386. Rooney, I. A., and B. P. Morgan. 1992. Characterization of the membrane attack complex inhibitory protein CD59 antigen on human amniotic cells and in amniotic fluid. *Immunology* **76**:541–547.

387. Vakeva, A., M. Jauhiainen, C. Ehnholm, T. Lehto, and S. Meri. 1994. High density lipoproteins can act as carriers of glycophosphoinositol lipid anchored CD59 in human plasma. *Immunology* **82**:28–33.

388. Meri, S., P. Nattila, and R. Renkonken. 1993. Regulation of CD59 expression on the human endothelial cell line EAhy 926. *Eur. J. Immunol.* **23**:2511–2516.
389. Gordon, D. L., H. Papazaharoudakis, T. A. Sadlon, A. Arellano, and N. Okada. 1994. Upregulation of human neutrophil CD59, a regulator of the membrane attack complex of complement, following cell activation. *Immunol. Cell Biol.* **72**:222–229.
390. Holguin, M. H., C. B. Martin, J. H. Weis, and C. J. Parker. 1993. Enhanced expression of the complement regulatory protein membrane inhibitor of reactive lysis (CD59) is regulated at the level of transcription. *Blood* **82**:968–977.
391. Davies, M. E., A. Horner, B. E. Loveland, and I. F. McKenzie. 1994. Upregulation of complement inhibitors MCP (CD46), DAF (CD55) and protectin (CD59) in arthritic joint disease. *Scand. J. Rheumatol.* **23**:316–321.
392. Rooney, I. A., A. Davies, D. Griffifths, J. D. Williams, M. Davies, S. Meri, P. J. Lachmann, and B. P. Morgan. 1991. The complement inhibiting protein protectin (CD59 antigen) is present and functionally active on glomerular epithelial cells. *Clin. Exp. Immunol.* **83**:251–256.
393. Dalmasso, A. P., G. M. Vercellotti, J. L. Platt, and P. H. Bach. 1991. Inhibition of complement-mediated endothelial cell cytotoxicity by decay accelerating factor: Potential for prevention of xenograft hyperacute rejection. *Transplantation* **52**:530–533.
394. Charreau, B., D. A. Cassard, L. Tesson, B. LeMauff, J. M. Navenot, D. Blanchard, D. Lublin, J. P. Soulillou, and I Anegon. 1994. Protection of rat endothelial cells from primate complement-mediated lysis by expression of human CD59 and/or decay accelerating factor. *Transplantation* **58**:1222–1229.
395. Kennedy, S. P., S. A. Rollins, W. V. Burton, P. J. Sims, A. L. Bothwell, S. P. Squinto, and G. B. Zavoico. 1994. Protection of procine aortic endothelial cells from complement-mediated cell lysis and activation by recombinant human CD59. *Transplantation* **57**:1494–1501.
396. Akami, T., R. Sawada, N. Minato, M. Naruto, A. Yamada, J. Imanishi, M. Matsuo, I. Nakai, M. Okamoto, and H. Nakajima. 1992. Cytoprotective effect of CD59 on transplantation injury. *Transplant Proc.* **24**:485–487.
397. Walsh, L. A., M. Tone, and H. Waldmann. 1992. Transfection of human CD59 complementary DNA into rat cells confers resistance to human complement. *Eur. J. Immunol.* **21**:847–850.
398. Schubert, J., P. Vciechowski, P. Delany, H. J. Tischler, W. Kolanus, and R. E. Schmidt. 1990. The PIG-anchoring defect in NK lymphocytes of PNH patients. *Blood* **76**:1181–1187.
399. Rother, R. P., S. A. Rollins, J. Mennone, A. Chodera, S. A. Fidel, M. Bessler, P. Hollmen, and S. P. Squinto. 1994. Expression of recombinant transmembrane CD59 in paroxysmal nocturnal hemoglobinuria B cells confers resistance to human complement. *Blood* **84**:2604–2611.
400. Hughes, T. R., S. J. Piddlesden, J. D. Williams, R. A. Harrison, and B. P. Morgan. 1992. Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement. *Biochem. J.* **284**:169–176.
401. van den Berg, C. W., R. A. Harrison, and B. P. Morgan. 1993. The sheep analogue of human CD59. Purification and characterization of its complement inhibitory activity. *Immunology* **78**:349–357.
402. Rollins, S. A., J. Zhao, H. Ninoyima, and P. J. Sims. 1991. Inhibition of homologous complement by CD59 is mediated by a species-selective recognition conferred through binding to C8 within C5b-8 or C9 within C5b-9. *J. Immunol.* **146**:2345–2351.

403. Ninoyima, H., and P. J. Sims. 1992. The human complement regulatory protein CD59 binds to the alpha chain of C8 and to the "b" domain of C9. *J. Biol. Chem.* **267:**13675–13680.

404. Tomlinson, S., B. Whitlow, and V. Nussenzweig. 1994. A synthetic peptide from complement component C9 binds to CD59 and enhances lysis of human erythrocytes by C5b-9. *J. Immunol.* **152:**1927–1934.

405. Chang, C. P., T. Hulser, J. Zhao, T. Wiedmer, and P. J. Sims. 1994. Identity of a peptide domain of C9 that is bound by the cell-surface complement inhibitor CD59. *J. Biol. Chem.* **269:**26424–26430.

406. Ninoyima, H., B. H. Stewart, S. A. Rollins, J. Zhao, A. L. M. Bothwell, and P. J. Sim. 1992. Contribution of the N-linked carbohydrate of erythrocyte antigen CD59 to its complement inhibitory activity. *J. Biol Chem.* **267:**8404–8410.

407. van den Berg, C. W., and B. P. Morgan. 1994. Complement inhibitory activities of CD59 and analogues from rat, sheep and pig are not homologously restricted. *J. Immunol.* **152:**4095–4101.

408. Korty, P. E., C. Brando, and E. M. Shevach. 1991. CD59 functions as a signal for human T cell activation. *J. Immunol.* **146:**4092–4098.

409. Morgan, B. P., C. W. van den Berg, E. V. Davies, M. B. Hallett, and V. Horejsi. 1993. Cross-linking of CD59 and of other glycosylphosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase. *Eur. J. Immunol* **23:**2841–2850.

410. Stefanova, I., V. Horejsi, I. J. Ansotegui, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* **254:**1016–1019.

411. Deckert, M., J. Kubar, D. Zoccola, G. Bernard-Pomier, P. Angelisova, V. Horejsi, and A. Bernard. 1992. CD59 molecule: A second ligand for CD2 in T cell adhesion. *Eur. J. Immunol.* **22:**2943–2947.

412. Hahn, W. C., E. Menu, A. L. Bothwell, P. J. Sims, and B. E. Bierer. 1992. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand, CD59. *Science* **256:**1805–1807.

413. Menu, E., B. C. Tsai, A. L. Bothwell, P. J. Sims, and B. E. Bierer. 1994. CD59 costimulation of T cell activation. CD58 dependence and requirement for glycosylation. *J. Immunol.* **153:**2444–2456.

414. Zalman, L. S., L. M. Wood, and H. J. Muller-Eberhard. 1986. Isolation of a human erythrocyte membrane protein capable of inhibiting expression of homologous complement transmembrane channels. *Proc. Natl. Acad. Sci. USA* **83:**6975–6979.

415. Schonermark, S., E. W. Rauterberg, M. L. Shin, S. Loke, D. Roelcke, and G. M. Hansch. 1986. Homologous species restriction in lysis of human erythrocytes: A membrane-derived protein with C8-binding capacity functions as an inhibitor. *J. Immunol.* **136:**1772–1776..

416. Watts, M. J., J. R. Dankert, and B. P. Morgan. 1990. Isolation and characterization of a membrane attack complex inhibiting protein present in human serum and other biological fluids. *Biochem. J.* **265:**471–477.

417. Hansch, G. M., P. F. Wella, and A. Nicholson-Weller. 1988. Release of C8 binding protein (C8bp) from the cell membrane by phosphatidylinositol specific phospholipase C. *Blood* **72:**1089–1092.

418. Hansch, G. M., S. Schonermark, and D. Roelcke. 1987. Paroxysmal nocturnal hemoglobinuria Type III: Lack of an erythrocyte membrane protein restricts the lysis by C5b-9. *J. Clin. Invest* **80:**7–12.

419. Zalman, S. 1992. Homologous restriction factor. *Curr. Top. Microbiol. Immunol.* **178:**87–100.
420. Chenoweth, D. E. 1986. Complement mediators of inflammation. *In* Immunobiology (G. Ross, Ed.), pp. 63–86. Academic Press, New York.
421. Hugli, T. E. 1986. Biochemistry and biology of anaphylatoxins. *Complement* **3:**11–127.
422. Morgan, E. L. 1986. Modulation of the immune response by anaphyatoxins. *Complement* **3:**128–136.
423. Hugli, T. E. 1989. Structure and function of C3a anaphylatoxin. *Curr. Top. Microbiol. Immunol.* **153:**181–208.
424. Gerard, C., and N. P. Gerard. 1994. C5a anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* **12:**775–808.
425. Liszewski, M. K., and J. P. Atkinson. 1993. The complement system. *In* Fundamental Immunology (W. E. Paul, Ed.), pp. 917–939. Raven Press, New York.

ADVANCES IN IMMUNOLOGY, VOL. 61

V(D)J Recombination Pathology

Klaus Schwarz and Claus R. Bartram[1]

Section of Molecular Biology, Pediatrics II, University of Ulm, D-89070 Ulm, Germany

I. Introduction

The genome of a mammalian cell is likely to experience a spectrum of DNA damages every day. One type of DNA lesion constitutes the formation of double-strand breaks (DSBs).[2] Unrepaired DSBs either are lethal or contribute to the induction of chromosomal rearrangements in eukaryotes. It has been estimated that a cell suffers about eight DSBs per day (Lukacsovich *et al.*, 1994). Even a single unrepaired DSB in a dispensable plasmid can serve as a signal for cell death in yeast (Bennett *et al.*, 1993), underscoring the importance of mechanisms for efficient DSB repair. This can be achieved through the recombination between the broken DNA and an intact homologous sequence. Another mode of DSB repair is through DNA end-joining, without homologous recombination. Taking into account the enormous inherent risk, that every DSB could be eventually lethal, it is very amazing that the diversity of the immune system is based on a process that necessitates DNA DSBs.

The cognate immune system recognizes and responds to a virtually infinite number of antigens through the interaction of immunoglobulin (Ig) or T cell receptor (TCR) molecules expressed by B and T lymphocytes, respectively. Ig and TCR chains consist of two structural domains: the constant region mediates effector functions, and the variable domain forms the antigen binding pocket. By a site-specific recombination event, known as V(D)J recombination, the variable domains are assembled during lymphocyte development from a set of subgenic elements classified as V (variable), D (diversity), and J (joining) families. Since each of the V elements can in principal join to any of the D and J elements, a finite

[1] Present address: Institute of Human Genetics and Anthropology, University of Heidelberg, 69120 Heidelberg, Germany.

[2] Abbreviations used: bHLH, basic helix–loop helix; CDK, cyclin-dependent kinase; CHO, Chinese hamster ovary; CK II, casein kinase II; D, diversity; DN, double negative; DSB, double-strand break; IL, interleukin; IL-Rγ_C interleukin receptor common gamma chain; J, joining; kb, kilobases; kDa, kiloDalton; RAG, recombinase activating gene; RSS, recombination signal sequence; SP, single positive; TdT, terminal deoxynucleotidyl transferase; V, variable; XLA, X-linked agammaglobulinemia.

number of subgenes can establish the enormous antigen receptor diversity. Every functional B or T cell must achieve a successful recombination not on one but on two different loci. In a complex process five V, (D), and J elements must be correctly identified, tagged, cut, and joined, necessitating six site-directed, coordinate DSBs and their ultimate resolution. Recently, several articles discussed the mechanistics of antigen receptor rearrangement and regulation (Alt *et al.*, 1992a,b; Chen and Alt, 1993; Feeney, 1992; Ferguson and Thompson, 1992; Gellert, 1992a,b; Kallenbach and Rougeon, 1992; Lewis, 1994a; Lieber, 1991, 1992; Lin and Desiderio, 1995; Schatz and Chun, 1992; Schatz *et al.*, 1992; Sell, 1992; Taccioli *et al.*, 1992; Van Dyk and Meek, 1992).

In this chapter we focus on components of the V(D)J recombination machinery that might be related to diseases in humans and animals.

II. V(D)J Recombination

A comprehensive review on the subtleties of V(D)J recombination appeared recently (Lewis, 1994a). We therefore restrict ourselves to a brief introduction into the principles of V(D)J recombination.

Seven loci encoding the Ig heavy (IgH) and κ and λ light chains as well as the T cell receptor (TCR) α, β, γ, or δ chains undergo somatic DNA rearrangements during lymphocyte development (Fig. 1A). This process assembles a complete variable domain from V, D, and J elements. (D segments being present only at IgH, TCR-β, and δ loci) (Fig. 1B). The loci share a similar, evolutionarily conserved organization (Blackwell and Alt, 1988; Davis, 1988; Raulet, 1989). However, the precise number and organization of these segments vary considerably between different loci and among species. Exons encoding the constant regions lie downstream of the clusters of variable elements.

A conserved consensus recombination signal sequence (RSS) consisting of a palindromic heptamer, a less conserved spacer sequence, and an AT-rich nonamer lies adjacent to each of the coding elements (Max *et al.*, 1979; Sakano *et al.*, 1979; Ramsden *et al.*, 1994). The V(D)J recombination is directed by a pair of these joining signals, one with a 12 (±1)-base pair and the other with a 23 (±1)-bp spacer (Fig. 1B) (Early *et al.*, 1980; Sakano *et al.*, 1981). The germline organization of V, D, and J segments reflects this rule: the RSS of all members belonging to any of the V, D, or J families carry the same configuration of signals, so that the joining possibilities are restricted to productive segment recombination. Thus, for example, V-to-V inversions are excluded from the repertoire, since each V element shows the same signal sequence composition. RSS are necessary and sufficient to guide V(D)J recombination in artificial extrachromosomal

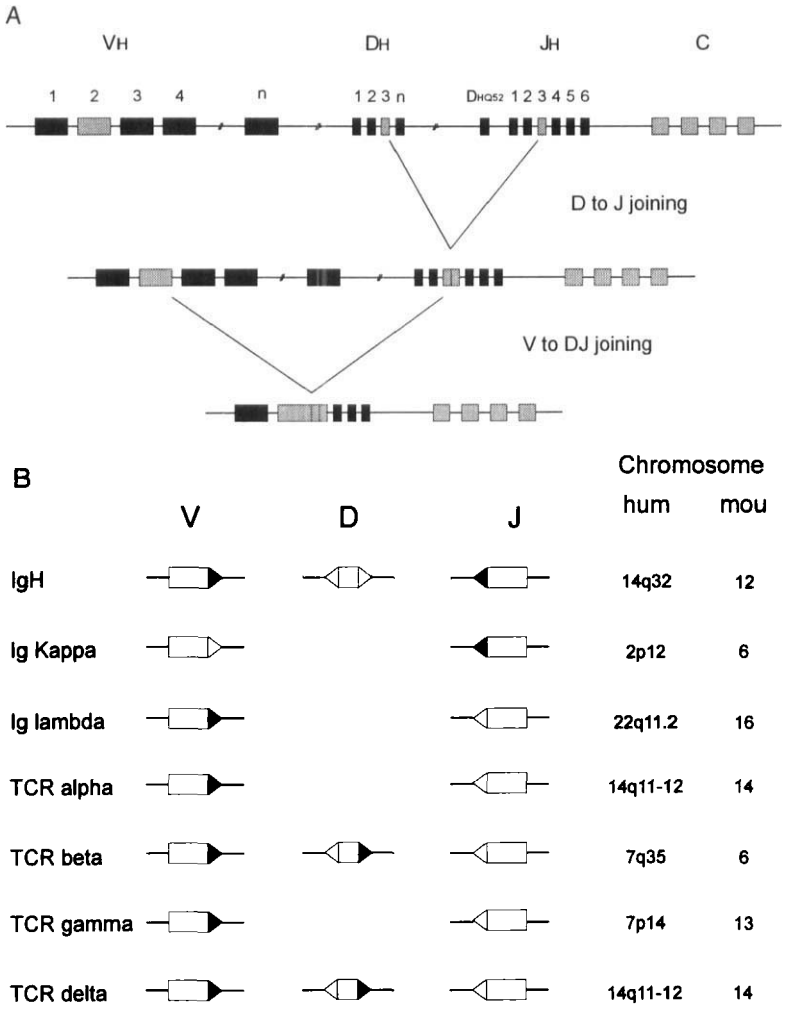


FIG. 1. (A) Schematic diagram of the rearrangement process on the human IgH locus. D to J joining is followed by V to DJ recombination. Each rectangle represents V, D, or J modules. (B) Principal structural design of V, D, and J segments at various antigen receptor loci. The 12mer spacer signals are sketched as open triangles. The 23mer spacers are drawn as filled in triangles.

substrates as well as in the genomic background, have been highly conserved throughout evolution, and can be used in an interchangeable way (Aguilera *et al.*, 1987; Hesse *et al.*, 1989). The optimal RSS has been defined by mutation analyses (Hesse *et al.*, 1989, Ramsden *et al.*, 1994)

(Fig. 2A). Since natural signals hardly ever match the optimized sequence, this may disadvantage individual segments in the recombination process.

The standard products of V(D)J recombination result from the ligation of the two coding elements (coding joints) and the two heptamers of the RSS (signal joint), respectively. Whether a rearrangement leads to DNA deletion or inversion depends on the orientation of the two RSS with respect to each other (Fig. 2B). The majority of rearrangements are associated with deletions. Inversions were noticed at the TCRβ, TCRδ, and Igκ loci (Malissen *et al.*, 1986; Kormann *et al.*, 1989; Feddersen and van Ness, 1985). Analysis of artificial recombination substrates has revealed only a slight preference of deletions over inversions (Hesse *et al.*, 1987; Lieber *et al.*, 1988).

V(D)J rearrangements can result in alternative or "nonstandard" products such as "hybrid" and "open-and-shut" junctions (Fig. 2C) (Lewis, 1994a, and references therein). Hybrid joints can arise in up to 30% of all rearrangements based on extrachromosomal substrates. In this reaction the signal from one gene segment joins to the coding end of another. In an open-and-shut joint, which is less common, the signal and coding ends created by site-specific cleavage are modified before both are religated. The only major difference between standard and nonstandard joints appears to be the choice of the ends that are ligated.

Joining of coding ends is generally imprecise, with base losses and additions of about 10 to 15 nucleotides. This process helps to diversify the receptor repertoire but includes the risk of nonfunctional genes because of out of frame joining and/or the introduction of stop codons. Signal joints on the other hand, are recombined very precisely without base loss, and infrequently, base addition (Lieber *et al.*, 1988).

Random additions of nongermline-encoded nucleotides (N-nucleotides) at the coding junctions of antigen receptor genes are mediated through the enzyme terminal deoxynucleotidyl transferase (TdT) (Sakano *et al.*, 1981) (Section III.A.2). Template encoded (palindromic) P-nucleotides are found at junctions where a coding end has been joined without prior truncation. They represent short palindromic repeats of untrimmed DNA ends (Lafaille *et al.*, 1989). P insertions may be generated at V(D)J junctions, because coding ends bearing covalently closed termini, so called hairpins, are produced as joining intermediates which are consecutively resolved by endonucleolytic cuts (Lieber, 1991; Roth *et al.*, 1992a,b; Zhu and Roth, 1995).

Other mechanisms also contribute to junctional diversity. Truncation of a small and varying number of bases at coding ends can be seen in virtually every V(D)J junction database (Davis and Bjorkman, 1988). Bases are removed prior to N-nucleotide addition most likely through several differ-

ent exo- or endonucleolytic activities or through a protein that removes DNA overhangs generated by internal homology joining. Joining of cut DNA ends in mammalian cells is facilitated by short sequence homologies of free DNA ends (Roth and Wilson, 1986). Similar reaction products between two coding elements have been described in V(D)J end resolution, namely, in "canonical" junctions of $\gamma\delta$ T cells (Raulet, 1989), during fetal development (Feeney, 1992 and references therein) and in the absence of TdT expression (Komori *et al.*, 1993; Gilfillan *et al.*, 1993). Some inserts within coding junctions bear DNA segments that are deduced from the sequence of the two DNA ends. Lieber (1992) suggested an oligonucleotide capture mechanism to explain these unusual inserts. In DNA general end-joining, capture of oligonucleotides requires fully complementary ends relative to the target region on each of the double-stranded oligonucleotides (Roth *et al.*, 1991). An experiment to verify oligonucleotide capture in V(D)J recombination has not been carried out, but a summary of endogenous V(D)J junctions suggests that similar events may take place during V(D)J recombination (Carroll *et al.*, 1993).

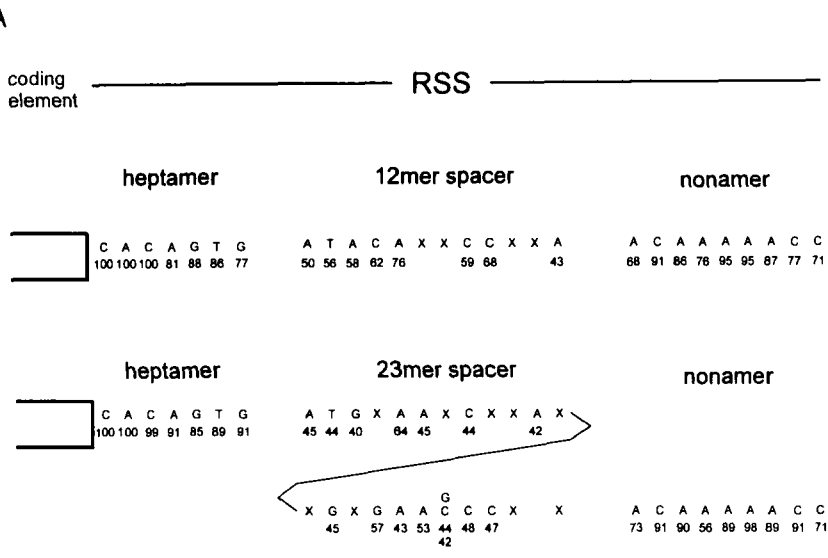
A miminalistic model for the V(D)J recombination process thus has to take into account that a lymphocyte-specific nuclease cleaves at RSS in a site-directed manner (Lewis and Gellert, 1989; Lewis, 1994a; Lieber, 1991). After the cleavage step, V(D)J recombination may follow a general pathway(s) of DNA end joining (Lewis and Gellert, 1989; Roth *et al.*, 1995). According to this view, lymphocyte specificity would be provided by the nuclease that cleaves at RSS and by the addition of N-nucleotides through TdT. Teleologically speaking, it might be that the immune system takes the risk of site-directed DSBs to gain access to general DNA end modification processes that thus can support the generation of antigen receptor diversity.

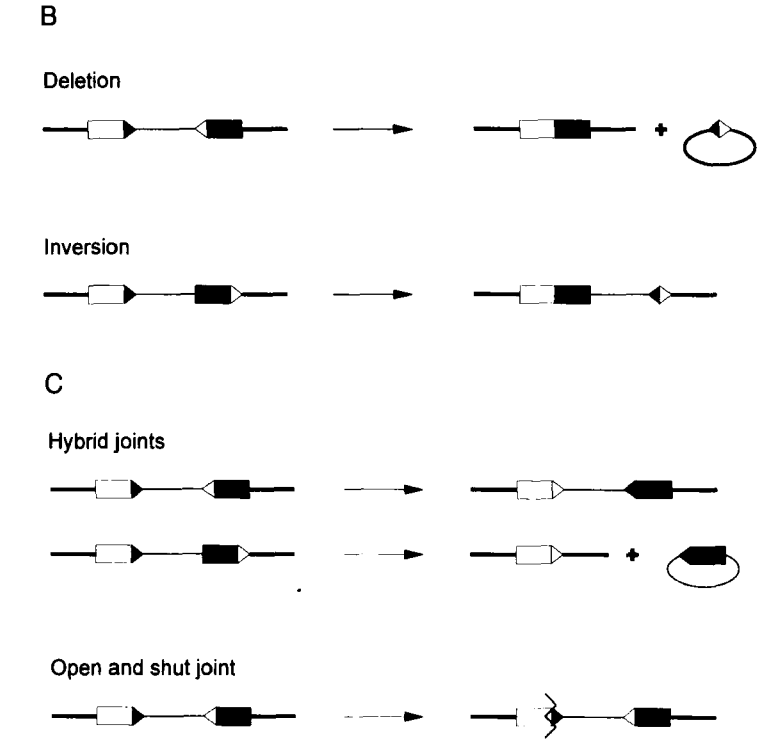
III. Components of the V(D)J Recombination Machinery

A. Lymphocyte-Specific Proteins

1. Recombinase Activating Genes 1 and 2 (RAG-1 and -2)

a. Organization and Function. The *RAG-1* and *RAG-2* genes were identified and cloned on the basis of their ability to rearrange an integrated recombination substrate upon transfection of genomic DNA in 3T3 fibroblasts (Schatz and Baltimore, 1988; Schatz *et al.*, 1989; Oettinger *et al.*, 1990). The two complementing genes show a unique organization. Their 3′ ends face each other and are separated by several kilobases (8 kb in mice). The coding sequences and the 3′ untranslated region of both genes are located on one exon. The open reading frames code in mice for a


A
coding element
RSS
heptamer
12mer spacer
nonamer
C A C A G T G
100 100 100 81 88 86 77
A T A C A X X C C X X A
50 56 58 62 76 59 68 43
A C A A A A A C C
68 91 88 76 95 95 87 77 71
heptamer
23mer spacer
nonamer
C A C A G T G
100 100 99 91 85 89 91
A T G X A A X C X X A X
45 44 40 64 45 44 42
G
X G X G A A C C C X X
45 57 43 53 44 48 47
42
A C A A A A A C C
73 91 90 56 89 98 89 91 71


B
Deletion
Inversion
C
Hybrid joints
Open and shut joint

119-kDa RAG-1 and a 66-kDa RAG-2 protein, respectively. The overall genomic organization as well as the amino acid sequences of RAG-1/2 are highly conserved throughout evolution: human, chicken, rabbit, and *Xenopus RAG-1/2* genes were isolated and the conserved 3′ part of RAG-1 was characterized in addition for shark, paddlefish, axolotl, and goldfish (Bernstein *et al.*, 1994; Carlson *et al.*, 1991; Fuschiotti *et al.*, 1993; Greenhalgh *et al.*, 1993; Ichihara *et al.*, 1992; Schatz *et al.*, 1989). No convincing homologs have been found in lower organisms. The mouse genes localize to chromosome 2p, while the human *RAG-1/2* locus maps to chromosome 11p13 (Oettinger *et al.*, 1992; Sherrington *et al.*, 1992; Ichihara *et al.*, 1992; Schwarz *et al.*, 1994). RAG-1 and RAG-2 reveal no sequence similarity to one another nor to any known protein. Putative functional domains appear not essential for V(D)J recombination (Section III.A.1.b).

As predicted by the distribution of V(D)J recombinase activity, *RAG-1/2* genes are coexpressed only within tissues and cells known to undergo V(D)J rearrangement (Boehm *et al.*, 1991; Guy-Grand *et al.*, 1992; Oettinger *et al.*, 1990; Schatz *et al.*, 1989; Turka *et al.*, 1991). Moreover, RAG-1/2 expression analysis might identify extrathymic pathways of T cell differentiation such as in human decidua (Hayakawa *et al.*, 1994). Concordant expression of RAG-1 and RAG-2 in human leukemic cells grossly mirrors the developmental block of the respective malignant clone (Bories *et al.*, 1991; Yoneda *et al.*, 1993; Urniel *et al.*, 1993). Also, discordant expression of RAG-1 and RAG-2 was reported. RAG-1 transcripts appear to be present in the murine central nervous system (Chun *et al.*, 1991), but no functional defect was assigned to RAG-$1^{-/-}$ mice (Section III.A.2). RAG-2 expression in avian bursal lymphocytes (in the absence of RAG-1) prompted speculations about RAG-2 involvement in gene conversion (Carlson *et al.*, 1991). However, a bursal-derived, transformed B cell line was still capable of gene conversion after inactivation of both *RAG-2* alleles (Takeda *et al.*, 1992). The level of RAG transcripts decreases during continuous cell culture of freshly isolated pre-B cells or mature B cell clones transfected with *RAG-1* and *RAG-2* (Rathbun *et al.*, 1993), raising caveats about the use of transformed pre-B cell lines as models for studying the control of V(D)J recombination activity, especially since differences in RAG-1 expression influence greatly the result of V(D)J recombination (Gallo *et*

FIG. 2. (A) The recombination signal structure. The consensus sequence for a RSS 3′ of its flanking sequence is depicted. The frequency of conserved bases (above 40%) in endogenous substrates at each position is indicated (Ramsden *et al.*, 1994). (B) Standard reaction products of deletional and inversional recombination. (C) "Nonstandard" V(D)J joining products. The outcome of hybrid joints depends on their relative genomic orientation.

al., 1994). To circumvent the latter problem cell lines have been generated containing heat shock inducible *RAG-1/2* genes and integrated recombination substrates under the control of different enhancing elements (Oltz *et al.*, 1993). These cell lines served for the unequivocal demonstration for the coupling of antigen receptor locus transcription and V(D)J recombination.

RAG-1/2 expression must be regulated at two levels that guarantee: (1) a developmental restriction of V(D)J recombination to B and T cell precursors and (2) a cell-cycle-dependent regulation in recombination competent lymphocytes (Lin and Desiderio, 1993, 1995; Schlissel *et al.*, 1993). The RAG-2 protein is differentially phosphorylated by one or more CDKs (cyclin-dependent kinase), resulting in a preferential RAG-2 accumulation in G1, due to interference with the RAG-2 degradation pathway (Lin and Desiderio, 1994). Down-regulation of RAG-2 protein occurs in avian thymocytes before the cells enter S phase, suggesting that RAG-2 function may be limited to noncycling cells, thus preventing DNA damage and cell cycle arrest through the V(D)J recombination machinery (Ferguson *et al.*, 1994). Onset and shutdown of V(D)J recombination during lymphocyte development are primarily regulated through RAG-1/2. RAG RNA levels drop in immature T and B cells upon crosslinking antigen receptors (Turka *et al.*, 1991; Ma *et al.*, 1992). Successful interaction of the TCR with major histocompatibility complex molecules during selection in the thymus down-regulates the expression of RAG-1/2 in cortical thymocytes (Bargulya *et al.*, 1992; Brändle *et al.*, 1992, 1994; Campbell and Hashimoto, 1991). Although it remains an open question why RAG expression persists after assembly of functional antigen receptors, it remains a possibility that continued RAG expression may permit the subsequent editing of antigen receptors (Gay *et al.*, 1993; Tiegs *et al.*, 1993).

RAG proteins are expressed mainly in the nucleus (Lin and Desiderio, 1993; Silver *et al.*, 1993), yet the physiological role of RAG-1/2 remains enigmatic. Neither RAG-1 nor RAG-2 proteins exhibit sequence specific DNA binding or cleavage activity. No interaction between RAG-1 and RAG-2 or of either protein with itself was detected in the yeast two-hybrid assay (Cuomo *et al.*, 1994; Schwarz *et al.*, unpublished). However, RAG-1 specifically interacts with Rag cohort factor 1 (Rch1) and the human homolog of the yeast protein SRP1 (suppressor of temperature-sensitive mutation of RNA polymerase I) (Cuomo *et al.*, 1994; Cortes *et al.*, 1994). SRP1 and Rch1 are members of a larger subfamily of proteins, that share Armadillo (Arm) repeats (Pfeifer *et al.*, 1994). *Xenopus* importin (Görlich *et al.*, 1994), another member of the Arm family, is required to mediate the first step of nuclear protein import. Because of sequence similarities between importin, Rch1, and SRP1 it is conceivable that Rch1 and SRP1 could play a role in nuclear protein import or nuclear matrix organization,

eventually building a core of a supramolecular structure for the V(D)J recombination machinery.

b. RAG-1/2 Mutational Analysis. As mentioned above, the function(s) of RAG-1/2 are unknown. RAG-1 and RAG-2 might encode components of the recombinase itself, or they might regulate recombinase expression. The protein sequences do not provide any indication as to RAG-1/2 function(s) (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). However, there are recognizable motifs that could imply functional abilities. In RAG-1 a Cys–His motif, similar yet noncanonical to zinc fingers, a nuclear localization signal, and a sequence similar to the active site of topoisomerases have been noted (Schatz *et al.*, 1989; Wang *et al.*, 1990). RAG-2 contains a very acidic stretch of amino acids, which is reminiscent of the acidic domain of some transcription factors (Oettinger *et al.*, 1990). Other regions in RAG-2 show a distant sequence similarity to a cAMP-dependent protein kinase target site, a tRNA ligase (*Escherichia coli*), and a methylase.

Clues to functionally important parts of RAG-1 and RAG-2 can be obtained through mutational analyses with the help of RAG-1/2 expression vectors and artificial, extrachromosomal substrates (Hesse *et al.*, 1987). Such mutational studies have assessed the critical regions for V(D)J recombination in RAG-1 (Silver *et al.*, 1933; Kallenbach *et al.*, 1993; Sadofsky *et al.*, 1993) and in RAG-2 (Silver *et al.*, 1993; Lin and Desiderio, 1993; Cuomo and Oettinger, 1994; Sadofsky *et al.*, 1994).

An amino acid alignment of RAG-1 in five species (human, mouse, rabbit, chicken, and *Xenopus*) revealed a striking natural boundary of conserved versus nonconserved regions in RAG-1. Murine amino acid 384 seems to separate a less conserved N-terminal fragment from a highly conserved C-terminal segment. RAG-1 genes with deletions up to the first 383 amino acids recombine signal joint substrates normally in transfected fibroblasts. A deletion which surpasses this border and disposes 437 amino acids, abolishes V(D)J rearrangement function. The Cys–His motif (amino acids 290–328) is thus dispensable for RAG-1 recombinase function. Similar results have been obtained upon the introduction of point mutants into the Cys–His motif (Sadofsky *et al.*, 1993; Silver *et al.*, 1993).

Carboxy-terminal truncation of the C-terminal 31 residues to amino acid 1009 had little measurable effect. However, further truncation to amino acid 994 eliminated recombination activity. Therefore, a region encompassing amino acid 384 to 1009 (of 1041 residues) defines a RAG-1 core that could not be further truncated without loss of function. Nine of 10 small insertions/deletions, dispersed over the core region, eliminated V(D)J recombinase capability. An exception was a mutant affecting amino acid 608 to 611 (Sadofsky *et al.*, 1993). A double mutant targeting the putative

nuclear localization signal was not different from wild-type function (Silver *et al.*, 1993).

Wang *et al.* (1990) described an amino acid sequence homology between the yeast gene *HPR1* and the portion of *RAG-1* from residue 472 onward. HPR1 bears some homology with topoisomerases. In particular the tyrosine which relates to murine RAG-1 tyrosine 998 suggested a potential topoisomerase active site. However, single substitution of position 998 as well as double mutants of residues 994 and 998 did not effect recombination (Silver *et al.*, 1993; Kallenbach *et al.*, 1993; Sadofsky *et al.*, 1993). These analyses rule out the participation of either of these tyrosines in forming a topoisomerase-like covalent bond to DNA. The region surrounding those tyosine residues appears to be essential for RAG-1 function, since a deletion of residues 994 to 998, as well as another one from residue 995 to the C-terminal end, prevents V(D)J recombination. At the protein level the deletion mutants seem to be transported to the nucleus and are stable toward degradation (Sadofsky *et al.*, 1993).

A similar analysis of the protein region of RAG-1 has addressed an interaction with human SRP1. The N-terminal region of RAG-1 (amino acids 1–288) without the zinc finger was sufficient for a stable interaction with hSRP1, but no interaction was observed with RAG-1 proteins lacking the N-terminal region (Cortes *et al.*, 1994). V(D)J recombination capability of RAG-1 mutants was tested only with signal joint extrachromosomal substrates in a fibroblastoid background; it is possible that in a natural setting other RAG-1 elements may well be required for the antigen-receptor rearrangement, such as those defined by SRP1 interaction.

Mutational analysis also defines a core region of RAG-2 that is functional in V(D)J recombination. Mutations and deletions as well as insertions in the N-terminal part eliminate almost all activity. Although the RAG-2 protein shows extensive evolutionary conservation across its total length, 25% of the C-terminus, including an acidic region putatively involved in transcription, can be deleted with the remaining 75% of RAG-2 retaining V(D)J recombination activity (Sadofsky *et al.*, 1994; Cuomo *et al.*, 1994). Mutants in the intermediate part of RAG-2 exhibit variant results. Alterations within the regions showing similarities to a cAMP protein kinase, a tRNA ligase, or a methylase have no influence on V(D)J recombination capability (Silver *et al.*, 1993). Thus, the core RAG-2 region appears to be sufficient for the basic recombination reaction, including the formation of signal and coding joints by deletional and inversional recombination on artificial as well as integrated substrates. Truncation of the C-terminal part of RAG-2 removes the coding elements with the highest phylogenetic conservation. This contrasts with the situation in RAG-1, where the most highly conserved segment plays an essential role in V(D)J recombination. It is tempting to speculate that both genes contain core regions required

for the recombination and other regions with a yet undefined function. Candidates could be determinants of interaction with chromosomes or the nuclear matrix, the control of rearrangement of the antigen receptor loci or the regulation of the recombination itself (Sadofsky *et al.*, 1994; Cuomo *et al.*, 1994).

Phosphorylation of murine RAG-2 occurs *in vivo* at two major sites after transfection in fibroblastoid cells (Lin and Desiderio, 1993, 1995). These sites conform to consensus sites for casein kinase II (CK II) and act as targets for CK II phosphorylation *in vitro*. Mutation of one of these sites, S356, does not affect RAG-2 nuclear localization or steady state RAG-2 levels. It reduces but does not abolish the activation of V(D)J recombination with artificial substrates. The cyclin-dependent kinase $p34^{cdc2}$ phosphorylates RAG-2 *in vitro*. Alanine substitution mutation at amino acid T490 stabilizes the RAG-2 protein and increases the half-life of RAG-2 in transfected fibroblastoid cells 20-fold, but does not affect nuclear localization as well as V(D)J activity. These data suggest that T490 is a component of the RAG-2 degradation pathway targeted by a kinase. The RAG-2 phosphorylation at T490 might coordinate the activity of RAG-2 with other potentially cell-cycle-regulated proteins of the V(D)J recombinase (Lin and Desiderio, 1995; Schlissel *et al.*, 1993).

c. Knockout Mice. *RAG-1* and *-2* are essential components involved in the V(D)J recombination process. Gene-targeted mice with an inactivation of the *RAG-1* or the *RAG-2* gene have no mature B and T cells due to their inability to initiate V(D)J recombination (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992). In contrast to *scid* mice, the mutant RAG-1/2$^{-/-}$ phenotype is not "leaky." B cell differentiation in the bone marrow of RAG-deficient mice is arrested at the large CD43$^+$/B220$^+$ pro-B cell stage and Ig genes remain in the germline configuration. Thymocyte maturation is blocked at the large Thy1$^+$/IL-2Rα^+/CD4$^-$/CD8$^-$ double-negative (DN) level. The thymi of both mutant mouse strains contain reduced numbers of thymocytes (3×10^6 compared with about 1×10^8 in the thymus of a normal young adult mouse) (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992). The developmental block of lymphocytes in these mice can be partially rescued through the introduction of functionally assembled Ig or TCRβ genes (Pfeffer and Mak, 1994, and references therein). Apparently no other hematopoietic compartment is defective in RAG $^{-/-}$ mice nor was any neuroanatomical or behavioral abnormality detectable.

2. *Terminal Deoxynucleotidyl Transferase (TdT)*

Addition of novel nucleotides at V(D)J junctions falls into two categories: template-independent N regions (Alt and Baltimore, 1982) and template-dependent P (palindromic) nucleotides (Lafaille *et al.*, 1989).

Terminal deoxynucleotidyl transferase has been implicated in the addition of N regions (Alt and Baltimore, 1982). The template-independent polymerization activity that randomly polymerizes deoxyribonucleoside triphosphates onto initiator DNA in cell-free systems (Bollum and Chang, 1986) and its preference for dG utilization both correlate well with the G/C richness of signal, coding, and hybrid joints (Lieber *et al.*, 1988; Lewis *et al.*, 1988). N inserts can be incorporated in coding and signal joints, in the latter case, the frequency of inserts correlates with *in vivo* TdT levels (Lieber *et al.*, 1988). In mice, two alternatively spliced TdT forms have been identified, each one introducing N regions into V(D)J joints (Landau *et al.*, 1987; Kallenbach *et al.*, 1992). TdT is expressed only in organs where T and B cell development occur, i.e. in cortical immature thymocytes and in bone marrow cells (Bogue *et al.*, 1992). The expression pattern of TdT follows closely RAG-1 expression, with functional activity in immature cells and a turn-off in mature lymphocytes (Bogue *et al.*, 1992). The abundance of N regions in V(D)J junctions in adult as compared to fetal lymphocytes correlates with the TdT expression in precursors of the former, in contrast to the latter lymphocyte population (Feeney, 1990, 1991; Aguilar and Bellmont, 1991). TdT protein biosynthesis and expression of TdT RNA do not appear earlier than 3 to 5 days postnatally (Bogue *et al.*, 1992). TdT seems to be associated with the nuclear matrix (Pandey *et al.*, 1989). However, TdT expression and N nucleotide addition are not required for V(D)J recombination. Fibroblastoid cells as well as other nonlymphocytes do not express TdT activity (Landau *et al.*, 1987). When artificial V(D)J substrates in conjunction with RAG-1/2 expression vectors were introduced into fibroblastoid cells or other cell lines, V(D)J joining efficiency and fidelity were not hampered. In addition, occasional insertions of 1–3 nucleotides were detected at the junctions (Lieber *et al.*, 1988; Schatz *et al.*, 1992; Kallenbach *et al.*, 1992; Taccioli *et al.*, 1993). Such base additions may be generated by the same mechanisms by which nucleotide addition occurs at recombinant junctions, when transfected linear DNA is joint in mammalian cells (Roth *et al.*, 1989).

An unequivocal evaluation of the role of TdT in V(D)J recombination and emergence of the immunologic repertoire became possible through gene-targeted inactivation mutagenesis (Komori *et al.*, 1993; Gilfillan *et al.*, 1993). Mutations generated by RAG-2-deficient blastocyst complementation addressing only lymphocyte development (Chen *et al.*, 1993c; Komori *et al.*, 1993) and by a germline-transmitted TdT defect (Gilfillan *et al.*, 1993) reached nearly identical conclusions. The occurrence of junctional N regions was lowered dramatically, without total elimination, and a fetal-like repertoire of IgH and TCR junctions mediated by homologous end-

joining was expressed. Thus, TdT constitutes a tissue-specific integral but not obligate component of the V(D)J recombinase machinery. If expressed, it qualitatively modifies the outcome of the V(D)J reaction and affects the resulting variable region repertoire in at least two ways. First, N region additions diversify the antigen binding pocket substantially; second, TdT apparently seems to block homology-directed recombinations even in the absence of end modifications. Homozygous mutant $TdT^{-/-}$ mice breed well and appear to be healthy in conventional animal facilities, they are of normal size and do not have increased infection rates (Gilfillan *et al.*, 1993). A more detailed immunological analysis should determine if a relatively restricted fetal/perinatal or a highly diverse adult repertoire is more critical for the development and well-being of the animals.

In humans, loss of TdT activity has not been assessed or intensively searched for.

B. Proteins Shared in DNA Double-Strand Break Repair and V(D)J Recombination

1. V(D)J Recombination and DNA Double-Strand Breaks

A number of DNA-damaging agents, ionization being one, generate DNA double-strand breaks. Cellular response to ionizing radiation is complex as indicated by the existence of nine complementation groups among X-ray-sensitive rodent cell mutants (Jeggo *et al.*, 1991; Thacker and Wilkinson, 1991; Zdzienicka *et al.*, 1992). Ionizing radiation of mammalian cells results in single-strand breaks, base damage, and double-strand breaks. Three different complementation groups for DSB repair mutants have been identified, indicating that at least three genes encode components of that DNA repair pathway (Zdzienicka *et al.*, 1992). In V(D)J recombination double-stranded blunt signal sequences at the Ig and TCR loci have been demonstrated (Schlissel *et al.*, 1993; Roth *et al.*, 1992a,b) and studies of V(D)J recombination and DSB repair are rapidly converging.

Through the introduction of RAG-1/2 expression vectors into virtually every cell type, it became possible to activate V(D)J recombination and study the V(D)J joining mechanism. This technique allows the analysis of the V(D)J reaction in the context of X-ray-sensitive and/or double-strand break repair mutants. Several groups searched for V(D)J joining deficiencies in DNA double-strand break defective hamster cell lines (Taccioli *et al.*, 1993, 1994a,b; Pergola *et al.*, 1993).The Chinese hamster ovary (CHO) mutants *xrs6* and XR-1 belong to two separate complementation groups. When tested with artificial substrates, coding and signal joint formation was very low and signal joints had more truncations at their ends as compared to wild type (Taccioli *et al.*, 1993; Pergola *et al.*, 1993). The

V(D)J defect in *xrs*-6 cells was complemented by human chromosome 2, by fusion to *scid* cells, or by XR-1/*xrs6* hybrids. Radiosensitivity of *xrs6* was also complemented by introduction of chromosome 2, which implies that specific chromosomal segments correct the V(D)J recombination defect and ionizing radiosensitivity, providing strong evidence that these two processes share common gene products.

Correction of the XR-1 defect in radiosensitivity and V(D)J recombination was obtained by substitution of human chromosome 5 (Taccioli *et al.*, 1993). All together, these data indicated (1) that the *xrs*, XR-1, and *scid* mutations encode separate proteins and (2) that each protein is involved in DNA DSB repair and V(D)J recombination.

Another CHO mutant, V-3 of the third DSB repair complementation group, is defective in joining coding ends, but shows almost normal activity regarding signal end recombination (Pergola *et al.*, 1993). The V-3 defect may be allelic to the *scid* mutation, since fusion products of both representative cell lines could not correct the radiation sensitivity or the V(D)J defect (Taccioli *et al.*, 1994a). Analysis of the structure of the V-3 coding joints revealed the occurrence of large insertions homologous to the back bone of the vectors employed and open-and-shut events. Such structures have not been observed in any of the other mutations that affect V(D)J recombination (Taccioli *et al.*, 1994a). It is possible that these differences reflect manifestations of different mutations on the same genetic locus or may be explained by differences in genetic background (*scid* mouse versus hamster).

2. *Murine scid and DNA-PK$_{CS}$*

The murine *scid* mutation was defined as an autosomal recessive mutation, that results in a general (but often not complete) absence of mature B and T cells due to an impairment in the V(D)J recombination process (Bosma *et al.*, 1983). It remains to be established whether correct rearrangement events observed in older *scid* mice are due to spontaneous revertants or rather reflect a low-frequency "leakiness" of the *scid* mutation. In immature lymphocytes of the *scid* mouse, DSB initiation is not affected, but recombination is defective at the level of coding joint formation, with extensive deletions reaching 5′ and 3′ of the normal V(D)J recombination sites of TCR and Ig genes (Hirayashi *et al.*, 1987; Kim *et al.*, 1988; Malyun *et al.*, 1988; Okazaki *et al.*, 1988; Schuler *et al.*, 1986; Witte *et al.*, 1987). Signal joints are in general less affected. Since coding but not signal ends are forming hairpin structures before modification and joining (Roth *et al.*, 1992a,b), it has been speculated that the *scid* mutation might impair the resolution or increase the production of hairpins. Impaired resolution of hairpins could eventually result in intermediate products and induce

premature death in lymphocyte progenitors. Contradictory to that assumption, a test of the hairpin-resolving ability in *scid* versus wild-type cells showed no differences upon introduction of artificial hairpins into transformed cell lines (Lewis, 1994b), indicating that none of the enzymatic operations involved in hairpin resolution or end-joining appear to be missing in *scid* mice (Lewis, 1994b, and references therein). Taken together these results suggest that the *scid* mutation somehow protects hairpins of coding ends from nuclease attack.

Furthermore, both lymphoid and nonlymphoid *scid* cells are hypersensitive to killing by ionizing radiation, owing to a DNA DSB repair defect (Biedermann *et al.*, 1991; Fulop and Phillips, 1990; Hendrickson *et al.*, 1991; Taniguchi *et al.*, 1993). In Chinese hamster ovary cells expressing transfected *RAG* genes, mutations in at least three complementation groups are associated with impaired radioresistance, V(D)J recombination, and DSB repair. Complementation group 7, which includes the V-3 mutant cells, corresponds to the mouse *scid* gene (Taccioli *et al.*, 1994a), which is located on chromosome 16. A human gene that restores the V(D)J recombination, normal radioresistance, and DSB repair to *scid* cells has been mapped to human chromosome 8q11 (Banga *et al.*, 1994; Itoh *et al.*, 1993; Kirchgessner *et al.*, 1993; Komatsu *et al.*, 1993, 1995; Kurimasa *et al.*, 1994). Moreover, a yeast artificial chromosome clone spanning the *DNA* PK_{CS} locus, which maps to this region, complements the V-3 defects of radioresistance and V(D)J recombination. Thus *DNA* PK_{CS} represents a strong candidate gene for the *scid* defect (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995). Although it is not yet possible to conclude with certainty that the mutations in V-3 and *scid* are located within the *DNA* PK_{CS} gene, the data indicate a crucial role of DNA PK_{CS} in V(D)J recombination and DNA DSB repair.

DNA PK is a mammalian protein Ser/Thr kinase which binds directly to DNA. Although physiologically relevant substrates have not been defined unequivocally, numerous *in vitro* interaction factors (Table I) have been characterized (Anderson, 1993, and references therein). DNA PK is a trimolecular complex. The catalytic subunit is a large 350-kDa protein (p350) which is targeted to DNA by the human autoimmune antigen KU, a heterodimeric polypeptide composed of 70- and 86-kDa subunits (Gottlieb and Jackson, 1993; Dvir *et al.*, 1992).

In vitro, DNA PK requires double-stranded DNA ends or other DNA double-helix discontinuities for activation (Gottlieb and Jackson, 1993; Morozov *et al.*, 1994). After binding, DNA PK translocates into the DNA molecule to assess distal targets. The wide variety of substrates phosphorylated *in vitro* argues against a role for DNA PK in controlling specific gene expression; rather the activation of DNA PK by DNA discontinuities

TABLE I
IN VITRO DNA PK SUBSTRATES ACCORDING TO ANDERSON (1993) WITH MODIFICATIONS

DNA-Binding Proteins	Non-DNA-Binding Proteins
SV40 large tumor antigen (TAg)	Heat shock protein 90 (hsp90)
Tumor suppressor protein p53	Microtubule-associated protein τ
Ku autoantigen (p70 and p86)	Casein
RNA polymerase II CTD domain	Phosvitin
RNA polymerase	DNA PK_{CS}
Serum response factor (SRF)	
Transcription factor cJun	
Transcription factor cFos	
Transcription factor Oct1 (POU domain)	
Transcription factor Sp1	
Transcription factor cMyc	
Transcription factor CTF/NF-I	
Transcription factor TFIID	
Chicken progesterone receptor	
Replication factor A (RPA)	
Topoisomerases I and II	
Xenopus histone 2A.X	
Adenovirus-2 72-kDa DNA-binding protein	
Bovine papillomavirus E2 protein	
Polyomavirus VP-1	
Unidentified HeLa 110-kDa polypeptide	
Unidentified HeLa 52-kDa polypeptide	

suggests a role in DNA damage recognition. DNA PK could also play a role in coordinating the use of DNA segments as templates for different enzymes, including those involved in transcription, replication, and repair. Several speculations not mutually exclusive have been put forward as to how DNA PK might function in DNA DSB repair and V(D)J recombination.

DNA PK binds to DNA termini to prevent nucleotide loss through exonucleases. DNA PK might regulate the activities of components of the DNA repair and recombination apparatus. Phosphorylation events mediated by a DNA end-restricted kinase recruit or inactivate components of the repair machinery only at genomic breaks, thereby ensuring an ordered sequence of events. DNA PK_{CS} might act indirectly through the activation of transcription factors or suppressors which regulate repair and recombination constituents. An attractive role for DNA PK might be the alignment of DNA ends to promote their ligation and/or the recruitment of additional factors to DNA ends serving eventually as a scaffolding protein together with Arm proteins (Blunt *et al.*, 1995). DNA PK_{CS} is essential only for coding joints, thus signal joining can proceed through DNA PK_{CS}-

independent pathways. In light of the persisting coding-joint hairpins in *scid* lymphocytes one might envisage that DNA PK_{CS} activates or recruits an endonuclease that opens up hairpin intermediates. Another possibility is that DNA PK_{CS} directly or indirectly disassembles parts of the recombination machinery to make hairpin ends accessible for further processing (Blunt *et al.*, 1995). Finally , DNA PK_{CS} may be involved in base gains and/or losses that occur at coding ends prior to ligation.

3. *KU 70/86*

The human KU autoantigen was originally recognized in patients with scleroderma–polymyositis overlap syndrome (Mimori *et al.*, 1981). KU is a heterodimeric protein composed of equimolar amounts of 86- and 70-kDa subunits (Mimori *et al.*, 1981; Reeves, 1985; Yaneva *et al.*, 1985).

Human and murine cDNAs for both subunits have been cloned (Reeves and Sthoeger, 1989; Yaneva *et al.*, 1989; Mimori *et al.*, 1990; Chan *et al.*, 1989; Porges *et al.*, 1990; Falzon and Kuff, 1992). One must bear in mind, that KU antigen is probably not a homogenous protein: its 70-KDa subunit was shown to be produced by a gene family, and thus a cell could synthesize different variants of the dimer for different purposes (Griffith *et al.*, 1992). KU is evolutionarily conserved; a DNA-end-binding protein with KU-like properties was purified from yeast (Feldmann and Winnacker, 1993).

KU 70 is located on human chromosome 22q13, and KU 86 is on 2q33-35 (Cai *et al.*, 1994). KU is expressed ubiquitously in normal tissues. Immunolocalization of the KU 70/86 polypeptides has demonstrated a cell-cycle-dependent staining of nucleoli versus the nucleoplasma (Reeves, 1987; Yaneva and Jhiang, 1991; Hiyashiura *et al.*, 1992; Li and Yeh, 1992). KU binds to the termini of duplex DNAs, regardless of their chemical features and sequences, and then slides along the termini in several copies like beads on a string, with a 25-bp periodicity and without energy requirement (Mimori and Hardin, 1986; May *et al.*, 1991; Blier *et al.*, 1993; Csordàs-Toth *et al.*, 1993; Woodgett, 1993).

KU was shown to be a regulator of DNA PK (Lees-Miller *et al.*, 1990; Dvir *et al.*, 1993; Gottlieb and Jackson, 1993; Woodgett, 1993) and to recruit DNA PK_{CS} to the DNA template for phosphorylating components of transcription complexes. An ATPase activity, which stimulates DNA polymerase α activity on primed, single-stranded DNA templates by overcoming a lag in the initiation of synthesis, was assigned to KU (Cao *et al.*, 1994). This DNA-dependent ATPase activity could provide energy for the DNA sliding mechanism. KU was also identified as human DNA helicase II (HDH II). *In vitro*-synthesized KU 70/86 heterodimer provides helicase activity. The ability of KU to unwind duplex regions points to a possible role for HDH II/KU in some form of strand exchange (Tuteja *et al.*, 1994).

CHO cell lines of complementation group 5 are both impaired in V(D)J recombination and DSB repair. *Xrs6* cells are defective in functional KU and lack detectable DNA PK activity (Getts and Stamato, 1994; Rathmell and Chu, 1994a,b; Taccioli *et al.*, 1994b; Finnie *et al.*, 1995). Furthermore, radiosensitivity and V(D)J recombination defects of *xrs6* cells are complemented by expression of the *KU86* cDNA, indicating that KU 86 is the product of the X-ray cross-complementing group 5 gene (XRCC5) that complements the defects in this group (Smider *et al.*, 1994; Taccioli *et al.*, 1994b; Boubnov *et al.*, 1995). The direct consequence of the KU defect is a concomitant deficiency in the kinase activity of DNA PK. These data suggest that KU/DNA PK functions as a complex *in vivo*, as it does *in vitro*, and indicate that the kinase activity requires KU. In addition, these results imply that no other endogenous proteins can target p350 to double-stranded DNA and cooperate with it to yield DNA PK activity (Finnie *et al.*, 1995). In principle, similar consequences as discussed for V(D)J recombination in XRCC7 mutants apply to the KU 86 defect. KU/DNA PK binds to DNA termini and protects them from nuclease digestion or assists in the alignment of free DNA ends. Such DNA protection might require the whole DNA PK complex or could be fulfilled by KU alone. Other possible functions of KU/DNA PK upon DNA binding include the activation and phosphorylation of factors involved in DNA repair or recombination, the inactivation of transcription factors, in order to make DNA breaks accessible to the repair/recombination machinery, and a role as DNA free-end-sensor signaling to cells that a DNA damage has occurred.

The cooperative binding of KU 70/86 onto DNA makes a KU 70-mediated DNA DSB repair/V(D)J recombination defect highly likely. A novel cell line, sxi-1, is deficient in DNA end binding. The complete phenotype of sxi-1 cells became reversible by transfection of a *KU70* cDNA (Lee and Hendrickson, 1995), much like the effect of *KU86* transfection into *xrs6* cells.

The XR-1 mutation, the third complementation group known to affect V(D)J recombination in CHO cells, has not been unravelled as of yet; it will not come as a surprise when additional proteins eventually have to be included in the DNA repair/V(D)J recombination circuit.

C. Biochemically Defined Proteins

In cell-free systems attempts have been made to dissect V(D)J recombinase activity into distinct steps. The isolation of factors that specifically bind, cut, polymerize onto, or ligate V(D)J ends has been initiated.

Several different signal binding proteins have been described on their virtue of protein/RSS interaction (Aguilera *et al.*, 1987; Halligan and Desiderio, 1987; Hamaguchi *et al.*, 1989; Kottmann *et al.*, 1992; Miyake

et al., 1990; Muegge *et al.*, 1993; Shirakata *et al.*, 1991; Wu *et al.*, 1993). None of these factors totally fulfills the criteria for a specific V(D)J RSS binding protein as defined by Lewis (1994a), i.e., (1) better binding to a RSS containing DNA fragment than to DNA lacking a RSS, (2) less avidity to noncanonical RSS variants, (3) lymphocyte restricted expression, and (4) putative enzymatic cutting or/and joining activity.

Detection of a site-specific nuclease activity has been reported (Desiderio and Baltimore, 1984; Kataoka *et al.*, 1984; Hope *et al.*, 1986) but has not been characterized in detail.

The DNA polymerase β gene *pol*β has been shown to be one of various enzymes involved in the DNA repair machinery (Fry, 1983). Inactivation of *pol*β at the germline level creates a lethal phenotype. Conditioned inactivation in T cells and T progenitors provides no direct evidence for a role of *pol*β in TCR gene rearrangement (Gu *et al.*, 1994).

The repair of some types of DNA double-strand breaks is thought to proceed through DNA flap structure intermediates. A DNA flap is a bifurcated structure composed of double-stranded DNA and a displaced single strand. A murine DNA structure-specific nuclease, *f*lap *e*ndo*n*uclease-1 (FEN-1), that cleaves only DNA flap strands, terminating with a 5′ single-stranded end, has been purified to homogeneity (Harrington and Lieber, 1994a) and the gene has been cloned (Harrington and Lieber, 1994b). The nucleotide sequence of FEN-1 is highly homologous to the *Saccharomyces cerevisiae* genes YKL 510 and RAD2, implicated in nucleotide excision repair. In addition to the flap endonuclease activity FEN-1 exhibited 5′-3′ double-strand-specific exonuclease activity, the latter one being sequence dependent. The prospect for FEN-1 function in DNA double-strand break repair is still not clear. Based on circumstantial evidence, FEN-1 might interact with polymerase ε. A high-molecular-weight complex which is capable of carrying out homologous recombination and double-strand break repair has been purified from calf thymus (Jessberger and Berg, 1991; Jessberger *et al.*, 1993). This complex contains polymerase ε, DNA ligase III, a 3′-5′ exonuclease, and a 5′-3′ exonuclease.

Whether joining of coding and signal ends defines a lymphoid specific V(D)J recombination process or rather an activity of nonspecific ligase(s) remains to be established. A V(D)J joining protein, V(D)JP, has been isolated based on its nonamer-binding properties (Guillams *et al.*, 1994). After the expression of a fragment of V(D)JP in bacteria, joining signal-dependent ligation activity was detected. The removal of a RSS from either end of the two fragments to be joined, abolished ligation activity, which was detected in pre-B cell lines but not in fibroblasts (Guillams *et al.*, 1994). The function of the V(D)JP ligation activity within the V(D)J recombination process is unclear. The molecular complex, purified by Jessberger *et al.*

(1993; see above) involves ligase III. The notion that this ligase is involved in V(D)J recombination is attractive, especially after ligase I has been excluded as constituent of the V(D)J recombinase machinery (Hsieh *et al.*, 1994; Petrini *et al.*, 1994).

D. Structural and *cis*-Acting Elements of Antigen Receptor Loci

Mutant mouse strains have been established to investigate the role of structural and *cis*-acting Ig gene elements in the control mechanisms of rearrangement and development,

Deletion of the J_H-locus blocks the transition from the large $CD43^+$ to the small $CD43^+$ murine pre-B cell stage (Ehlich *et al.*, 1993; Chen *et al.*, 1993a). Heavy chains are not rearranged in those progenitors. The κ light chain locus rearranges completely independently from the presence of DJ_H or VDJ_H rearrangements. In a mutant mouse strain in which the Ig heavy chain intron enhancer ($E\mu$) was removed, V_H to DJ_H recombination was severely impaired as judged from quantitative PCR analysis, while D_H to J_H rearrangement stayed almost normal (Serwe and Sablitzky, 1993). These results are in line with data showing that the V(D)J recombination machinery is modulated through *cis*-regulatory elements within the intron enhancer (Ferrier *et al.*, 1984, 1990), and predict the existence of other *cis*-regulatory element(s) which are required to activate the V(D)J recombinase.

The production of the μ membrane heavy chain during pre-B cell differentiation might mediate allelic exclusion by preventing further V_H to DJ_H rearrangements (Weaver *et al.*, 1985; Rusconi and Köhler, 1985). Mice with a disruption of the membrane exon of the Ig μ chain demonstrate, that in the absence of the membrane-bound μ heavy chain, pre-B cell allelic exclusion is impaired (Kitamura *et al.*, 1991; Kitamura and Rajewski, 1992).

The control of the κ light chain rearrangement has been addressed in mice being deficient for either *cis*-acting DNA elements or coding modules of the κ light chain gene (Zou *et al.*, 1993; Chen *et al.*, 1993b). C_κ-, $J_\kappa C_\kappa$-, or intronic E_κ enhancer (iE_κ)-deficient homozygous mice produce only λ light chain B cells, in about 10-fold normal quantity, while total B cells are reduced to 50%. In $J_\kappa C_\kappa$- and iE_κ-deficient mice κ-chain rearrangement is completely abolished. However, in C_κ-defective animals, $V_\kappa J_\kappa$ rearrangement occurs. Thus κ-chain rearrangement is not a prerequisite for λ recombination, although rearrangement of the κ locus usually precedes rearrangement of the λ locus.

There are no defined human counterparts for the defects mentioned. Deletions in chromosome 14q32 of the heavy chain constant region genes are common, occurring in 5–10% of the caucasoid population. Moreover,

unequal crossing-over frequently affects the heavy chain locus. Individuals who are homozygous for such deletions lack the relevant isotypes and subclasses (Rosen *et al.*, 1992). However, most such families were found during screening of entirely healthy blood donors, although a few individuals with these defects presented with recurrent pyogenic infections. Families characterized by the expression of λ but not κ light chains have been reported in which circulating B cells were normal. Point mutations in the κ chain gene were present in one of those families (Rosen *et al.*, 1992).

The importance of TCRβ rearrangement for the differentiation of $CD4^-/CD8^-$ DN to DP thymocytes was demonstrated in animals with a TCRβ deletion (Mombaerts *et al.*, 1991, 1992b). Thymocyte numbers were low and no TCR was expressed. TCRα rearrangements and full size TCRα transcripts can be found in TCRβ mutants, challenging the theory that TCRβ expression constitutes a prerequisite for TCRα rearrangement. A developmental arrest in TCRα constant-region-mutant mice occurs late in thymocyte ontogeny (Mombaerts *et al.*, 1992b; Philcott *et al.*, 1992). Thymi of these mice reveal normal numbers of DN and DP precursors with a marked reduction of CD4 and CD8 SP cells. TCRα rearrangements were observed at a reduced level in these mice; TCRβ recombinations were not affected. Development of δ T cells was not impaired in either the TCRα or -β knockout animals. Vice versa, a targeted disruption of the TCRδ constant region has no apparent effect on the development of thymocytes expressing TCRαβ receptors (Itahara *et al.*, 1993), while the resultant $TCR\delta^{-/-}$ mouse strain has no detectable surface expression of any γδ products. A human immunodeficiency based on the loss of structural or *cis*-acting DNA elements at the TCR loci has not been reported.

IV. V(D)J Recombination Defects in Humans

A. Severe Combined Immunodeficiency (SCID)

Human severe combined immunodeficiency (SCID) comprises a group of genotypically as well as phenotypically heterogeneous diseases (Rosen *et al.*, 1992; Fischer, 1992, and references therein). Without bone marrow transplantation, affected infants usually die from severe and persistent infections. Significant progress has been made in the characterization of the molecular basis underlying several SCID entities (Table II). X-linked SCID caused by a defect in the common chain of the interleukin 2, 4, 7, 9, 13, and 15 receptors (IL-$R\gamma_c$) (Noguchi *et al.*, 1993; Matthews *et al.*, 1995) and the autosomal recessively inherited adenosine deaminase deficiency (Hirschhorn, 1995) are observed in about 40 and 20% of patients, respectively. Rare cases of SCID result from abnormalities of the enzyme purine nucleoside phosphorylase, inappropriate expression of HLA class

TABLE II
HUMAN SEVERE COMBINED IMMUNODEFICIENCY ENTITIES

X-linked SCID
Interleukin receptor common gamma chain (IL-Rγ_C)*
Autosomal recessive SCID
Adenosine deaminase (ADA) deficiency*
Purine nucleoside phosphorylase (PNP) deficiency*
MHC class II defects
CIITA transactivator*
RFX5: p75* and p36*
Reticular dysgenesis
TCR/CD3 defects
CD3 ε*
CD3 γ*
Lymphokine deficiencies
IL-2 deficiency*
Multiple lymphokine deficiency (NF-AT1)
Signal transduction defects
ZAP70 tyrosine kinase*
Jak 3*
Selectively disturbed calcium flux in lymphocytes
V(D)J recombinase
RAG-1*
RAG-2*

Note. Asterisks denote identification of the molecular defect in the respective disorders.

II molecules, defective interleukin-2 production, impairments in the T cell receptor/CD3 complex, or disturbances of signal transduction pathways within T cells. A common feature of all these SCID entities is the presence of B cells (B^+SCID) in the peripheral blood of respective patients. Approximately 25–30% of SCID patients lack evidence of peripheral B cells (B^-SCID), thus representing a candidate group for V(D)J recombination defects. A critical point in human SCID is the fact that a high percentage of SCID patients acquires maternal T cells by maternofetal transfusion (Pollack *et al.*, 1982; Wahn *et al.*, 1991; Knobloch *et al.*, 1991). Therefore, the mere presence of T cells does not exclude a lack of V(D)J recombinase activity.

A first hint to V(D)J recombination defects in B^- SCID came from the description of an unusual rearrangement at the IgH locus of a cell line which placed a D_{LR1} element 60 nucleotides downstream from J_{H4} on one allele (Ichihara *et al.*, 1988). However, Thompson et al. (1990, 1992) established EBV-transformed B cell lines of B^-SCID patients and identi-

fied germ-line configurations at the J_H and J_κ loci. These authors therefore argued against a V(D)J recombinase defect. However, the possibility of contaminating maternal T cells was not excluded in this study.

A systematic approach to the V(D)J recombination potential of SCID patients was initiated by Schwarz *et al.* (1991). PCR products of the IgH D_{HQ52}–J_H region were analyzed. While B^+SCID patients exhibited a normal recombination pattern, all six B^-SCID patients tested showed a grossly altered rearrangement pattern characterized by either a total or partial absence of regular D_{HQ52}–J_H recombinations and/or the presence of abnormal rearrangements. The latter events were caused by deletions surpassing the boundaries of immunoglobulin coding elements. These results were confirmed by an independent study that identified subtle differences in the V(D)J recombination capability of B^-SCID patients (Abe *et al.*, 1994). One of the patients did not exhibit any recombination event in the D_{HQ52}–J_H assay. A second patient initiated DJ recombination, but had a very low level of V(D)J products. These differences in V(D)J recombination capability either may reflect allelic variants of a single gene defect or may be due to the fact that different genes may cause a B^-SCID phenotype. The V(D)J products of both studies were reminiscent of those obtained in the RAG-1/2 knockout or in *scid* mouse models.

In EBV-transformed pre-B cells of B^-SCID patients the IgH enhancer sequence, located between the J_H and μ genes, was hypomethylated and an abundant germ-line Cμ was transcribed (Kamachi *et al.*, 1993). These results suggest that the chromatin structure of the IgH gene locus in these cell lines is accessible to the V(D)J recombinase and is able to participate in the DNA rearrangement. Since no RAG-1/2 transcripts were detected in these cell lines the failure of the assembly of a competent V(D)J recombinase, which includes RAG proteins, could explain the phenotype in these patients. Taken together, the studies strongly suggest that V(D)J recombination can be disturbed in human B^-SCID patients at several differentiation levels.

1. RAG-1/2 Mutations

An attempt to identify human RAG translocations or deletions either at the cytogenetic level or by Southern blot analyses failed (Schwarz, unpublished). Therefore a screening of the human genomic RAG-1/2 locus by single stranded conformation polymorphism (SSCP) analysis (Orita *et al.*, 1989) was initiated. The study design with overlapping PCR cassettes of 350–380 bp should allow the detection of about 80% of the mutations, if present.

Previous studies had identified three DNA polymorphisms in the RAG-1 gene and one polymorphism in the RAG-2 gene (Table III) (Nomdedéu

TABLE III
DNA POLYMORPHISMS OF HUMAN RAG-1/2 GENES

Gene	Nucleotide	AA Change
RAG-1	C579T	A156V[a]
	G858A	R249H[a]
	A2992G	None[b]
RAG-2	G1223A	V81[a]

Note. Base and amino acid numbering according to Schatz *et al.* (1992) and Ichihara *et al.* (1992).
[a] Schwarz *et al.* (1995a).
[b] Nomdedeu *et al.* (1995).

et al., 1995; Schwarz *et al.*, 1995a). After transfection into human fibroblasts, the amino acid exchanges A156V, R249H in RAG-1, and V8I in RAG-2 proved to be functionally competent in the recombination of signal and coding ends with artificial substrates (Schwarz *et al.*, 1995a).

When we compared 14 B^-SCID patients to 16 B^+SCID children, B^+ SCID cases exhibited no RAG mutation, while 6 B^-SCID patients from five independent families had a RAG-1 and/or a RAG-2 mutation (Schwarz *et al.*, 1995b). One patient was characterized by a deletion of RAG-1 and RAG-2 on the paternal allele, and a functionally effective RAG-2 mutation (besides a RAG-1 polymorphism) on the maternal chromosome (Table IV). Missense and nonsense mutations as well as deletions are indicated in Table IV. A mutationional hot-spot was not observed. All mutations were inherited, i.e., the parents were heterozygous for one allele (Table IVA). Neither the parents of the RAG-1/2 deficient children nor heterozygous siblings had any immunological or neurological abnormalities in line with the results obtained in $RAG^{-/-}$ mice. Taking into account that only 80% of all mutations are detectable by this approach, about 50% of B^- SCID patients, i.e. 15% of all SCID cases, may bear RAG mutations. In turn, defects in other genes involved in the differentiation pathway of lymphocytes, such as the *ikaros* gene (Georgopoulos *et al.*, 1994), or in the V(D)J recombination process and its regulation might also cause a B^-/T^-SCID phenotype.

When tested with artificial substrates, the altered RAG-1/2 proteins showed recombination values which were typically two to three orders of magnitude below wild type for coding and signal joint formation (Table IVB). The rare signal coding joints recovered with the RAG-1/2 mutants did not exhibit any obvious difference to wild-type recombination products. The marked reduction in mature B and T cells in the peripheral blood of

TABLE IVA

RAG-1/2 MUTATIONS IN HUMAN SCID PATIENTS: MOLECULAR CHARACTERIZATION OF RAG-1/2 MUTATIONS

Patients	Allele	Nucleotide	AA Change
RAG-1			
C.T.	m	G 2276 to A	E 722 K
	p	G 2432 to T	E 774 stop
K.V.	m	T 2926 to G	Y 938 stop
	p	T 2926 to G	Y 938 stop
L.M.	m	C 579 to T	A 156 V(polym)
	p	del	—
M.T.	m	C2801 to T	R 897 stop
	p	G 1983 to A	R 624 H
RAG-2			
J.Je and J.Jo.	m	G 2634 to A	C 478 Y
	p	G 2634 to A	C 478 Y
L.M.	m	G 1887 to A	R 229 Q
	p	del	—

Note. The maternal (m) or paternal (p) origin of the mutation is indicated. Nucleotides are numbered for RAG-1 according to Schatz *et al.* (1992) and for RAG-2 according to Ichihara *et al.* (1992).

TABLE IVB

FUNCTIONAL CONSEQUENCES OF RAG-1/2 MUTATIONS AS MEASURED WITH ARTIFICIAL V(D)J RECOMBINATION SUBSTRATES FOR SIGNAL (SJ) AND CODING JOINTS (CJ) IN 293-C18 FIBROBLASTS (SCHWARZ *ET AL.*, 1995b)

Expression Vectors	Joints	Reduction (Fold)
wt RAG-2/wt RAG-2	sj	1,0
	cj	1,0
(E7228)Rag-1/wt RAG-2	sj	330
	cj	>200
(Y938Stop)RAG-1/wt RAG-2	sj	160
	cj	>1400
(R624H)Rag-1/wt RAG-2	sj	>140
	cj	580
wt RAG-1/(C478Y) RAG-2	sj	80
	cj	740
wt RAG-1/(R229Q) RAG-2	sj	170
	cj	>510

these patients (Table V) may thus be conclusively explained by the failure of V(D)J recombination reflected by the large reduction for coding and signal joint formation. The common immunological hallmark of all the RAG-defective patients is the absence of B cells. Maternal T cells are often observed in SCID children, as discussed earlier. The absence of T cells is thus not a prerequisite for considering a RAG mutation in SCID patients. One of the patients studied (L. M., Table V) had a low number of T cells. These cells were of patient origin as judged by HLA typing, fingerprinting, and mutation analysis (Schwarz *et al.*, 1995b). Whether the presence of T cells reflects leakiness of the variant RAG allele as in murine *scid* or whether the oligoclonal T cell population was due to reversions of the point mutation in the RAG-2 gene remains to be established at a clonal level.

2. *DNA Repair Defects in SCID Patients*

The presence of DNA repair defects in SCID patients has thus far not been evaluated in detail. SCID patients with a defect in the IL-Rγ_c or in RAG-1/2 exhibit a normal radiosensitivity pattern (Cavazzana-Calvo *et al.*, 1993; Schwarz, unpublished results). Increased radiosensitivity of granulo-

TABLE V
IMMUNOPHENOTYPES OF PATIENTS WITH RAG MUTATIONS AT PRESENTATION.

	J. Jo.	J. Je.	L. M.	K. V.	M. T.	C. T.
Leukocytes (G/liter)	12.4	8.5	19.6	5.0	13.0	3.5
Granulocytes	6.1	2.9	16.9	1.0	8.6	1.6
Monocytes	0.3	2.2	1.0	0.0	0.8	1.2
Lymphocytes	6.0	3.4	1.7	1.5	3.6	0.7
B cells (CD 20) (% of MNC)	1	0	0	0	0	0
T cells (% of MNC)						
CD 2	68	46	83	nd	nd	2
CD 3	70	0	59	15	15	0
TCRα/β	64	1	48	nd	10	2
TCR-γ/δ	nd	0	18	nd	0	nd
CD 4	46	0	45	5	4	nd
CD 8	21	26	8	10	16	nd
CD 16	19	64	4	67	61	4
Maternal T cells	Yes	No	No	Yes	Yes	No
Immunoglobulins (g/liter)					ps	ps
IgG	0.91	6.5	2.1	0.17	8.92	8.13
IgA	0.07	0.1	0.1	0.07	0.25	0.0
IgM	0.25	1.0	0.08	0.09	0.46	0.0

Note. G/liter, Giga per liter (10^9 cells per liter); MNC, mononuclear cells; g/liter, grams per liter; U/liter, units per liter; nd, not determined; ps, postsubstitutionem.

cyte/macrophage colony-forming units (GM-CFU) has been reported in 3 B^-/T^-SCID patients (Cavazzana-Calvo *et al.*, 1993). A fibroblast cell line from one of those patients exhibited a twofold increase in radiosensitivity. A fourth B^-SCID patient with oligoclonal patient-derived T cells and the features of Omenn's syndrome (i.e., SCID with lymphnodes, reticulohistocytic infiltration, skin rash, and hypereosinophilia) likewise revealed an increased radiosensitivity in GM-CFU assays.

In a recent study 5 out of 20 diploid fibroblast cell lines derived from B^-SCID children without RAG-1/2 defects reacted more sensitive than wild-type cells to γ-irradiation in a clonogeneic assay (Schwarz, unpublished results). Immunologically, those patients exhibited a heterogeneous phenotype. Three of them were B^-/T^-SCID patients, a fourth alymphocytotic patient also lacked granuloyctes (reticular dysgenesis), and the fifth case showed reduced, albeit maximally activated, patient- derived T cell numbers

B. Primary Immunodeficiencies Other Than SCID

The World Health Organization (WHO) recognizes 17 specific primary immunodeficiency diseases (Rosen *et al.*, 1992), which can be subdivided into three groups (Table VI). Combined immunodeficiencies have been discussed above. The remaining diseases go along with T and/or B cells. Therefore their recombinase machinery should by definition be intact, although its regulation may be hampered at various levels. Bruton's agammaglobulinemia may represent a case in point (Bruton, 1952) and will be discussed in more detail.

1. Bruton's (X-linked) Agammaglobulinemia

X-linked agammaglobulinemia (XLA) was the first disorder of humoral immunity to be described (Bruton, 1952). Affected boys remain healthy during the first 6 to 9 months of life because of protection by maternally transmitted IgG antibodies. The disorder is usually diagnosed quite early in life as a result of repeatedly acquired infections with high-grade pathogens. The patients show drastically reduced numbers of circulating mature B lymphocytes and very little immunoglobulins of any isotype (Conley, 1985). The bone marrow contains normal or slightly decreased numbers of B cell precursors expressing TdT, membrane CD19, and CD10 (Campana *et al.*, 1990). The number of pre-B lymphocytes identified by cytoplasmic IgH chain Cμ expression is normal or decreased (Campana *et al.*, 1990; Pearl *et al.*, 1978; Landreth *et al.*, 1985). The XLA gene maps to Xq22 (Kwan *et al.*, 1986) and is caused by defects of the *BTK* (Bruton tyrosine kinase) gene (Vetrie *et al.*, 1993; Tsukada *et al.*, 1993). BTK together with ITK and TEC are members of a newly identified family

TABLE VI
PRIMARY IMMUNE DEFICIENCY DISORDERS RECOGNIZED BY THE WORLD HEALTH ORGANIZATION

Predominantly antibody deficiency disorders
X-linked agammaglobulinemia (Bruton)
X-linked hypogammaglobulinemia with growth hormone deficiency
Immunoglobulin deficiency with increased IgM
Immunoglobulin heavy chain deletions
Kappa chain deficiency
Selective deficiency of IgG subclasses
Common variable immunodeficiency
IgA deficiency
Transient hypogammaglobulinemia of infancy
Combined immune deficiency disorders
Severe combined immune deficiency (X-linked and autosomal recessive)
Adenosine deaminase deficiency
Purine nucleoside phosphorylase deficiency
MHC class II deficiency
Reticular dysgenesis
Other well-defined immune deficiency disorders
Wiskott–Aldrich syndrome
Ataxia telangiectasia
DiGeorge anomaly

of SRC-related protein tyrosine kinases, with an N-terminal pleckstrin homology region, a single SH3 and SH2 region, and a putative autophosphorylation site (Desiderio, 1994, and references therein).

BTK is expressed throughout B cell differentiation, from early precursor to mature B cell stages, in myeloid as well as in macrophage cell lines. Plasma cells and T cells do not express BTK (Smith, 1994; De Weers *et al.*, 1993).

Initially, partial or absent V(D)J recombinase activity has been suggested in "minor" or "major" forms of XLA, since partial DJ rearrangements have been observed in cell lines of XLA patients (Schwaber and Chen, 1988). The partial gene rearrangements derived from the XLA patients are now regarded as characteristics of pre-B cells, rather than being pathognomonic for XLA. Support for an intact V(D)J recombinase machinery in XLA is provided by complete IgH and IgL chain rearrangements both in bone marrow and in EBV-transformed B lymphoblastoid cell lines derived from the peripheral blood of patients (Mensink *et al.*, 1986; Timmers *et al.*, 1991, 1993; Anker *et al.*, 1989; Milili *et al.*, 1993), and T cell lines exhibiting normal TCR rearrangements (Campana *et al.*, 1990). Moreover, all mecha-

nisms known to generate Ig diversity seem to operate during the differentiation of XLA B lymphocytes. Unique features of $V_H(D)J_H$ regions in XLA, such as overrepresentation of particular V_H genes and restricted N diversity, both suggesting a fetal-like immunological repertoire, as well as a high percentage of unconventional D-to-D fusions, might argue for a regulatory role of BTK in the Ig gene rearrangement process (Timmers *et al.*, 1991, 1993; Milili *et al.*, 1993).

V(D)J assays with artificial substrates may be useful to address why XLA patients exhibit a restricted N-region repertoire in spite of the expression of TdT. In normal human B cell development IL-7 leads to a down-regulation of RAG-1/2 and TdT. Expression of RAG-1/2 can be rescued by CD19 crosslinking. However, IL-7 fails to induce TdT down-regulation in pro-B cells of XLA patients, while RAG-1/2, although reduced through IL-7, cannot be restored through CD19 signals to normal levels (Cooper, personal communication). These data indicate that regulation of the V(D)J recombinase may be involved in the arrest of B lymphocyte differentiation in XLA.

The idea that the V(D)J recombination process can be regulated in a B-cell specific manner is strongly supported by results derived from E2A and PAX-5 knockout mice (Bain *et al.*, 1994; Zhuang *et al.*, 1994; Urbánek *et al.*, 1994) or Id transgenic mice (Sun, 1994). The E2A gene encodes the basic helix–loop helix (bHLH) transcription factors E12, E47, and ITF-1, while PAX-5 encodes a paired domain-containing transcription factor called B cell lineage-specific activator protein (BASP). Id, a HLH protein, can form heterodimers with bHLH proteins and blocks their binding to DNA (Benezra *et al.*, 1990). Loss of transcription factor activity has the same effect in all these models: the B cell development is arrested at the CD43+ pro-B cell stage, immunoglobulin genes in these precursors do not initiate DJ rearrangement, and RNA expression of RAG -1/2, λ_5, mb1, and CD19 is down-regulated. Possibly, the E2A product, E47, activates recombination by binding to DNA. Alternatively, E47 expression could activate other genes whose products in turn stimulate recombination. E2A proteins differentially regulate the production of specific types of immunoglobulin germline transcripts that may be involved in the initiation of IgH gene recombination.

$E2A^{-/-}$ homozygotes represent only one-tenth of the murine offspring in the first postnatal week, while the loss of mutant homozygotes was not seen at Embryonic Day 18.5 (Zhuang *et al.*, 1994). $E2A^{-/-}$ animals grow more slowly than wild-type or heterozygote littermates. By 2 weeks, the body weight of the surviving mutants is three to five times less than that of their littermates. The ratio of 1-week-old male to female $E2A^{-/-}$ mutants is about 8 : 1, leaving the impression of a sex-linked trait (Zhuang *et al.*,

1994). A detailed analysis of the hematopoiesis of $E2A^{-/-}$ animals reveals that B cells are absent, while other lineages including T cells, granulocytes, macrophages, and erythroid cells are intact. Given the features of $E2A^{-/-}$ mice with agammaglobulinemia, growth retardation, and a seemingly sex-inherited trait, it appears to be worthwhile to investigate E2A, PAX-5, or Id genes in those agammaglobulinemia patients who (1) exhibit growth retardation due to hormone deficiency (Fleisher *et al.*, 1980, Sitz *et al.*, 1990; Conley *et al.*, 1991; Monafo *et al.*, 1991), (2) lack pre-B cells (Landreth *et al.*, 1985), and (3) cannot be characterized by a BTK mutation.

In light of an emerging B-cell-specific V(D)J recombinase regulatory pathway, a T-cell-specific control of the V(D)J recombination machinery can be envisaged. Some of the B^+/T^-SCID patients are obvious candidates to test this hypothesis.

C. DNA Breakage Syndromes

A group of autosomal recessive diseases with hypersensitivity to DNA-damaging agents, resulting in high frequencies of chromosomal breaks and rearrangements together with an increased propensity to the development of leukemias and other malignancies, has been tentatively classified as DNA-repair disorders (Setlow, 1978) and provide a fascinating link to transcription control (Hanawalt, 1994). The inclusion of DNA-repair activities in V(D)J recombination marks those diseases as candidates for genes involved in the recombination pathway or in its regulation.

Ataxia telangiectasia (AT) is characterized by cerebellar ataxia with Purkinje cell degeneration, ocular and cutaneous telangiectasia, sinopulmonary disease, endocrine abnormalities, variable B and T cell deficiency, and high incidence of lymphoid malignancy with chromosomal translocations, often involving antigen receptor loci. AT cells are hypersensitive to the lethal and clastogenic effects of ionizing radiation and radiorestistant to DNA synthesis (McKinnon, 1987; Gatti *et al.*, 1991, and references therein). AT is subdivided in five different complementation groups (Jaspers *et al.*, 1988). The genes have been localized to chromosome 11q (Gatti *et al.*, 1988). V(D)J recombination with extrachromosomal V(D)J substrates appears completely normal in SV-40-transformed fibroblast lines of AT patients (Hsieh *et al.*, 1993) and in AT-like hamster cell mutants (Pergola *et al.*, 1993). Coding joint and signal joint formation as well as the frequencies of deletion and inversion rearrangements are not defective.

Fanconi's anemia (FA) patients may have skeletal abnormalities, such as microcephaly, microphtalmia, and absence of radii and thumbs, in addition to hyperpigmentation of the skin. The bone marrow fails progressively, and the incidence for acute myeloid leukemia is increased (Alter, 1993). Cells from patients with Fanconi's anemia have a high level of spontaneous

chromosomal aberrations compared with cells from unaffected individuals. This phenotype is more pronounced after induction by DNA crosslinking agents, such as mitomycin C, diepoxybutane, or photoactivated psoralens. Four genetic complementation groups (A–D) have been described. The FACC gene has been cloned and mapped to chromosome 9q. Its function, however, is still unknown (Strathdee *et al.*, 1992a,b). The FACA gene localizes on chromosome 20q (Mann *et al.*, 1991). V(D)J recombination analyses employing a deletional substrate in FACD lymphoblasts exhibited junctions consistent with normal V(D)J recombinase activity. Remarkably, when the substrates were transfected in the absence of RAG-1 or -2, no rearrangements were detected in normal cells, whereas in FA cells abnormally large deletions were observed (Papadopoulo *et al.*, 1995).

In Xeroderma pigmentosum (XP), skin changes are first noted during infancy and childhood. The fully expressed condition of XP includes a strong predisposition to sunlight-induced melanomas and basal cell and squamous cell carcinomas of the skin. In addition to nonneoplastic cutaneous and ocular abnormalities, XP patients manifest a variety of neurological defects, including microcephaly, progressive mental retardation, ataxia, abnormal reflexes, and deafness (Cleavers and Kraemer, 1989; Hoeijmarkers and Bootsma, 1990). Cell lines from XP patients are defective in the repair of pyrimidine dimers as well as other DNA lesions. The disease is genetically complex. Eight human [groups A–G plus a variant (V) form] and 11 hamster complementation groups which are defective in nucleotide excision repair have been identified (Tanaka and Wood, 1994). Most of these genes have been cloned (reviewed in Drapkin *et al.*, 1994, and Hoeijmarkers and Bootsma, 1994). When tested with extrachromosomal substrates UV-sensitive cell lines of complementation groups A–G showed a normal frequency and fidelity of V(D)J deletional recombination for signal and coding joints (Taccioli *et al.*, 1993).

Bloom's syndrome (BS) is characterized by erythema and telangiectasia of the face, photosensitivity, and dwarfism of prenatal onset. Features of the BS immunodeficiency include defects in T helper cells, aberrant immunoglobulin isotype serum profiles, reduction in the response of B cells to mitogenic stimuli, and a drastic reduction in the number of IgG-bearing lymphocytes in circulation (German, 1993). BS patients have an increased incidence of malignancy, a striking elevation of sister-chromatide exchanges, and a decrease in DNA ligase activity. Analysis of the DNA ligase I gene in BS revealed no mutation, implicating an eventual cofactor of ligase I as BS defect (Petrini *et al.*, 1991). The BS locus maps to chromosome 15q26.1 (German *et al.*, 1994). In contrast to BS, mutations in both alleles of the DNA ligase I gene leading to its functional inactivation have been described in only one individual (Webster *et al.*, 1992; Barnes

et al., 1992), who showed growth retardation, sun sensitivity, and a relatively late onset of severe immunodeficiency. SV-40-transformed fibroblasts of BS and DNA ligase I deficiency are essentially normal in V(D)J recombination of coding as well as signal joints upon transfection of deletion and inversion substrates (Hsieh *et al.*, 1993; Petrini *et al.*, 1994).

In summary, the clinically defined and partially molecularly characterized DNA breakage syndromes mentioned above exhibit no obvious deficiencies in V(D)J recombination with artificial substrates. The initiation of the V(D)J recombination process seems to be functional in DNA breakage syndrome patients, since B and T cells, albeit sometimes at lower numbers, are present. These analyses most likely exclude RAG-1/2, KU 70, KU 86, and DNA PK_{CS} defects in these disorders.

V. Conclusion

V(D)J recombination of antigen receptor genes constitutes the only site-specific recombination process presently known in mammals. Two lymphocyte-specific proteins, RAG-1 and -2, initiate this recombination process, while enzymes of the general DNA DSB repair pathway(s) are involved in the resolution of DNA ends (Fig.3).

Loss of RAG-1/2 gene function results in a severe combined immunodeficiency phenotype in humans as well as in mice. Patients with RAG mutations exhibit a spectrum of immunophenotypes that are defined by the individual genetic lesion. Murine *scid* represents a distinct V(D)J recombinase-associated defect characterized by a loss of DNA PK_{CS} function. KU 70/86, a heterodimeric protein, which targets DNA PK_{CS} to DNA ends, has also been implicated in V(D)J recombination by analysis of hamster cell lines defective in both V(D)J recombination and DNA DSB

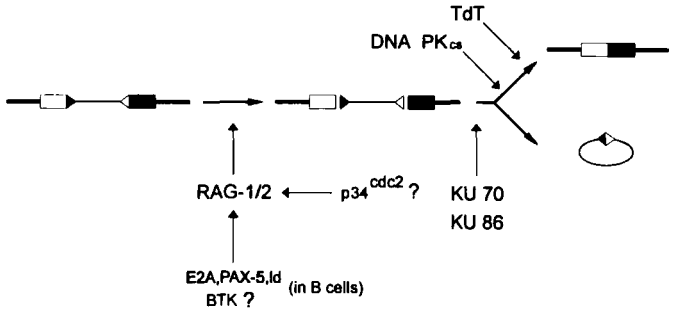


FIG. 3. Presently identified factors/regulators involved in V(D)J recombination.

repair. So far no human equivalents for DNA PK_{CS} or KU 70/86 mutants have been detected, although the existence of γ-irradiation-sensitive human SCID fibroblast lines suggests that some patients with DNA repair deficiency could eventually be subsumed in the genotypically heterogeneous human SCID phenotype.

The majority of primary human immunodeficiencies exhibit a functionally intact V(D)J recombinase machinery, but a hypothetical block in a B-cell-specific regulation pathway of the recombination process might contribute to the XLA phenotype. Molecular defects underlying human DNA breakage syndromes apparently do not influence the frequency and the fidelity of V(D)J recombination.

In the future, even greater scrutiny in the exact evaluation of immunodeficient patients will allow a refinement of additional activities involved in the V(D)J recombination process and its regulation, adding to the experiences gained by animal and cell line models. In turn, this development will lead to improvements in the diagnosis and subclassification of immunodeficient patients and may ultimately pave the way for the introduction of novel treatment strategies, including somatic gene therapy.

Note Added in Proof

Recently it has been shown that the essential cut at the RSS is mediated by RAG-1 and -2 (Van Gent *et al.*, 1995) and might be dependent on the physical interaction of RAG-1 and -2 (Leu and Schak, 1995).

Acknowledgments

The authors thank Professor E. Kleihauer for his continuous support and dedicate this work to him on the occasion of his retirement from the chair of pediatrics at the University of Ulm. We gratefully acknowledge close collaborations and discussions with W. Friedrich, T. E. Hansen-Hagge, J. W. G. Janssen, M. R. Lieber, and A. Schulz. We thank Regina Reitinger for excellent assistance in all phases of manuscript preparation. This work is supported by grants from the Deutsche Forschungsgemeinschaft and Sonderforschunjsbereich 322.

References

Abe, T., Tsuge, J., Kamachi, Y., Torii, S., Utsumi, K., Akohori, Y., Ichihara, Y., Kurosawa, Y., and Matsuoka, H. (1994). *J. Immunol.* **152,** 5504.

Aguilar, L. K., and Bellmont, J. W. (1991). *J. Immunol.* **146,** 1348.

Aguilera, R. J., Akira, S., Okazaki, K., and Sakano, H. (1987). *Cell* **51,** 909.

Alt, F. W., and Baltimore, D. (1982). *Proc. Natl. Acad. Sci. USA* **79,** 4118.

Alt, F. W., Oltz, E. M., Young, F., Gorman, J., Taccioli, G., and Chen, J. (1992a). *Immunol. Today* **13,** 306.

Alt, F. W., Rathbun, G., Oltz, E., Taccioli, G., and Shinkai, Y. (1992b). *Ann. N.Y. Acad. Sci.* **651,** 277.

Alter, B. P. (1993). *Br. J. Haematol.* **85,** 9.

Anderson, C. W. (1993). *TIBS* **18,** 433.

Anker, R., Conley. M. E., and Pollock, B. A. (1989). *J. Exp. Med.* **169,** 2109.

Bain, G., Maandag, E. C. R., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Drop, J., Schlissel, F. S., Feeney, A. J., Van Roon, M., Van der Valk, M., Te Riele, H. P. J., Berns, A., and Murre, C. (1994). *Cell* **79,** 885.

Banga, S. S., Hall, K. T., Sandhu, A. K., Weaver, D. T., and Athwal, R. S. (1994). *Mutat. Res.* **315,** 239.

Barnes, D. E., Tomkinson, A. E., Lehmann, A. R., Webster, A. D. B., and Lindahl, T. (1992). *Cell* **69,** 495.

Benezra, R., Davis, R. L., Lockshou, D., Turner, D. L., and Weintraub, H. (1990). *Cell* **61,** 49.

Bennett, C. B., Lewis, A. L., Baldwin, K. K., and Resnick, M. A. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 5613.

Bernstein, R. M., Schluter, S. F., Lake, D. F., and Marchalonis, I. J. (1994). *Biochem. Biophys. Res. Commun.* **205,** 687.

Biedermann, K. A., Sun, J., Giaccia, A. J., Tosto, L. M., and Brown, J. M. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 1394.

Blackwell, T. K., and Alt, F. W. (1988). In Molecular Immunology (B. D. Hames and D. M. Glover, Eds.), Vol. 1. IRL, Washington, DC.

Blier, P. R., Griffith, A. J., Craft, J., and Hardin, J. A. (1993). *J. Biol. Chem.* **268,** 7594.

Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C. M., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., Jeggo, P. A., and Jackson, S. P. (1995). *Cell* **80,** 813.

Boehm, T., Gonzales-Sarmiento, R., Kennedey, M., and Rabbits, T. H. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 3927.

Bogue, M., Gilfillan, S., Benoist, C., and Mathis, D. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 11011.

Bollum, F. J., and Chang, L. M. S. (1986). *Adv. Cancer Res.* **47,** 37.

Borgulya, P., Kishi, H., Kematsu, Y., and von Boehmer, H. (1992). *Cell* **69,** 529.

Bories, J. C., Cayuela, J. M., Loseau, P., and Sigeaux, F. (1991). *Blood* **78,** 2053.

Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). *Nature* **301,** 527.

Bosma, G. C., Fried, M., Custer, R. P., Caroll, A., Gibson, D. M., and Bosma, T. J. (1988). *J. Exp. Med.* **167,** 1016.

Boubnov, N. V., Hall, K., Wills, Z., Lee, S. E., Ming He, D., Benjamin, D. M., Palaski, C. R., Band, H., Reeves, W., Hendrickson, E. A., and Weaver, D. T. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 890.

Brändle, D., Müller, C., Rülicke, T., Hengartner, H., and Pircher, H. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 9529.

Brändle, D., Müller, S., Müller, C., Hengartner, H., and Pircher, H. (1994). *Eur. J. Immunol.* **24,** 145.

Bruton, O. C. (1952). *Pediatrics* **9,** 722.

Cai, Q.-Q., Plet, A., Imbet, J., Lafage-Pochitaloff, M., Cerdan, C., and Blanchard, J.-M. (1994). *Cytogenet. Cell Genet.* **65,** 221.

Campana, D., Farrant, J., Inandor, N., Webster, A. D. B., and Janossy, G. (1990). *J. Immunol.* **145,** 1675.

Campbell, J. J., and Hashimoto, Y. (1991). *J. Immunol.* **150,** 1307.

Cao, Q. P., Pitt, S., Leszyk, J., and Beril, E. F. (1994). *Biochemistry* **33,** 8548.

Carlson, L. M., Oettinger, M. A., Schatz, D. G., Masteller, E. L., Hurley, E. A., McCormack, W. T., Baltimore, D., and Thompson, C. B. (1991). *Cell* **64,** 201.

Carroll, A. M., Slack, J. K., and Ma, X. (1993). *J. Immunol.* **150,** 2222.

Cavazzana-Calvo M., Le Deist, F., De Saint Basile, G., Papadopoulo, D., Villartay, J. P., and Fischer, A. (1993). *J. Clin. Invest.* **91,** 1214.

Chan, J. Y. C., Lerman, M. I., Prabhaker, B. S., Isozaki, O., Santisteban, P., Kuppers, R. C., Oates, E. L., Notkins, A. L., and Kohn, L. D. (1989). *J. Biol. Chem.* **264,** 3651.
Chen, J., and Alt, F. W. (1993). *Curr. Op. Immunol.* **5,** 194.
Chen, J., Trounstine, M., Alt, F. W., Young, F., Kurahara, C., Loring, J. F., and Huszar, D. (1993a). *Int. Immunol.* **5,** 647.
Chen, J., Trounstine, M., Kurahara, C., Young, F., Kuo, C. C., Xu, Y., Loring, J. F., Alt, F. W., and Huzar, D. (1993b). *EMBO J.* **12,** 821.
Chen, J., Lansford, R., Stewart, V., Young, F., and Alt, F. W. (1993c). *Proc. Natl. Acad. Sci. USA* **90,** 4528.
Chen, J., Shinkai, Y., Young, F., and Alt, F. W. (1994). *Curr. Op. Immunol.* **6,** 313.
Chun, J. J. M., Schatz, D. G., Oettinger, M. A., Jaenisch, R., and Baltimore, D. (1991). *Cell* **64,** 189.
Cleavers, J. E., and Kraemer, K. H. (1989). *In* The Metabolic Basis of Inherited Diseases, 6th ed. (C. A. Scriver, A. L., Beandet, W. S. Sly, and D. Vale, Eds.), p. 2949. McGraw-Hill, New York.
Conley, M. E. (1985). *J. Immunol.* **134,** 3070.
Conley, M. E., Burks, A. W., Herrod, H. G., and Puck, J. M. (1991). *J. Pediatr.* **119,** 392.
Cortes, P., Ye, Z.-S., and Baltimore, D. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 7633.
Csordàs-Toth, E., Marusic, L., Ochem, A., Patthy, A., Pongor, S., Giacca, M., and Falaschi, A. (1993). *Nucleic Acids Res.* **21,** 3257.
Cuomo, C. A., Kirch, S., Gyuris, J., Breut, R., and Oettinger, M. A. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 6156.
Cuomo, C. A., and Oettinger, M. A. (1994). *Nucleic Acids Res.* **22,** 1810.
Davis, M. M. (1988). *In* Molecular Immunology (B. D. Hames and D. M. Glover, Eds.), p. 1. IRL, Washington, DC.
Davis, M. M., and Bjorkman, P. J. (1988). *Nature* **334,** 395.
Desiderio, S. (1994). *Curr. Op. Immunol.* **6,** 248.
Desiderio, S., and Baltimore, D. (1984). *Nature* **308,** 860.
De Weers, M., Verschuren, M. C. M., Kraakman, M. E. M., Mensink, R. G. J., Schuurmann, R. K. B., Van Dongen, J. J. M., and Hendriks, R. W. (1993). *Eur. J. Immunol.* **23,** 3109.
Drapkin, R., Sancar, A., and Reinbeg, D. (1994). *Cell* **77,** 9.
Dvir, A., Peterson, S. R., Knuth, M. W., Lu, H., and Dynan, W. S. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 11910.
Dvir, A., Stein, L. A., Calore, B. L., and Dynan, W. S. (1993). *J. Biol. Chem.* **268,** 10440.
Early, P., Huang, H., Calame, K., and Hood, L. (1980). *Cell* **19,** 981.
Ehlich, A., Schaerle, S., Gu, H., Kitamura, D., Müller, W., and Rajewski, K. (1993). *Cell* **72,** 695.
Falzon, M., and Kuff, E. L. (1992). *Nucleic. Acids Res.* **20,** 20.
Feddersen, R. M., and van Ness, B. G. (1985). *Proc. Natl. Acad. Sci. USA* **82,** 4793.
Feeney, A. J. (1990). *J. Exp. Med.* **172,** 1377
Feeney, A. J. (1991). *J. Exp. Med.* **174,** 115.
Feeney, A. J. (1992). *Int. Rev. Immunol.* **8,** 133.
Feldmann, H., and Winnacker, E. L. (1993). *J. Biol. Chem.* **268,** 12895.
Ferguson, S. E., and Thompson, C. B. (1992). *Curr. Biol.* **3,** 51.
Ferguson, S. E., Accanitti, M. A., Wang, D. D., Chen, C.-L., and Thompson, C. B. (1994). *Mol. Cell. Biol.* **14,** 1994.
Ferrier, P., Krippl, B., Furley, A. J. W., Blackwell, T. K., Suh, H., Mendelsohn, M., Winoto, A., Cook, W. D., Hood, L., Constantini, F., and Alt. F. W. (1984). *Cold Spring Harbor Symp. Quant. Biol.* **54,** 191.

Ferrier, P., Krippl, B., Blackwell., T. K., Furley, A. J. W., Suh, H., Winoto, A., Cook, W. D., Hood, L., Constantini, F., and Alt, F. W. (1990). *EMBO J.* **9,** 117.
Finnie, N. J., Gottlieb, T. M., Blunt, T., Jeggo, P. A., and Jackson, S. P. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 320.
Fischer, A. (1992). *Immunodefic. Rev.* **3,** 83.
Fleisher, T. A., White, R. M., Border, S., Nissley, S. P., Blaese, R. M., Mulvihill, J. J., Olive, G., and Waldmann, T. (1980). *N. Engl. J. Med.* **302,** 1429.
Fry, M. (1983). *In* Enzymes of Nucleic Acid Synthesis and Modification (S. T. Jacob, Ed.), p. 39. CRC Press, Boca Raton, FL.
Fulop, G. M., and Phillips, R. A. (1990). *Nature* **347,** 479.
Fuschiotti, P., Harindranath, N., Mage, R. G., McCormack, W. T., Dhanarajan, P., and Roux, K. H. (1993). *Mol. Immunol.* **30,** 1021.
Gallo, M. L., Pergola, F., Daniels, G. A., and Lieber, M. R. (1994). *J. Biol. Chem.* **269,** 22188.
Gatti, R. A., Beskel, J., Boder, E., Braedt, G., Charmley, P., Concannon, P., Ersoy, F., Foroud, T., Jaspers, N. G. J., Lange, K., Lathrop, G. M., Leppert, M., Nakamura, Y., O'Connell, P., Paterson, M., Salser, W., Sanal, O., Silver, J., Sparkers, R. S., Susi, E., Weeks, D. E., Wie, S., White, R., and Yoder, F. (1988). *Nature* **336,** 577.
Gatti, R. A., Boder, E., Vinters, H. V., Sparkers, R. S., Norman, A., and Lange, K. (1991). *Medicine* **70,** 99.
Gay, D., Sanders, T., Camper, S., and Weigert, M. (1993). *J. Exp. Med.* **177,** 1009.
Gellert, M. (1992a). *Annu. Rev. Genet.* **26,** 425.
Gellert, M. (1992b). *Trends Genet.* **8,** 408.
Georgopoulos, K., Bigby, M., Wang, J. H.,Molnar, A., Wu, P., Winandy, S., and Sharpe, A. (1994). *Cell* **79,** 143.
German, J. (1993). *Medicine* **72,** 393.
German, J., Roe, A. M., Leppert, M. F., and Ellis, N. A. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 6669.
Gerstein, R. M., and Lieber, M. R. (1993). *Nature* **363,** 625.
Getts, R. C., and Stamato, T. O. (1994). *J. Biol. Chem.* **269,** 15981.
Gilfillan, S., Dierich, A., Lemeur, M., Benoist, L., and Mathis, D. (1993). *Science* **261,** 1175.
Godfrey, D. J., and Zlotnik, A. (1993). *Immunol. Today* **14,** 547.
Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994). *Cell* **79,** 767.
Gottlieb, T. M., and Jackson, S. P. (1993). *Cell* **72,** 131.
Greenhalgh, P., Olesen, C. E. M., and Steiner, L. A. (1993). *J. Immunol.* **151,** 3100.
Griffith, A. J., Craft, J., Evans, J., Mimori, T., and Hardin, J. A. (1992). *Mol. Biol. Reprod.* **16,** 91.
Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewski, K. (1994). *Science* **265,** 103.
Guillams, T. G., Teng, M., and Halligan, B. D. (1994). *Biochem. Biophys. Res. Commun.* **202,** 1134.
Guy-Grand, D., Vanden Broecke, C., Briottet, C., Malassis-Seris, M., Selz, F., and Vassalli, P. (1992). *Eur. J. Immunol.* **22,** 105.
Halligan, B. D., and Desiderio, S. V. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 7019.
Hamaguchi, Y., Yamamoto, Y., Iwanari, H., Maruyama, S., Furukawa, T., Matsumani, N., and Honjo, T. (1992). *J. Biochem.* **112,** 314.
Hanawalt, P. C. (1994) *Science* **266,** 1957.
Harrington, J. J., and Lieber, M. R. (1994a). *EMBO J.* **13,** 1235.
Harrington, J. J., and Lieber, M. R. (1994b). *Genes Dev.* **8,** 1344.
Hayakawa, S., Saito, S., Nemoto, N., Chishima, F., Akiyama, K., Skiraishi, H., Hayakawa, J., Karasaki-Suzuki, M., Fujii, F. T., Ichijo, M., Sakurai, J., and Satoh, K. (1994). *J. Immunol.* **153,** 4934.

Hendrickson, E. A., Qin, X.-A., Bump, E. A., Schatz, D. G., Oettinger, M., and Weaver, D. T. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 4061.
Hesse, J. E., Lieber, M. R., Gellert, M., and Mizuuchi, K. (1987). *Cell* **49,** 775.
Hesse, J. E., Lieber, M. R., Mizuuchi, K., and Gellert, M. (1989). *Genes Dev.* **3,** 1053.
Hirayashi, K., Nishikawa, S., Kina, T., Hatanaka, M., Habu, S., Nomura, T., and Katsura, Y. (1987). *Eur. J. Immunol.* **17,** 1051.
Hirschhorn, R. (1993). *Ped. Res.* **33**(Suppl. 1), 35.
Hiyashiura, M. Shimizu, Y., Tanimoto, M., Morita, T., and Yagura, T. (1992). *Exp. Cell. Res.* **201,** 444.
Hoeijmarkers, J. H. J., and Bootsma, D. (1990). *Cancer Cells* **2,** 311.
Hoeijmarkers, J. H. J., and Bootsma, D. (1994). *Nature* **371,** 654.
Hope, T. J., Aguilera, F. J., Minie, M. E., and Sakano, H. (1986). *Science* **231,** 1141.
Hsieh, C.-L., Arlett, C. F., and Lieber, M. R. (1993). *J. Biol. Chem.* **268,** 20105.
Ichihara, Y., Matsuoka, H., Tsuge, J., Okada, J., Torii, S., Yasui, H., and Kurosawa, Y. (1988). *Immunogenetics* **27,** 330.
Ichihara, Y., Hirai, M., and Kurosawa, Y. (1992). *Immunol. Lett.* **33,** 277.
Itahara, S., Mombaerts, P., Lafaille, J., Iacomini, J., Nelson, A., Clarke, A. R., Hooper, M. L., Farr, A., and Tonegawa, S. (1993). *Cell* **72,** 337.
Itoh, M., Hamatami, K., Komatsu, K., Araki, R., Takayama, K., and Abe, M. (1993). *Radiat. Res.* **134,** 364.
Jaspers, N. G., Gatti, R. A., Baan, C., Liussen, P. C. M. L., and Bootsma, D. (1988). *Cytogenet. Cell Genet.* **49,** 259.
Jeggo, P. A., Tesmer, J., and Chen, D. J. (1991). *Mutat. Res. DNA Repair* **254,** 125.
Jessberger, R., and Berg, P. (1991). *Mol. Cell. Biol.* **11,** 445.
Jessberger, R., Podust, V., Hubscher, U., and Berg, P. (1993). *J. Biol. Chem.* **268,** 15070.
Kallenbach, S., and Rougeon, F. (1992). *Res. Immunol.* **143,** 873.
Kallenbach, S., Doyen, N., D'Andon, M. F., and Rougeon, F. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 2799.
Kallenbach, S., Brinkmann, T., and Rougeon, F. (1993). *Int. Immunol.* **5,** 231.
Kamachi, Y., Ichihara, Y., Tsuge, J., Tokuichiro, A., Torii, S., Kurosawa, Y., and Matsuoka, H. (1993). *Eur. J. Immunol.* **23,** 1401.
Kataoka, T., Kondo, S., Nishi, M., Kodaira, M., and Honjo, T. (1984). *Nucleic Acids Res.* **12,** 5995.
Kim, M. G., Schuler,W., Bosma, M. J., and Maren, K. B. (1988). *J. Immunol.* **141,** 1341.
Kirchgessner, C. U., Tosto, L. M., Biedermann, K. A., Koracs, N., Aranjo, D., Stanbridge, E. J., and Brown, J. M. (1993). *Cancer Res.* **53,** 6011.
Kirchgessner, C. U., Patil, C. K., Evans, J. W., Cuomo, C. A., Fried, L. M., Carter, T., Oettinger, M. A., and Brown, J. M. (1995). *Science* **267,** 1178.
Kitamura, D., Roes, J., Kuhn, R., and Rajewski. K., (1991). *Nature* **350,** 423.
Kitamura, D., and Rajewski, K. (1992). *Nature* **356,** 154.
Kmiel, T., Pattengale, P., and Weinberg, K. (1993). *Leukemia* **7,** 435.
Knobloch, C., Goldmann, S. F., and Friedrich, W. (1991). *J. Immunol.* **146,** 4157.
Komatsu, K., Okta, T., Jinno, Y., Nikawa, N., and Okumara, Y. (1993). *Human. Mol. Genet.* **2,** 1031.
Komatsu, K., Kubota, N., Gallo, M., Okumara, Y., and Lieber, M. R. (1995). *Cancer Res.* **55,** 1774.
Komori, T., Okada, A., Stewart, V., and Alt, F. W. (1993). *Science* **261,** 1171.
Kormann, A. J., Maruyama, J., and Raulet, D. H. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 267.
Kottmann, A. H., Brack, C., Eibel, H., and Köhler, G. (1992). *Eur. J. Immunol.* **22,** 2113.

Kurimasa, A., Nagata, Y., Shimizu, M., Eni, M., Nakamura, Y., and Oshimura, M. (1994). *Human. Genet.* **93,** 21.

Kwan, S. P., Kunkel, L., Bruns, G., Wedgwood, R. J., Latt, S., and Rosen, F. S. (1986). *J. Clin. Invest.* **77,** 649.

Lafaille, J. J., De Cloux, A., Bonneville, M., Takagaki, Y., and Tonegawa, S. (1989). *Cell* **59,** 859.

Landau, N. R., Schatz, D. G., Rosa, M., and Baltimore, D. (1987). *Mol. Cell. Biol.* **7,** 3237.

Landreth, K., Engelhard, D., Anasetti, C., Kapoor N., Kincade, P. W., and Good, R. A. (1985). *J. Clin. Immunol.* **5,** 84.

Lee, S., and Hendrickson, E. A. (1995). *J. Cell. Biochem. Suppl.*, 328. [Abstract C5-446]

Lees-Miller, S. P., Chen, Y. R., and Anderson, C. W. (1990). *Mol. Cell. Biol.* **10,** 6472.

Leu, T. M. J., and Schatz, D. G. (1995). *Mol. Cell. Biol.* **15,** 5657.

Lewis, S., Hesse, J. E., Mizuuchi, K., and Gellert, M. (1988). *Cell* **55,** 1099.

Lewis, S., and Gellert, M. (1989). *Cell* **58,** 585.

Lewis, S. M. (1994a). *Adv. Immunol.* **56,** 27.

Lewis, S. M. (1994b). *Proc. Natl. Acad. Sci. USA* **91,** 1332.

Li, L. L., and Yeh, N. H. (1992). *Exp. Cell. Res.* **199,** 262.

Lieber, M. R., Hesse, J., Mizuuchi, K., and Gellert, M. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 8598.

Lieber, M. R. (1991). *FASEB J.* **4,** 2934.

Lieber, M. R. (1992). *Cell* **70,** 873.

Lin, W.-C., and Desiderio, S. (1993). *Science* **260,** 953.

Lin, W.-C., and Desiderio, S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 2733.

Lin, W.-C., and Desiderio, S. (1995). *Immunol. Today* **16,** 279.

Lukacsovich, T., Yang, D., and Waldman, A. S. (1994). *Nucleic Acids Res.* **22,** 5649.

Ma, A., Fisher, P., Dildrop, R., Oltz, E., Rathbun, G., Achacoso, P., Stall, A., and Alt, F. W. (1992). *EMBO J.* **11,** 2727.

Malissen, M., McCoy, C., Blanc, D., Tracy, J., Deveaux, C., Schmitt-Verhulst, A. M., Fitch, F., Hood, L., and Malissen, B. (1986). *Nature* **319,** 28.

Malyun, B. A., Blackwell, T. K., Fulop, G. M., Rathbun, G. A., Furley, A. J. W., Ferrier, P., Heinke, L. B., Phillips, R. A., Yancopoulos, G. D., and Alt, F. W. (1988). *Cell* **54,** 453.

Mann, W. R., Venkatraj, V. S., Allen, R. G., Liu, Q., Olsen, D. A., Adler-Brecher, B., Mao, J.-I., Weffenbach, B., Sherman, S. L., and Auerbach, A. D. (1991). *Genomics* **9,** 329.

Matthews, D. J., Clark, P. A., Herbert, J., Morgan, G., Armitage, R. J., Kinnon, C., Minty, A., Grabstein, K. H., Caput, D., Ferrara, P., and Callard, R. (1995). *Blood* **85,** 38.

Max, E. E., Seidmann, J. G., and Leder, P. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 3450.

May, G., Sutton, C., and Gould, H. (1991). *J. Biol. Chem.* **266,** 3052.

McKinnon, P. J. (1987). *Human Genet.* **75,** 197.

Mensink, E. J. B. M., Schuurmann, R. K. B., Schot, J. D. L., and Alt, F. W. (1986). *Eur. J. Immunol.* **16,** 963.

Milili, M., Le Deist, F., De Saint-Basile, G., Fischer, A., Fougereau, M., and Schift, C. (1993). *J. Clin. Invest.* **91,** 1616.

Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S., and Homma, M. (1981). *J. Clin. Invest.* **268,** 611.

Mimori, T., and Hardin, J. A. (1986). *J. Biol. Chem.* **261,** 10375.

Mimori, T., Okosono, Y., Hama, N., Suwa, A., Akizuki, M., Homma, M., Griffith, A. J., and Hardin, J. A. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 1777.

Miyake, S., Sugiyama, H., Torii, Y., Fukuta, T., and Kishimoto, S. (1990). *J. Immunogenet.* **17,** 67.

Mombaerts, P., Clarke, A. R., Hooper, M. L., and Tonegawa, S. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 3084.

Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. (1992a). *Cell* **68,** 869.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itahora, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L., and Tonegawa, S. (1992b). *Nature* **360,** 225.

Monafo, V., Maghnie, M., Terracciano, L., Valtora, A., Massa, M., and Severi, F. (1991). *Acta Paediatr. Scand.* **80,** 563.

Morozov, V. E., Falzon, M., Anderson, C. W., and Kuff, E. L. (1994). *J. Biol. Chem.* **269,** 16684.

Muegge, K., West, M., and Durum, S. K. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 4151.

Noguchi, M., Huafang, Y., and Rosenblatt, H. M. (1993). *Cell* **73,** 147.

Nomdedéu, J. F., Lasa, A., Seminago, R., Baiget, M., and Soler, J. (1995). *Leukemia* **9,** 229.

Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990). *Science* **248,** 1517.

Oettinger, M. A., Stanger, B., Schatz, D. G., Glaser, T., Call, K., Housman, D., and Baltimore, D. (1992). *Immunogenetics* **35,** 97.

Okazaki, K., Nishikawa, S., and Sakano, H. (1988). *J. Immunol.* **141,** 1348.

Oltz, E. M., Alt, F. W., Lin, W.-C., Chen, J., Taccioli, G., Desiderio, S., and Rathbun, G. (1993). *Mol. Cell. Biol.* **13,** 6223.

Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989). *Genomics* **5,** 874.

Pandey, V. N., Dare, V. P., and Patil, M. S. (1989). *Mol. Biol. Reprod.* **13,** 179.

Papadopoulo, D., Andreau, J.-C., Doyen, N., Kallenbach, S., Laquerbe, A., Rougeon, F., and Moustacchi, E. (1995). *J. Cell. Biochem. Suppl.*, 318. [Abstract C5-407]

Pearl, R. E., Vogler, L. B., Okos, A. J., Crist, W. M., Lawton, A. R., and Cooper M. (1978). *J. Immunol.* **120,** 1169.

Pergola, F., Zdzienicka, M. Z., and Lieber, M. R. (1993). *Mol. Cell. Biol.* **13,** 3464.

Peterson, S. R., Kurimasa, A., Oshimara, M., Dynan, W. S., Bradbury, E. M., and Chen, D. J. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 3171.

Petrini, J. H. H., Huwiler, K., and Weaver, D. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 7615.

Petrini, J. H. J., Donovan, J. W., Dimare, C., and Weaver, D. (1994). *J. Immunol.* **152,** 176.

Pfeffer, K., and Mak, T. W. (1994) *Annu. Rev. Immunol.* **12,** 367.

Pfeifer, M., Berg, S., and Reynolds, A. B. (1994). *Cell* **76,** 789.

Philcott, K. L., Viney, J. L., Kay, G., Rastan, S., Gardiner, E. M., Chae, S., Hayday, A. C., and Owen, M. J. (1992). *Science* **256,** 1448.

Pollack, M. S., Kirkpatrick, D., Kapoor, N., Dupont, B., and O'Reilly, R. J. (1982). *N. Engl. J. Med.* **307,** 662.

Porges, A. J., Ny, T., and Reeves, W. H. (1990). *J. Immunol.* **145,** 4222.

Ramsden, D. H., Baetz, K., and Wu, G. E. (1994). *Nucleic Acids Res.* **22,** 1785.

Rathbun, G., Oltz, E. M., and Alt, F. W. (1993). *Int. Immunol.* **5,** 997.

Rathmell, W. K., and Chu, G. (1994a). *Mol. Cell. Biol.* **14,** 4741.

Rathmell, W. K., and Chu, G. (1994b). *Proc. Natl. Acad. Sci. USA* **91,** 7623.

Raulet, D. H. (1989). *Annu. Rev. Immunol.* **7,** 175.

Reeves, W. H. (1985). *J. Exp. Med.* **161,** 18.

Reeves, W. H. and Sthoeger, Z. M. (1984). *J. Biol. Chem.* **264,** 5047.

Reeves, W. H. (1987). *Rheumatology* **14**(Suppl. 13), 97.

Rosen, F. S., Wedgwood, R. J., Eibl, M. C. Griscelli, C., Seligmann, M., Aiuti, F., Kishimoto, T., Matsaamoto, S., Kakkalin, L. N., Hanson, L. A., Hitzig, W. H., Thompson, R. A., Cooper, M. D., Good, R. A., and Waldmann, T. A. (1992). *Immunodefic. Rev.* **3,** 195.

Roth, D. B., and Wilson, J. H. (1986). *Mol. Cell. Biol.* **6,** 4295.

Roth, D. B., Chang, X. C., and Wilson, J. H. (1989). *Mol. Cell. Biol.* **9,** 3049.

Roth, D. B., Proctor, G. N., Stewart, L. K., and Wilson, J. H. (1991). *Nucleic Acids Res.* **19,** 7201.
Roth, D. B., Menetski, J. P., Nakajima, P., Bosma, M. J., and Gellert, M. (1992a). *Cell* **70,** 983.
Roth, D. B., Nakajima, P., Menetski, J. P., Bosma, M. J., and Gellert, M. (1992b). *Cell* **69,** 41.
Roth, D. B., Zhu, C., and Gellert, M. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 10788.
Roth, D. B., Lindahl, T., and Gellert, M. (1995). *Curr. Biol.* **5,** 496.
Rusconi, S., and Köhler, G. (1985). *Nature* **314,** 350.
Sadofsky, M. J., Hesse, J. E., McBlane, F. J., and Gellert, M. (1993). *Nucleic Acids Res.* **21,** 5644.
Sadofsky, M. J., Hesse, J. E., and Gellert, M. (1994). *Nucleic Acids Res.* **22,** 1805.
Sakano, H., Hüppi, K., Heinrich, G., and Tonegawa, S. (1979). *Nature* **280,** 288.
Sakano, H., Kurosawa, Y., Weigert, M., and Tonegawa, S. (1981). *Nature* **290,** 562.
Sakano, H., Maki, R., Kurosawa, Y., Roeder, W., and Tonegawa, S. (1989). *Nature* **286,** 676.
Schatz, D. G., and Baltimore, D. (1988). *Cell* **53,** 107.
Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989). *Cell* **59,** 1035.
Schatz, D. G., and Chun, J. J. M. (1992). *N. Biol.* **4,** 188.
Schatz, D. G., Oettinger, M. A., and Schlissel, M. S. (1992). *Annu. Rev. Immunol.* **10,** 359.
Schlissel, M. S., Constantinescu, A., Murrow, T., Baxter, M., and Peng, A. (1993). *Genes. Dev.* **7,** 2520.
Schuler, W., Weiler, J. J., Schuler, A., Phillips, R. A., Rosenberg, N., Mak, T. W., Kearney, J. F., Perry, R. P., and Bosma, M. J. (1986). *Cell* **46,** 963.
Schwaber, J., Molgaaard, H., Orkin, S. H., Gould, H. J., and Rosen, F. S. (1983). *Nature* **304,** 355.
Schwaber, J., and Chen, R. H. (1988). *J. Clin. Invest.* **81,** 2004.
Schwarz, K., Hansen-Hagge, T. E., Knobloch, C., Friedrich, W., Kleihauer, E., and Bartram, C. R. (1991). *J. Exp. Med.* **174,** 1039.
Schwarz, K., Hameister; H., Gessler, M., Grzeschik, K.-H., Hansen-Hagge, T. E., and Bartram, C. R. (1994). *Human Genet.* **93,** 215.
Schwarz, D., Gauss, G., Ludwig, L., Lindner, D., Pannicke, U., Hansen-Hagge, T. E., Lieber, M. R., and Bartram, C. R. (1995a), submitted for publication.
Schwarz, D., Gauss, G., Ludwig, L., Lindner, D., Pannicke, U., Friedrich, W., Seger, R. A., Hansen-Hagge, T. E., Kleihauer, E., Lieber, M. R., and Bartram, C. R. (1995b), submitted for publication.
Sell, S. M. (1992). *Comput. Chem.* **12,** 125.
Serwe, M., and Sablitzky, F. (1993). *EMBO J.* **12,** 2321.
Setlow, R. B. (1978). *Nature* **271,** 713.
Sherrington, P. D., Forster, A., Seawright, A., van Heysingen, V., and Rabbitts, T. H. (1992). *Genes Chrom. Cancer* **5,** 404.
Shinkai, Y., Rathbun, G., Lam, K.-P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charon, J., Detta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). *Cell* **68,** 855.
Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K. M., Loh, D. Y., Reinherz, E. L., and Alt, F. W. (1993). *Science* **259,** 822.
Shirakata, M., Hüppi, K., Usuda, S., Okazaki, K., Yoshida, K., and Sakano, H. (1991). *Mol. Cell. Biol.* **11,** 4528.
Silver, D. P., Spanopoulou, E., Mulligan, R. C., and Baltimore, D. (1993). *Proc. Natl. Acad. Sci. USA* **20,** 6100.
Sitz, K. V., Burks, A. W., Williams, L. W., Kemp, S. F., and Steele, R. W. (1990). *J. Pediatr.* **116,** 292.
Smider, V., Rathmell, W. K., Lieber, M. R., and Gilbert, C. (1994). *Science* **266,** 288.

Smith, C. I. E., Baskin, B., Humire-Greift, P., Zhou, J.-N., Olsson, P. G., Mamiar, H. S., Kjellen, P., Lambris, J. D., Christenensson, B., Hammarstom, L., Bentley, D., Vetrie, D., Islam, K. B., Vorechovský, J., and Sideras, P. (1994). *J. Immunol.* **152,** 557.

Strathdee, C. A., Duncan, A. M. V., and Buchwald, M. (1992a). *Nat. Genet.* **1,** 196.

Strathdee, C. A.,. Gavish, H., Shannon, W. R., and Buchwald, M. (1992b). *Nature* **356,** 763.

Sun, X.-H. (1994). *Cell* **79,** 893.

Taccioli, G. E., Rathbun, G., Shinkai, Y., Oltz, E. M., Chen, H.-L., Whitmore, G., Stamato, T., Jeggo, P., and Alt, F. W. (1992). *Curr. Top. Microbiol. Immunol.* **182,** 107.

Taccioli, G. E., Rathbun, G., Oetz, E., Stamato, T., Jeggo, P. A., and Alt, F. A. (1993). *Science* **260,** 207.

Taccioli, G. E., Cheng, H.-L., Varghese, A. J., Whitmore, G., and Alt, F. W. (1994a). *J. Biol. Chem.* **269,** 7439.

Taccioli, G. E., Gottlieb, T. M., Blunt, T., Priestly, A., Demengeot, J., Mizuta, R., Lehmann, A., Alt, F. W., Jackson, S. P., and Jeggo P. A. (1994b). *Science* **265,** 1442.

Takeda, S., Musteller, E. L., Thompson, C. B., and Buerstedde, J.-M. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 4023.

Tanaka, K., and Wood, R. D. (1994). *TIBS* **13,** 83.

Taniguchi, S., Hirabayashi, Y., Inoue, T., Kamisawa, M., Sasaki, H., Komatsu, K., and Mori, K. J. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 4354.

Thacker, J., and Wilkinson, R. E. (1991). *Mutat. Res. DNA Repair* **254,** 135.

Thompson, A., Hendriks, R. W., Kraakmann, M. E. M., Horning, F., Langlois-van den Bergh, R., Vossen, J. M., Weemaes, C. M. R., and Schuurmann, R. K. B. (1990). *Eur. J. Immunol.* **20,** 2051.

Thompson, A., Timmers, E., Kenter, M. J. H., Kraakmann, M. E. M., Hendriks, R. W., and Schuurmann, R. K. B. (1992). *Eur. J. Immunol.* **22,** 3167.

Tiegs, S. L., Russell, D. M., and Nemazee, D. (1993). *J. Exp. Med.* **177,** 1009.

Timmers, E., Kentner, M., Thompson, A., Kraakman, M. E. M., Berman, J. E., Alt, F., and Schuurmann, R. K. B. (1991). *Eur. J. Immunol.* **21,** 2355.

Timmers, E., Hermans, M. M., Kraakmann, M. E. M., Hendriks, R. W., and Schuurmann, R. K. B. (1993). *Eur. J. Immunol.* **23,** 619.

Tsukada, S., Saftran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Kusak, J., Spardes, R. S., Kubagawa, H., Mohandes, T., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E., and Witte, O. N. (1993). *Cell* **72,** 279.

Turka, L. A., Schatz, D. G., Oettinger, M. A., Chun, J. J. M., Gorka, C., Lee, K., McCormack, W. T., and Thompson, C. B. (1991). *Science* **253,** 778.

Tuteja, N., Tuteja, R., Ochem, A., Taneja, P., Huang, N. W., Simoncsits, A., Susic, S., Rahman, K., Marusic, L., Chen, J., Zhang, J., Wang, S., Pongor, S., and Falaschi, A. (1994). *EMBO J.* **13,** 4991.

Urbánek, P., Wang, Z.-Q., Fetka, O., Wagner, E. F., and Busslinger, M. (1994). *Cell* **79,** 901.

Urniel, T., Pattengale, P., and Weinberg, K. (1993). *Leukemia* **7,** 435.

Van Dyk, L., and Meek, K. (1992). *Int. Rev. Immunol.* **8,** 123.

Van Gent, D. C., McBlane, J. F., Ramsden, D. A., Sadofsky, M. J., Hesse, J. E., and Gellert, M. (1995). *Cell* **81,** 925.

Vetrie, D., Vorechovsky, J., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C. I. E., and Bentley, D. R. (1993). *Nature* **361,** 226.

Wahn, V., Yokota, S., Meyer, K. L., Janssen, J. W., Hansen-Hagge, T. E., Knobloch, C., Koletzko, S., Stein, H., Friedrich, W., and Bartram, C. R. (1991). *J. Immunol.* **147,** 2934.

Wang, J. C., Caron, P. R., and Kim, R. A. (1990). *Cell* **62,** 403.

Weaver, D., Constantini, F., Imanishi-Kari, T., and Baltimore, D. (1985). *Cell* **42,** 117.

Webster, A. D. B., Barnes, D., Arlett, C. F., Lehmann, A., and Lindahl, T. (1992). *Lancet* **539,** 1508.
Witte, P. L., Burrows, P. D., Kincadae, P. W., and Cooper, M. D. (1987). *J. Immunol.* **138,** 2698.
Woodgett, J. R. (1993). *Curr. Biol.* **3,** 449.
Wu, L.-C., Mak, C.-H., Dear, N., Boehm, T., Foroni, L., and Rabbitts, T. H. (1993). *Nucleic Acids Res.* **21,** 5067.
Yaneva, M., Ochs, R., McRorie, D. K., Zweig, S., and Busch, H. (1985). *Biochem. Biophys. Acta* **841,** 22.
Yaneva, M., Wen, J., Ayala, A., and Cook, R. (1989). *J. Biol. Chem.* **264,** 13407.
Yaneva, M., and Jhiang, S. (1991). *Biochem. Biophys. Acta* **1090,** 181.
Yoneda, N., Tatsumi, E., Kawano, S., Matsuo, Y., Minowada, J., and Yomaguchi, N. (1993). *Blood* **82,** 207.
Zdzienicka, M. Z., van Wessel, N., and van Der Schans, G. P. (1992). *Mutat. Res.* **131,** 309.
Zhu, C., and Roth, D. B. (1995). *Immunity* **2,** 101.
Zhuang, Y., Soriano, P., and Weintraub, H. (1994). *Cell* **79,** 875.
Zou, Y. R., Takeda, S., and Rajewski, K. (1993). *EMBO J.* **12,** 811.

ADVANCES IN IMMUNOLOGY, VOL. 61

Major Histocompatibility Complex Class II Deficiency: A Disease of Gene Regulation

VIKTOR STEIMLE, WALTER REITH, AND BERNARD MACH

Department of Genetics and Microbiology, University of Geneva Medical School, CH-1211 Geneva, Switzerland

I. Introduction

Major histocompatibility complex (MHC) class II deficiency, often referred to as the bare lymphocyte syndrome (BLS), is a rare primary immunodeficiency that results from a complete absence MHC class II (HLA-DR, -DP, and -DQ) expression at the surface of all the cell types and tissues that should normally express them (*1,2*). MHC class II molecules are heterodimeric (α and β chain) transmembrane glycoproteins playing a pivotal role in the control of the immune response: they present peptide antigens to $CD4^+$ T lymphocytes, thereby leading to T cell activation (*3–5*). Because of this crucial role, the deficiency in MHC class II expression in BLS leads to a severe immunodeficiency, which is caused by the absence of $CD4^+$ T cell-dependent cellular and humoral immune responses (*1,2,6*). The resulting clinical manifestations, immunological features, and outcome have been reviewed elsewhere (*1,2,6*). Briefly, affected patients are prone to severe and recurrent viral, bacterial, fungal, and protozoal infections, mainly of the respiratory system and gastrointestinal tract (*6*). This leads to malabsorption, failure to thrive, and generally to death in early childhood. Currently the only curative treatment consists of bone marrow transplantation (*6,7*).

BLS is a disease of MHC class II gene regulation. This was first suggested by the observation that no expression of HLA class II genes was detected at either the protein or mRNA levels, and that the defect concerned the α and β chain genes of all three HLA class II isotypes, namely HLA-DR, -DQ, and -DP (*8–10*). Family studies demonstrating that the genetic lesions causing BLS do not cosegregate with the MHC allowed us to conclude that mutations affecting trans-acting regulatory factors are responsible for the disease (*9*). Subsequently, cell fusion studies demonstrated that the MHC class II genes of BLS patients are indeed intact and can be expressed into functional proteins (*11–13*). The lack of mRNA expression was shown to be due to a lack of MHC class II gene transcription (*14*). The realization that BLS is a disease of gene regulation raised the hope that elucidation of the genetic basis for BLS would contribute to our comprehension of the mechanisms controlling expression of MHC class II genes. The classifi-

cation of BLS patients into several different complementation groups by means of cell fusion experiments demonstrated that the disease was genetically heterogeneous (*11–13,15*), and indicated that several distinct MHC class II regulatory factors could be identified by means of a systematic molecular analysis of the defects affecting BLS patients in the different complementation groups.

This chapter focuses on the progress that has been made in the elucidation of the molecular defects responsible for the BLS disease. Two essential MHC class II regulatory factors, CIITA and RFX5, have been isolated and shown to be mutated in BLS patients. Analysis of these two regulatory factors has made a major contribution to our understanding of the molecular mechanisms governing transcription of MHC class II genes.

II. Regulation of MHC Class II Gene Expression

The level of MHC class II expression is a key parameter in T cell activation, and MHC class II gene regulation is therefore an essential aspect of the control of the immune response (*4*). Moreover, excessive or ectopic expression of MHC class II molecules has been implicated in pathological situations characterized by aberrant activation of $CD4^+$ T lymphocytes (*16,17*). This has led to an interest in the molecular basis of MHC class II gene regulation in the context of autoimmune diseases. In addition to the biological importance of MHC class II gene regulation for the immune response, two features make the system of more general interest to the field of gene regulation as a whole. First, MHC class II genes exist as a family of related α and β chain genes (IE and IA in mouse, and HLA-DR, -DQ, and -DP in man), and all of these genes are in general regulated in a coordinate fashion. Second, expression of MHC class II genes can be constitutive, induced, or repressed in a cell-type-specific manner. These two characteristics make the regulation of MHC class II gene expression a good model system for the study of regulatory mechanisms that confer a complex pattern of expression to a family of coexpressed genes.

Constitutive expression of MHC class II molecules is largely restricted to epithelial cells in the thymus and to professional antigen presenting cells, such as B lymphocytes and dendritic cells. The majority of other cell types are MHC class II negative. However, in many of these, expression of MHC class II genes can be induced by a variety of stimuli, particularly by interferon gamma (IFN-γ). In addition to the constitutive and inducible modes of expression, MHC class II genes are known to be subjected to an active repression mechanism in plasma cells. The different modes of MHC class II gene expression are controlled primarily at the level of

transcription. In transient transfection experiments, a 150-bp promoter region situated immediately upstream of the transcription initiation site is sufficient for both constitutive and IFN-γ inducible transcription [for reviews and references, see Refs. (*18–20*)]. Systematic and extensive functional dissection of this promoter region has led to the identification of the X, X2, and Y "boxes," highly conserved cis-acting elements present in the promoters of all MHC class II genes (*18–20*). All three sequences are required for optimal constitutive and inducible expression, and they appear to function together as a single unit [reviewed in Refs. (*21,22*)].

A bewildering variety of different protein complexes have been identified in nuclear extracts and/or cloned on the basis of their ability to bind *in vitro* to the X, X2, or Y boxes of MHC class II promoters [reviewed in Refs. (*18,19,22*)]. Cloned proteins include the multimeric Y-box binding factor NF-Y (*23–25*), a family of X box binding proteins referred to as RFX1 to RFX4 (*26,27*) and several different X2 box binding proteins (*28–32*). In addition, several nuclear complexes distinct from these cloned proteins have been detected (*14,22,33*). Among the latter, the X box binding complex RFX (*14*) and the X2 box binding complex X2bp (*33*) are of particular interest in the context of MHC class II gene regulation (Fig. 1), and will be discussed below. The identification and analysis of the various MHC class II promoter binding proteins has provided relatively little information on the precise molecular mechanisms responsible for either the constitutive or the inducible mode of MHC class II gene expression. Fortunately, the analysis and elucidation of the molecular defects affecting BLS patients and *in vitro*-generated MHC class II regulatory mutants has been more informative. This genetic approach has led to the isolation of two crucial regulatory factors, and has thus made an important contribution to our current understanding of the mechanisms controlling expression of MHC class II genes.

III. Biochemical and Genetic Heterogeneity in BLS

Three independent approaches (*in vitro* binding studies, mapping of DNase I hypersensitive sites, and *in vivo* footprint experiments) have revealed the existence of two distinct biochemical phenotypes in BLS patients. The first approach consisted of a comparison of the MHC class II promoter binding complexes detected in nuclear extracts from MHC class II positive B cell lines and B cell lines derived from BLS patients. A multimeric X box binding complex called RFX was found to be specifically deficient in the majority of BLS cell lines (*14,34–37*). In these patients, binding activity of RFX is undetectable while other MHC class II promoter binding proteins, such as the X2 box binding complex X2bp or the Y box

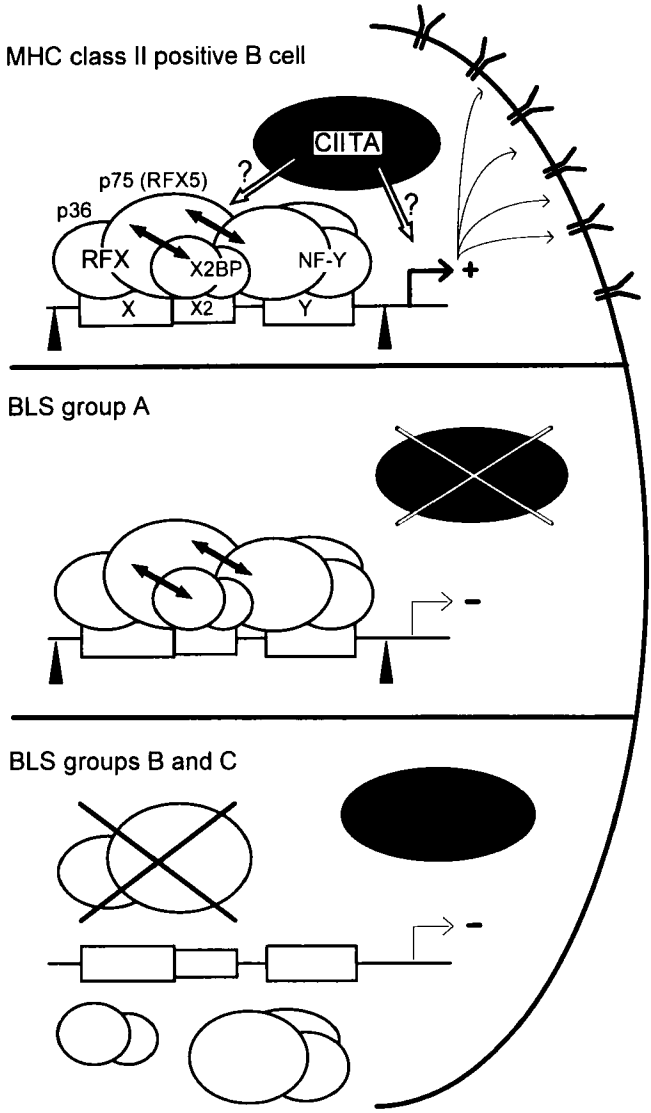


FIG. 1. Biochemical and molecular defects in MHC class II deficiency. The top of the figure represents MHC class II promoter occupancy and transcription status in normal MHC class II positive B cells. The open boxes represent the X, X2, and Y boxes of the proximal MHC class II promoter. MHC class II promoter binding complexes RFX with its subunits p36 and p75 (RFX5), X2bp, and NF-Y are shown. Solid double-headed arrows illustrate cooperative binding between RFX and X2bp, and RFX and NF-Y, respectively. CIITA is shown above the DNA binding complexes. Activation of MHC class II transcription through CIITA is indicated by the open arrows. Solid arrowheads indicate the presence of

binding protein NF-Y, bind normally *in vitro* (*14,34,36,37*). In the remaining patients, on the other hand, binding activity of RFX is indistinguishable from that detected in normal MHC class II positive B cell lines (*34,36,37*). The existence of two biochemical phenotypes was subsequently confirmed by the two other approaches. First, two DNase I hypersensitive sites that normally flank the promoter in MHC class II positive cells are missing in RFX-deficient BLS cells (*38*), indicating that the chromatin structure at the promoter is altered in such patients. Second, *in vivo* footprint experiments demonstrated that MHC class II promoters are unoccupied in RFX-deficient BLS cells (*39,40*). In contrast, both the presence of the DNase I hypersensitive sites (unpublished data) and promoter occupation (*39,40*) are unaffected in BLS patients that do not exhibit the RFX binding defect (Fig. 1).

The existence of the two biochemical phenotypes implied that the BLS disease has a genetically heterogeneous origin. This was formally demonstrated by means of somatic cell fusion experiments which classified BLS patients into at least three different genetic complementation groups (A, B, and C) (*11–13*). In these experiments three *in vitro*-generated MHC class II negative mutants, RJ2.2.5 (*41*) and RM2 and RM3 (*42*), were also analyzed and assigned to BLS complementation group A (*11–13*). There is a tight correlation between the two biochemically defined phenotypes and the heterogeneity in the genetic origin of BLS (*21,22,37*). Patients in complementation groups B and C all exhibit the characteristic deficiency in RFX binding activity, the absence of the DNase I hypersensitive sites, and a bare promoter. On the other hand, patients in complementation group A are indistinguishable from wild-type cells with respect to binding of RFX, the presence of the DNase I hypersensitive sites, and promoter occupation (Fig. 1).

IV. CIITA, the Gene Mutated in Complementation Group A

A genetic complementation approach was developed to isolate the regulatory genes that are defective in BLS patients and *in vitro*-generated B

---

two DNase I hypersensitive sites flanking the MHC class II promoters found in normal B cells. The middle section of the figure represents BLS complementation group A, in which CIITA is mutated. The defect in CIITA does not influence RFX binding *in vitro*, promoter occupancy *in vivo*, or the presence of the DNase I hypersensitive sites. The bottom of the figure represents BLS complementation groups B and C, which are biochemically indistinguishable. They are characterized by a defect in RFX-binding, which leads to a bare promoter and the absence of DNase I hypersensitve sites. In BLS complementation group C the defect is located in the RFX5 (p75) subunit of RFX.

cell lines. A series of mammalian expression vectors was constructed. These vectors contain the EBV origin of replication (Ori P) to permit maintenance as episomes in mammalian cells, a highly efficient cDNA cloning cassette, and features allowing several alternative selection strategies (*43*). Size-selected B cell cDNA expression libraries were prepared in these vectors, and transfected into the MHC class II negative regulatory mutants. Transfectants reexpressing MHC class II genes were selected by sorting with anti-MHC class II antibodies and magnetic beads. The plasmids responsible for restoring the MHC class II positive phenotype were rescued from these cells, amplified in bacteria, and retransfected into the mutant cells for analysis.

This genetic complementation strategy was particularly well suited for elucidating the molecular defect affecting cells in BLS complementation group A, in which no biochemical differences with wild-type cells are apparent. Complementation of an *in vitro*-generated MHC class II negative cell line (RJ2.2.5) from BLS complementation group A led to the isolation of a cDNA encoding CIITA (MHC *C*lass *II T*rans-*A*ctivator). CIITA is capable of restoring expression of all the endogenous MHC class II genes to wild-type levels in all cell lines in complementation group A (*43*) (Fig. 1). Mutations of the CIITA gene have been identified in the BLS cell lines BLS-2 and BCH, as well as in the *in vitro*-generated cell line RJ2.2.5 (*43*; S. Bontron *et al.*, manuscript in preparation). These mutations include exon skipping due to splice site mutations (BLS-2 and BCH), a point mutation (BCH), and genomic deletions (RJ2.2.5).

CIITA is a novel 1130-amino-acid protein exhibiting no significant homology to any other known proteins. It contains no obvious structural motifs other than a consensus sequence for an ATP/GTP binding cassette. In particular, CIITA contains no regions showing homology to known DNA binding motifs, and there is to date no evidence suggesting that it is a DNA binding protein recognizing the MHC class II promoter (*43*). This is consistent with the observation that *in vitro* and *in vivo* binding studies have failed to demonstrate a deficiency in an MHC class II promoter binding protein in cell lines from complementation group A. Yet CIITA does transactivate the minimal MHC class II promoter (44). Moreover, its N-terminal moiety contains features that are characteristic of transcription activation domains, namely regions rich in acidic amino acids or in Pro/Ser/Thr (*43*). In fact, the acidic region of CIITA has been shown to be able to function as a transcription activation domain in both yeast and mammalian cells (*45,46*). Taken together, these findings suggest that CIITA may be a non-DNA binding transcription factor or coactivator that is recruited to the MHC class II promoter by protein–protein interactions with DNA binding proteins (Fig. 1).

V. RFX5, the Gene Mutated in Complementation Group C

The same complementation strategy that led to the isolation of CIITA was subsequently applied to cell lines exhibiting the characteristic defect in RFX binding activity. Complementation of a BLS cell line (SJO) in group C led to the isolation of RFX5, a cDNA clone that is capable of restoring expression of all MHC class II genes to wild-type levels in all three cell lines (SJO, Ro, and TF) from BLS complementation group C (*47*) (Fig. 1). Mutations in the RFX5 gene have been identified in all three of these BLS patients, demonstrating that the genetic defects responsible for complementation group C indeed reside in the RFX5 gene (*47;* W. R. *et al.*, manuscript in preparation). These mutations include a point mutation generating a premature stop codon (Ro), and splice site mutations that lead to the use of cryptic splice sites and result in deletions within RFX5 mRNA (SJO and TF).

RFX5 derives its name from the fact that it represents the fifth member of a previously identified family of X box binding proteins called RFX1 to RFX4 (*26,27*). RFX5 contains a DNA binding motif that exhibits strong homology to the DNA binding domains identified in the other RFX family members. Outside of this DNA binding domain, RFX5 shows no homology to the other RFX proteins and it thus appears to be a distant member of the RFX family. The finding that RFX5 contains the DNA binding domain typical of other known X box binding proteins suggested that it was likely to be a subunit of RFX, the multimeric X box binding complex known to be deficient in BLS patients in groups B and C (*14,34,36*). This indeed turned out to be the case; electrophoretic mobility "supershift" assays with specific antibodies and peptide sequences derived from affinity-purified RFX have shown that RFX5 is in fact the 75-kDa subunit of the RFX complex (Fig. 1). The demonstration that the molecular defects underlying BLS complementation group C are mutations of the 75-kDa RFX5 subunit of the RFX complex has validated our previous interpretation that a deficiency in binding of RFX is responsible for the lack of MHC class II gene transcription in these patients (*14,34,37,47*).

VI. Function of CIITA

Correction of the MHC class II negative phenotype of BLS B cell lines by CIITA demonstrated that CIITA is essential for constitutive expression of MHC class II genes in B cells. Further studies also demonstrated that CIITA is the major regulator of MHC class II gene expression in other cell types. Analysis of the expression profile of CIITA revealed a complete correlation of CIITA and MHC class II expression, both qualitatively and

quantitatively (*43,44;* L. A. Otten *et al.*, manuscript in preparation). In particular, the CIITA gene is silent in MHC class II negative cell lines such as fibroblasts, but is switched on when these cells are induced to become class II positive by stimulation with IFN-γ (*44*). This IFN-γ-induced expression of CIITA precedes the activation of MHC class II gene transcription. Two key experiments revealed that CIITA is in fact an obligatory intermediate in the induction of MHC class II genes by IFN-γ. First, MHC class II gene expression cannot be induced by IFN-γ in fibroblasts derived from a CIITA-deficient patient, demonstrating that CIITA is strictly required for this induction and cannot be bypassed (*44*). Second, transfection of the CIITA cDNA under the control of a constitutive promoter into a variety of MHC class II negative cell lines is sufficient to render the transfected cells constitutively MHC class II positive in the absence of IFN-γ (*44*). On the basis of these results we proposed that activation of CIITA expression in response to intracellular signal(s) elicited by IFN-γ is an essential step in the pathway leading to induction of MHC class II gene expression (*44*). This model is consistent with two subsequent studies addressing the role of CIITA in IFN-γ-induced MHC class II expression (*48,49*).

As well as being essential for constitutive and IFN-γ-induced expression of MHC class II genes, CIITA also plays a key role in the extinction of these genes in plasma cells (*50*). Differentiation of B cells into plasma cells is accompanied by the extinction of MHC class II gene expression, a process believed to be due to a dominant repression mechanism (*51,52*). In MHC class II negative plasmacytoma cells, the CIITA gene is silent and MHC class II promoters are inactive and unoccupied *in vivo* (*50*). Transfection of CIITA into these cells is sufficient to induce promoter occupation and restore transcription of MHC class II genes (*50*). It is thus likely that silencing of MHC class II gene expression in plasmacytoma cells is mediated via repression of the CIITA gene.

In addition to the genes encoding the α and β chains of the HLA-DR, -DQ, and -DP molecules, CIITA also controls expression of the invariant (Ii) chain and DM genes involved in antigen presentation (*53,54*). For the moment, no additional genes are known to be under the control of CIITA. The promoters of the Ii and DM genes contain sequences homologous to the X, X2, and Y motifs found in the classical MHC class II genes. CIITA therefore appears to function exclusively at promoters containing these cis-acting elements.

VII. Function of RFX

RFX is essential for transcription of MHC class II genes. This is evident both from the MHC class II negative phenotype of RFX-deficient BLS

patients and from *in vitro* transcription experiments (*14,37,47*). These experiments demonstrated that the MHC class II promoter is transcriptionally silent in nuclear extracts from RFX-deficient BLS cells, but that wild-type activity can be restored by supplementing these extracts with affinity-purified RFX (*37*). This indicated that RFX is a true transcription factor. However, RFX does not function simply as a classical transcription activator *in vivo*. *In vivo* footprint experiments have shown that the entire MHC class II promoter, including the X2 and Y boxes as well as the X box, is unoccupied in RFX-deficient BLS cells. RFX thus appears to function as an "accessibility factor" required to allow X2 and Y box binding proteins to gain access to their cognate target sites in the MHC class II promoter. An indication of how RFX achieves this has been provided by studies examining protein–protein interactions between RFX and other MHC class II promoter binding proteins (*55,56*). These studies have shown that RFX binds cooperatively with the X2 box binding factor X2bp and the Y box binding factor NF-Y to form stable higher-order protein–DNA complexes consisting of RFX+X2bp, RFX+NF-Y, and RFX+NF-Y+X2bp (*55,56*) (Fig. 1). The interactions of RFX, X2bp, and NF-Y with their respective target sites are strongly stabilized in these higher-order protein–DNA complexes (*55,56*). Promoter mutations that destabilize the RFX+X2bp and RFX+NF-Y complexes have a strong negative effect on promoter activity (*55,56*). Taken together, these results indicate that cooperative binding interactions between RFX, X2bp, and NF-Y are required for stable occupation of the entire MHC class II promoter and hence its transcriptional activity. This now explains why the specific lack of RFX binding activity in BLS cells leads to a bare promoter phenotype. Cooperative binding between RFX and NF-Y probably also explains the observations that destruction of the Y box leads to reduced occupation of the X box (*57*).

The complete lack of MHC class II expression in RFX-deficient BLS patients tells us that RFX, like CIITA, is essential for the coordinate transcription of the α and β chain genes of all three MHC class II isotypes (HLA-DR, -DP, and -DQ). This appears to be in contradiction with the previous finding that RFX does not bind equally well to the X boxes of all MHC class II promoters. On its own, RFX binds efficiently to the X boxes of certain MHC class II promoters (e.g., DRA and DPA) but only very poorly or not at all to the X boxes of others (e.g., DQA, DRB1, and DRB3) (*56,58,59*). It is now clear, however, that the higher-order RFX+X2bp and RFX+NF-Y complexes can form efficiently on the promoters of the DQA, DRB1, and DRB3 genes (*56,59*, unpublished data). Due to the cooperative binding interactions with X2bp and NF-Y, RFX can thus be recruited as part of a multiprotein complex even to promoters containing low-affinity X box sites.

The dependence of promoter occupation on protein–protein interactions between RFX, X2BP, and NF-Y implies that the MHC class II promoter should be considered as a composite element consisting of several different interdependent binding sites. It is therefore not surprising that the MHC class II promoter behaves as a single functional unit in which all the different cis-acting elements not only are essential but also must be positioned correctly with respect to each other (*55,60,61*).

VIII. Conclusions, Unresolved Questions, and Perspectives

Elucidation of the molecular defects responsible for the lack of MHC class II expression in two types of BLS patients (complementation groups A and C) has led to the identification of CIITA and RFX5, two trans-acting factors controlling transcription of the MHC class II, Ii, and DM genes (*43,47,54*). The two factors fulfill two absolutely essential but very different roles. CIITA is a non-DNA binding trans-activator that behaves as a molecular switch controlling constitutive, inducible, and repressed MHC class II expression (*43,44,50*). RFX5 on the other hand is a subunit of a ubiquitously expressed DNA binding protein, RFX, which is a key participant in cooperative protein–protein interactions required for the formation of a stable multiprotein complex at the MHC class II promoter (*14,37,47,55,56*).

The majority of BLS patients belong to complementation group B (*15*), in which the affected gene has not been isolated. In addition, there exist a number of other MHC class II regulatory mutants. These include the *in vitro* generated mutant 6.1.6 (*62*), mutant cells refractory to induction by IFN-γ, new patients who may define new BLS complementation groups, and two recently described twins exhibiting a novel BLS phenotype in which the defect does not appear to affect all cell types and MHC class II genes to the same extent (*15,63–67*). Elucidation of the molecular defects responsible for these regulatory mutants remains an important challenge because it will lead to the identification of additional trans-acting factors and thus will further our comprehension of the molecular mechanisms governing transcription of MHC class II genes. Interestingly, many of the unresolved regulatory mutants, i.e., all of the BLS patients in group B (*14,22,34,37*), the 6.1.6 cell line (*35*), and one of IFN-γ induction mutants (*48*), exhibit the same biochemical defect as BLS patients in group C, namely a lack in RFX binding activity. Given the fact that mutations of the RFX5 subunit (p75) account for the lack of RFX binding activity in complementation group C, it seems likely that at least some of these RFX-deficient mutants could be due to mutations in the gene encoding a second subunit (p36) of the RFX complex (*37*). Alternatively, mutations

in a gene encoding a cofactor or modifying activity required for binding of RFX may be implicated.

In addition to the MHC class II negative phenotype, several BLS patients have been reported to display a reduced density of cell surface MHC class I expression (*6*). This is surprising, because the expression of MHC class I and class II genes is believed to be under the control of different regulatory mechanisms and trans-acting factors (*68*). Nevertheless, we have investigated a possible effect of CIITA and RFX5 on the level of MHC class I expression. Complementation of BLS cells in groups A and C with CIITA and RFX5, respectively, does not modify MHC class I expression (*47*, unpublished results). Moreover, transfection of CIITA into various other cell lines also has no effect on MHC class I expression (*44*). It thus seems unlikely that the reduction in MHC class I described in certain BLS patients is a direct consequence of mutations in the CIITA and RFX5 genes.

A very surprising observation is the relatively mild reduction of circulating $CD4^+$ T cell numbers found in BLS patients (*6*). According to current models for positive selection and maturation of T lymphocytes, this relatively normal population of $CD4^+$ T lymphocytes in the periphery should not be present in the absence of MHC class II expression in the thymus. An animal model for MHC class II deficiency has been obtained through the "knockout" of the MHC class II structural genes in mice (*69,70*). The most striking anomaly in these mice is the almost complete lack of $CD4^+$ T cells in the periphery (*69,70*). A possible explanation for this apparent discrepancy is that a low-level residual MHC class II expression in the thymus of BLS patients is sufficient to drive $CD4^+$ T cell development (*6,71*). Otherwise, restriction elements other than MHC class II, possibly MHC class I, would have to be postulated to explain the $CD4^+$ T cell population of BLS patients. RFX5- and CIITA-deficient mice will provide valuable model systems to investigate this question.

Three unusual features of RFX5 and CIITA are implicit in the nature of the BLS disease. First, the lack of MHC class II expression is essentially complete, indicating that RFX5 and CIITA are absolutely essential and that no bypass or alternative pathways can compensate for their absence. Second, all of the immunological anomalies and clinical consequences characteristic of BLS are attributable to the lack of MHC class II expression, indicating that the action of RFX5 and CIITA is highly specific for MHC class II genes. Third, despite the genetic heterogeneity in the cause of BLS, the disease is remarkably homogeneous in the sense that no phenotype distinctive of one complementation group has been described (*6*), suggesting that RFX5 and CIITA do not function independently of each other in other regulatory systems. Taken together, these three features suggest that experimental modulation of the synthesis or activity of CIITA

and RFX5 should have a highly specific and efficient effect on the level of MHC class II expression. The search for agents capable of affecting activation of MHC class II genes by CIITA and RFX5 might consequently lead to the development of novel immunomodulatory strategies.

ACKNOWLEDGMENT

We thank our colleagues in the laboratory who have contributed to the work described here. This work was supported by the Louis Jeantet Foundation and the Swiss National Science Foundation.

REFERENCES

1. Griscelli, C., Lisowska-Grospierre, B., and Mach, B. (1993). *In* "Immunodeficiencies" (F. S. Rosen and M. Seligman, Eds.), p. 141. Harwood Academic Publishers, Chur, Switzerland.
2. Griscelli, C., Lisowska-Grospierre, B., and Mach, B. (1989). *Immunodefic. Rev.* **1,** 135.
3. Benacerraf, B. (1981). *Science* **212,** 1229.
4. Janeway, C. A., Bottomly, K., Conrad, P., Conzen, S., Jones, B., Kaye, J., Katz, M., McVay, L., Murphy, D. B., and Tite, J. (1984). *Immunol. Today* **5,** 55.
5. Cresswell, P. (1994). *Annu. Rev. Immunol.* **12,** 259.
6. Klein, C., Lisowska Grospierre, B., LeDeist, F., Fischer, A., and Griscelli, C. (1993). *J. Pediatr.* **123,** 921.
7. Klein, C., Cavazzana-Calvo, M., Le Deist, F., Jabado, N., Benkerrou, M., Blanche, S., Lisowska-Grospierre, B., and Griscelli, C. (1995). *Blood* **85,** 580.
8. Lisowska-Grospierre, B., Charron, D. J., de Preval, C., Durandy, A., Griscelli, C., and Mach, B. (1985). *J. Clin. Invest.* **76,** 381.
9. de Preval, C., Lisowska-Grospierre, B., Loche, M., Griscelli, C., and Mach, B. (1985). *Nature* **318,** 291.
10. de Preval, C., Hadam, M. R., and Mach, B. (1988). *N. Engl. J Med.* **318,** 1295.
11. Hume, C. R., and Lee, J. S. (1989). *Hum. Immunol.* **26,** 288.
12. Benichou, B., and Strominger, J. L. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 4285.
13. Seidl, C., Saraiya, C., Osterweil, Z., Fu, Y. P., and Lee, J. S. (1992). *J. Immunol.* **148,** 1576.
14. Reith, W., Satola, S., Herrero Sanchez, C., Amaldi, I., Lisowska-Grospierre, B., Griscelli, C., Hadam, M. R., and Mach, B. (1988). *Cell* **53,** 897.
15. Lisowska-Grospierre, B., Fondaneche, M. C., Rols, M. P., Griscelli, C., and Fischer, A. (1994). *Hum. Mol. Genet.* **3,** 953.
16. Bottazzo, G. F., Todd, I., Mirakian, R., *et al.* (1986). *Immunol. Rev.* **94,** 137.
17. Sospedra, M., Obiols, G., Santamaria Babi, L. F., Tolosa, E., Vargas, F., Roura-Mir, C., Lucas-Martin, A., Ercilla, G., and Pujol-Borell, R. (1995). *J. Immunol.* **154,** 4213.
18. Benoist, C., and Mathis, D. (1990). *Annu. Rev. Immunol.* **8,** 681.
19. Glimcher, L. H., and Kara, C. J. (1992). *Annu. Rev. Immunol.* **10,** 13.
20. Mach, B., Steimle, V., and Reith, W. (1994). *Immunol. Rev.* **138,** 207.
21. Reith, W., Steimle, V., and Mach, B. (1995). *Immunol. Today* **16,** 539.
22. Mach, B., Steimle, V., Martinez-Soria, E., and Reith, W. (1996). *Annu. Rev. Immunol.,* in press.
23. Hooft Van Huijsduijnen, R., Li, X. Y., Black, D., Matthas, H., Benoist, C., and Mathis, D. (1990). *EMBO J.* **9,** 3119.
24. Mantovani, R., Pessara, U., Tronche, F., Li, X. Y., Knapp, A. M., Pasquali, J. L., Benoist, C., and Mathis, D. (1992). *EMBO J.* **11,** 3315.

25. Sinha, S., Maity, S. N., Lu, J., and De Crombrugghe, B. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 1624.
26. Reith, W., Herrero Sanchez, C., Kobr, M., Silacci, P., Berte, C., Barras, E., Fey, S., and Mach, B. (1990). *Genes Dev.* **4,** 1528.
27. Reith, W., Ucla, C., Barras, E., Gaud, A., Durand, B., Herrero Sanchez, C., Kobr, M., and Mach, B. (1994). *Mol. Cell. Biol.* **14,** 1230.
28. Liou, H. C., Boothby, M. R., and Glimcher, L. H. (1988). *Science* **242,** 69.
29. Liou, H. C., Boothby, M. R., Finn, P. W., Davidon, R., Nabavi, N., Zeleznik Le, N. J., Ting, J. P., and Glimcher, L. H. (1990). *Science* **247,** 1581.
30. Andersson, G., and Peterlin, B. M. (1990). *J. Immunol.* **145,** 3456.
31. Ono, S. J., Liou, H. C., Davidon, R., Strominger, J. L., and Glimcher, L. H. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 4309.
32. Ono, S. J., Bazil, V., Levi, B. Z., Ozato, K., and Strominger, J. L. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 4304.
33. Hasegawa, S. L., and Boss, J. M. (1991). *Nucleic Acids Res.* **19,** 6269.
34. Herrero Sanchez, C., Reith, W., Silacci, P., and Mach, B. (1992). *Mol. Cell. Biol.* **12,** 4076.
35. Stimac, E., Urieli-Shoval, S., Kempin, S., and Pious, D. (1991). *J. Immunol.* **146,** 4398.
36. Hasegawa, S. L., Riley, J. L., Sloan III, J. H., and Boss, J. M. (1993). *J. Immunol.* **150,** 1781.
37. Durand, B., Kobr, M., Reith, W., and Mach, B. (1994). *Mol. Cell. Biol.* **14,** 6839.
38. Gönczy, P., Reith, W., Barras, E., Lisowska-Grospierre, B., Griscelli, C., Hadam, M. R., and Mach, B. (1989). *Mol. Cell. Biol.* **9,** 296.
39. Kara, C. J., and Glimcher, L. H. (1991). *Science* **252,** 709.
40. Kara, C. J., and Glimcher, L. H. (1993). *Immunogenetics* **37,** 227.
41. Accolla, R. S. (1983). *J. Exp. Med.* **157,** 1053.
42. Calman, A. F., and Peterlin, B. M. (1987). *J. Immunol.* **139,** 2489.
43. Steimle, V., Otten, L. A., Zufferey, M., and Mach, B. (1993). *Cell* **75,** 135.
44. Steimle, V., Siegrist, C., Mottet, A., Lisowska-Grospierre, B., and Mach, B. (1994). *Science* **265,** 106.
45. Riley, J. L., Westerheide, S. D., Price, J. A., Brown, J. A., and Boss, J. M. (1995). *Immunity* **2,** 533.
46. Zhou, H. and Glimcher, L. H. (1995). *Immunity* **2,** 545.
47. Steimle, V., Durand, B., Barras, E., Zufferey, M., Hadam, M. R., Mach, B., and Reith, W. (1995). *Genes Dev.* **9,** 1021.
48. Chin, K., Mao, C., Skinner, C., Riley, J. L., Wright, K. L., Moreno, C. S., Stark, G. R., Boss, J. M., and Ting, J. P. (1994). *Immunity* **1,** 687.
49. Chang, C. H., Fontes, J. D., Peterlin, M., and Flavell, R. A. (1994). *J. Exp. Med.* **180,** 1367.
50. Silacci, P., Mottet, A., Steimle, V., Reith, W., and Mach, B. (1994). *J Exp. Med.* **180,** 1329.
51. Latron, F., Jotterand Bellomo, M., Maffei, A., Scarpellino, L., Bernard, M., Strominger, J. L., and Accolla, R. S. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 2229.
52. Dellabona, P., Latron, F., Maffei, A., Scarpellino, L., and Accolla, R. S. (1989). *J. Immunol.* **142,** 2902.
53. Chang, C. H., and Flavell, R. A. (1995). *J. Exp. Med.* **181,** 765.
54. Kern, I., Steimle, V., Siegrist, C.-A., and Mach, B. (1995). *Int. Immunol.* **7,** 1295.
55. Reith, W., Siegrist, C. A., Durand, B., Barras, E., and Mach, B. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 554.
56. Reith, W., Kobr, M., Emery, P., Durand, B., Siegrist, C. A., and Mach, B. (1994). *J. Biol. Chem.* **269,** 20020.

57. Wright, K. L., Vilen, B. J., Itoh Lindstrom, Y., Moore, T. L., Li, G., Criscitiello, M., Cogswell, P., Clarke, J. B., and Ting, J. P. (1994). *EMBO J.* **13,** 4042.
58. Kobr, M., Reith, W., Herrero Sanchez, C., and Mach, B. (1990). *Mol. Cell. Biol.* **10,** 965.
59. Emery, P., Mach, B., and Reith, W. (1993). *Hum. Immunol.* **38,** 137.
60. Vilen, B. J., Cogswell, J. P., and Ting, J. P. (1991). *Mol. Cell. Biol.* **11,** 2406.
61. Vilen, B. J., Penta, J. F., and Ting, J. P. (1992). *J. Biol. Chem.* **267,** 23728.
62. Gladstone, P., and Pious, D. (1978). *Nature* **271,** 459.
63. Loh, J. E., Chang, C., Fodor, W. L., and Flavell, R. A. (1992). *EMBO J.* **11,** 1351.
64. Mao, C., Davies, D., Kerr, I. M., and Stark, G. R. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 2880.
65. Peijnenburg, A., Godthelp, B., van Boxel-Dezaire, A., and van den Elsen, P. J. (1995). *Immunogenetics* **41,** 287.
66. Hauber, I., Gulle, H., Wolf, H. M., Maris, M., Eggenbauer, H., and Eibl, M. M. (1995). *J. Exp. Med.* **181,** 1411.
67. Wolf, H. M., Hauber, I., Gulle, H., Thon, V., Eggenbauer, H., Fischer, M. B., Fiala, S., and Eibl, M. M. (1995). *N. Engl. J. Med.* **332,** 86.
68. Ting, J. P., and Baldwin, A. S. (1993). *Curr. Op. Immunol.* **5,** 8.
69. Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. (1991). *Cell* **66,** 1051.
70. Grusby, M. J., Johnson, R. S., Papaioannou, V. E., and Glimcher, L. H. (1991). *Science* **253,** 1417.
71. Schuurman, H. J., van de Wijngaert, F. P., Huber, J., Schuurman, R. K., Zegers, B. J., Roord, J. J., and Kater, L. (1985). *Hum. Immunol.* **13,** 69.

ADVANCES IN IMMUNOLOGY, VOL. 61

TH1–TH2 Cells in Allergic Responses: At the Limits of a Concept

IWAN AEBISCHER AND BEDA M. STADLER

Institute of Immunology and Allergology, University of Bern, Inselspital, CH-3010 Bern, Switzerland

I. Introduction

Our knowledge on the complexity of effector functions performed by the immune system continues to increase as it has become obvious that a variety of different cell types, further divided into cell subpopulations, play characteristic parts in the orchestration of an immune response to a given antigen. We know that lymphocytes cooperate with each other as well as with other cells of hematopoietic and nonhematopoietic origin. We start to better understand the immunological language used as an increasing number of molecules are identified on lymphocyte surfaces and inside the cells, and secreted as mediator molecules. Some of them control lymphocyte development and lymphocyte effector functions, thereby regulating the ontogenetic development, from pluripotent to lymphoid effector cells, that is characterized by migration and subsequent interaction with a competent environment *in vivo*, as well as by the differentiation of uncommitted lymphocytes into differentiated mature and highly specific lymphocytes. Specificity of the immune response is functionally maintained by regulatory mechanisms designed to allow the clonal expansion and maturation of antigen-specific cells recognizing immunogenic nonself compounds.

The adaptive immune system, developed to recognize, memorize, and discriminate antigens as it operates, can also be dangerous, like all defense systems. Such unwanted pathological responses are the well known allergic reactions, e.g., to food or to ubiquitous and apparently inoffensive agents such as pollens. Like any defense system the immune system is an integral part of a highly organized organism that consists of operationally defined systems. In vertebrates, immunologists defined the immune system, neurologists the nervous system, and endocrinologists the endocrine system. *In vivo* all these systems cooperate and mutually influence each other.

In this chapter we summarize and challenge the current TH1–TH2 concept and present an integrative approach to better understand the observed heterogeneity of an individual response to allergens. The chapter also examines the rapidly expanding literature on neuroendocrine mechanisms controlling the onset, the extent, and the termination of an immune response. We document that the phenotypically unstable TH1–TH2 expression is especially prone to be influenced by other regulatory circuits.

II. The Regulation of Human IgE Synthesis

The physiological role of the immunoglobulin E (IgE) molecule is still not completely understood, even though a tight association can be observed between certain parasitic infestations and serum IgE levels in humans (Johansson, 1967; Stevens *et al.*, 1983; Fraser *et al.*, 1993; Kennedy *et al.*, 1990; Pinon *et al.*, 1990). New insights concerning the role of IgE were obtained from IgE knockout mice (Oettgen *et al.*, 1994). The absence of IgE did not influence the normal humoral immune response and apparently had no negative effect on the health of normal and even parasite-infested mice. Remarkably, in those knockout mice, even an anaphylactic shock induced by ovalbumin was observed.

A. The IgE Molecule: A Marker for All Heterogeneous Reactions in Allergy?

Since the discovery of human IgE in 1966 (Ishizaka *et al.*, 1966) its regulation remained a major topic in basic allergy research. IgE mediates the early events associated with IgE-dependent hypersensitivity disorders and IgE produced by allergic as well as normal individuals seems to recognize a wide spectrum of allergens. IgE antibodies can strongly bind via their Fc portion (Ogawa *et al.*, 1971) to receptors in the membrane of tissue mast cells, basophils, and Langerhans cells (Helm *et al.*, 1988; Bieber, 1994; Kinet, 1990). Crosslink of this antibody–receptor complex by means of allergens (Liu *et al.*, 1986; Lowman *et al.*, 1988a; Haydik and Ma, 1988) leads to degranulation and to the release of potent mediators such as histamine (Sheard *et al.*, 1967). Some clinical symptoms like asthma and rhinitis (Goetzl, 1980) are directly due to interactions of these mediators with target cells. But even though a large body of literature exists on the role of the IgE during the effector phase of allergic diseases there are still many open questions concerning the natural role of IgE antibodies on the one hand and the different regulatory mechanisms governing the etiology of allergic diseases in an individual on the other.

The immune response to environmental allergens is believed to depend on multiple factors including the genetic background of an individual and other environmental factors (Marsh *et al.*, 1982; Frick *et al.*, 1979; Ishizaki *et al.*, 1987; Busse, 1989; Molfino *et al.*, 1991; Lehrer *et al.*, 1984; Suoniemi *et al.*, 1987). Even among humans that are exposed to a similar environment only some individuals develop an IgE-mediated immune reaction to normally inoffensive antigens and develop various allergic manifestations.

The amount of specific IgE as well as the total IgE level in a patient's serum is often believed to reflect the extent of an allergic disease. However, serum IgE concentrations are highly fluctuating; seasonal and individual

variations are extreme (Johansson, 1983). Genetic, environmental, and eventually other factors may play an important role in the development of clinical symptoms. Generally, 60–70% of allergic patients have a serum IgE concentration higher than the mean value of the corresponding healthy population. Concentrations of IgE in serum of atopic dermatitis patients are usually strongly increased whereas patients with urticaria or other dermatoses have normal IgE levels. Chronic idiopathic urticaria was suggested to be an IgE-independent phenomenon due to auto-antibodies recognizing the FcεRI (Hide *et al.*, 1993).

The best example for the clinical role of IgE may be the amount of specific IgE in pollen-allergic patients that show extreme seasonal variations (Möller and Elsayed, 1990). High amounts of IgE were also found in a distinct clinical situation, termed hyper-IgE syndrome (Ring and Landthaler, 1989). In African children high IgE levels correlated with ascaris infestation (Johansson *et al.*, 1968). Other infestations with helminths were also reported to increase IgE serum concentrations (Hagel *et al.*, 1993; Abdel-Azim *et al.*, 1989). At present it is not clear whether this effect on increased IgE synthesis is due to a general adjuvant or an immunologically specific effect. Notably these parasite-infested patients do not develop allergic symptoms in connection with the parasite. However, the normal beneficial role of IgE *in vivo* is still unclear and is complicated by the observations that IgE level in sera of atopics and nonatopics is not a definitive predictive parameter for an allergic state.

Possibly one of the most decisive approaches to clarify the biological role of IgE is currently underway. Injected anti-IgE antibodies that abrogated IgE production in animal experiments are now in clinical evaluation. If these treatments provide a broad remedy for all allergic (IgE-dependent) diseases, then the role of IgE is confirmed beyond any doubt, or else parts of this chapter and most textbooks would have to be revised (Presta *et al.*, 1993; Vogel *et al.*, 1994; Stadler *et al.*, 1993; Shakib and Smith, 1994; Stämpfli *et al.*, 1994; Miescher *et al.*, 1994; Saban *et al.*, 1994).

B. IgE Synthesis and Constituents of the Cellular Microenvironment

Animal models *in vivo* and *in vitro* provide many interesting perspectives on IgE regulation. Most of the data on human IgE regulation were preceded by analogous data in murine systems. It became obvious that the regulation of IgE synthesis on a cellular level is a complex system with the participation of many different cell types including B cells, T cells, monocytes, and eventually basophils, mast cells, and eosinophils (Ishizaka, 1976; Romagnani, 1990; DeKruyff *et al.*, 1989; Vercelli *et al.*, 1989; Gauchat *et al.*, 1993; Brunner *et al.*, 1993; Wu *et al.*, 1991). Many studies demon-

strated that interactions with membrane-associated molecules on T cells as well as soluble factors play a central role in the regulation of human IgE synthesis (Yang *et al.*, 1988; Vercelli *et al.*, 1989; Armitage *et al.*, 1993a). Briefly, a direct contact between B cells and T cells or B cells and necessary cells is believed to be mandatory for the induction of IgE synthesis. The likely relevant molecules for IgE regulation are the CD40 ligand on activated T cells, basophils, eosinophils, or monocytes and the CD40 antigen (reviewed in this issue by J. Banchereau) on B cells (Spriggs *et al.*, 1992; Banchereau *et al.*, 1994; Horner *et al.*, 1995; Minty *et al.*, 1993; Punnonen *et al.*, 1992; Gauchat *et al.*, 1995).

The synthesis of IgE is directly dependent not only on the presence of surface molecules on cells interacting with B cells but also on a cocktail of different soluble cytokines or mediators. Many studies have demonstrated that T-cell-derived as well as monocyte-derived cytokines play a central role in the regulation of human IgE synthesis. The key cytokine, produced mainly by activated T cells, that seems to be mandatory for the isotype switch to IgE is interleukin-4 (IL-4) (Kopf *et al.*, 1993; Finkelman *et al.*, 1990; Vercelli *et al.*, 1990; Dugas *et al.*, 1993; Knoller *et al.*, 1989; Kimata *et al.*, 1992b; Punnonen *et al.*, 1993b; Pene *et al.*, 1988; Gauchat *et al.*, 1992b; Qiu *et al.*, 1990; Claassen *et al.*, 1990a,b). Recently IL-13 was described to have a similar effect as it induces *in vitro* IgE synthesis independent of IL-4 (McKenzie *et al.*, 1993; Punnonen and de Vries, 1994; de Vries *et al.*, 1993; Aversa *et al.*, 1993). However, *in vivo*, IL-13 is not able to bypass IL-4 in IL-4-deficient mice (Kopf *et al.*, 1993). IL-3, IL-5, IL-6, IL-9, and tumor necrosis factor-α (TNF-α) all enhance human IgE synthesis *in vitro* (Maggi *et al.*, 1989; Herrod, 1989; Rousset *et al.*, 1991a; Delespesse *et al.*, 1989), whereas IL-2, IL-8, IL-10, IFN-γ, and TGF-β are inhibitors (Knoller *et al.*, 1989; King and Nutman, 1993; Punnonen *et al.*, 1993a; Coffman *et al.*, 1993; Gauchat *et al.*, 1992b; Machold *et al.*, 1993). Thus, IgE synthesis is regulated by a very complex system (Fig. 1) involving not only many different cell types but also a whole cocktail of different cytokines as well as (most importantly) glucocorticoids, produced *in vivo* by adrenal gland cells (Stanton and Levine, 1988; Sarfati *et al.*, 1989; Wu *et al.*, 1991; Jabara *et al.*, 1991, 1993; Nüsslein *et al.*, 1992, 1993, 1994; Kimata *et al.*, 1995).

III. The Regulatory Potential of T Cells

Originally T cells were functionally distinguished from B cells by their capacity to provide help to antibody-producing B cells (Claman *et al.*, 1966; Mitchell and Miller, 1968). *In vivo*, most of the circulating T cells are resting T cells. Cytokine analysis by *in situ* hybridization revealed that

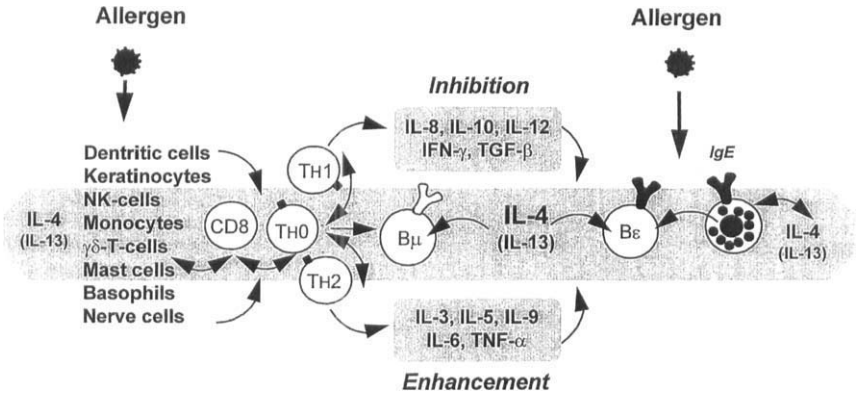


FIG. 1. The TH1–TH2 concept in a broader context. One of the most prominent features of the TH1–TH2 reciprocal regulation is the synthesis of IgE. TH2 cells produce mainly cytokines that induce and enhance while TH1 cells produce a series of cytokine that inhibit IgE synthesis (small shaded boxes). The central TH2 cytokine is IL-4 (with shaded area in the middle), which can also be produced by other cell types, such as basophils and mast cells. IL-4 production is under the stringent control of other cells of allergic inflammation, and it must be assumed that APCs and inflammatory cells play the decisive role for the polarization of a TH response. It can also be speculated that for the development of a TH2 microenvironment non-T cells may be responsible. It should also be noted that IL-4 can be replaced by IL-13, a cytokine that is produced by most TH cells. TH polarization must be regarded as a regulatory circuit that is tightly controlled by other systems, especially by the neuroendocrine system.

in normal and atopic individuals the level of IL-4 and IFN-γ production is nearly undetectable in human peripheral blood mononuclear cells (PBMNC) (Qiu *et al.*, 1988). Significant cytokine mRNA expression was detected only if cells were polyclonally stimulated with mitogens such as PHA, PWM, and anti-CD3 antibody (Gauchat *et al.*, 1991, 1990). Antigens such as tetanus toxin (TT) or allergens such as Der p I or pollen extracts can influence the proliferative response of PBMNC and also the expression of IL-4 and IFN-γ. However, the levels of induced IL-4 and IFN-γ mRNA did not correlate with the allergic state of the donors even if monoallergic donors were used, and the levels were minimal compared to the amount of cytokines produced by specific T cell clones (Wierenga *et al.*, 1990; Gauchat *et al.*, 1989, 1988; Brantschen *et al.*, 1989). In stimulated PBMNC cultures IL-4 and IFN-γ were coexpressed, and TT did not preferentially induce IFN-γ.

From such data it became obvious that the heterogeneity of the cellular microenvironment, as observed in PBMNC cultures, will always hamper the analysis of individual T cell responses. On the other hand it will remain

questionable whether data derived from experiments using single T cell clones will ever shed light on the complex events within a culture of different cell types.

A. The TH1–TH2 Concept

Nevertheless, the use of cloned T cell populations facilitated the understanding of the functional characteristics and the activation requirements of distinct T cell populations. $CD4^+$ T cells appeared to act on B cells mostly via the production of secreted cytokines (Glasebrook and Fitch, 1980; Prystowsky *et al.*, 1982), supporting the observation that in the course of an immune response the heterogeneity of the $CD4^+$ T cells seemed to determine whether a humoral or cell-mediated response will be induced (Parish, 1972). This concept was strengthened by the observation that previously established murine long-term T cell clones could be categorized into at least two distinct subsets, designated TH1 and TH2 (Mosmann *et al.*, 1986). TH1 clones mainly produced IL-2, IFN-γ, and lymphotoxin, factors characterizing a typical cell-mediated immune response (Mosmann *et al.*, 1986; Stevens *et al.*, 1988).

TH2 clones mainly produced IL-4, IL-5, and IL-10 to direct humoral immune responses on the one hand, and to promote allergic type responses in the mouse by the induction of IgE synthesis on the other (Coffman and Carty, 1986; Finkelman *et al.*, 1988; Mosmann and Coffman, 1989). Also, many mouse clones fit the TH1–TH2 patterns, but most clones derived from nonimmunized mice did not (Street *et al.*, 1990; Gajewsky and Fitch, 1988; Firestein *et al.*, 1989). Thus it became obvious that in the mouse an immune response to a particular antigen could induce a polarized TH (TH1 or TH2) response as assessed *in vitro* by generating long-term T cell clones that produce these distinct cytokine patterns.

B. Polarized TH1–TH2 Responses in Humans

The difficulty in reproducing the TH1–TH2 concept for the human system as it was established in cell cultures from immunized mice seems now resolved. The discrepancy is in part attributable to the very different model systems. In the mouse, $CD4^+$ T cell clones were often derived from secondary lymphoid organs of hyperimmunized mice (Stevens *et al.*, 1988; Sher and Coffman, 1992) and the stimulated with protein or peptide antigens. In contrast, in the human system the $CD4^+$ T cells that did not fit in the TH1–TH2 concept were usually obtained from the peripheral blood of nonimmunized normal individuals and were stimulated by mitogen (Sher and Coffman, 1992; de Vries *et al.*, 1991; Andersson *et al.*, 1990). Subsequently examples of polarized human T cell responses were indeed found in T cell clones derived from peripheral blood and lymph nodes of

individuals with chronic diseases (Romagnani, 1991; Wierenga *et al.*, 1990; Maggi *et al.*, 1991; Parronchi *et al.*, 1991; Haanen *et al.*, 1991; Yssel *et al.*, 1992a) and allergic reactions (Maggi *et al.*, 1991; Robinson *et al.*, 1992; Yssel *et al.*, 1992b, 1994; Spiegelberg *et al.*, 1994; Nanda *et al.*, 1994; Carballido *et al.*, 1992).

C. Cytokine Production by Human CD4⁺ T Cells

The characteristics of specific immune responses are influenced and to a large extent determined by cytokines secreted by various T cell clones. $CD4^+$ TH1 clones, specific for *Mycobacterium tuberculosis* or *Bordetella pertussis* antigens, produced predominantly IFN-γ, IL-2, and TNF-β, meaning those cytokines that are involved in a humoral immune response dominated by IgG2a synthesis or in a cell-mediated immune response (delayed type hypersensitivity (DTH), antibody-dependent cytotoxicity, and activation of macrophages) (Tsicopoulos *et al.*, 1992; Stout and Bottomly, 1989; Brewer *et al.*, 1994). $CD4^+$ TH2 clones, specific for allergens or *Toxocaria canis* antigens, synthesized IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, namely, cytokines efficiently stimulating B-cell-mediated humoral immune responses (isotype switching to IgG_4 and IgE) as well as growth and differentiation of eosinophils and mast cells (Wierenga *et al.*, 1993; Sperr *et al.*, 1994). In the mouse system the immune response to *T. canis* seems to be independent of IgE, as no Fcε receptors have been found on eosinophils (Jones *et al.*, 1994). However, in the absence of "polarizing" signals, TH0 clones were established with overlapping cytokine patterns (Parronchi *et al.*, 1991; Maggi *et al.*, 1991; Paliard *et al.*, 1988), indicating that the general T cell response is more complex than the TH1–TH2 paradigm suggested.

Whether these different subsets of human T cell clones originate from a common precursor T cell, as suggested in the mouse systems (Rocken *et al.*, 1992; Evavold *et al.*, 1992; Seder *et al.*, 1992b; Hsieh *et al.*, 1992; Swain *et al.*, 1988; Swain and Weinberg, 1990; Weinberg *et al.*, 1990), or whether these phenotypes are preferentially selected or even interchangeable, cannot be addressed in the human system as the study of T cell clones is restricted to the secondary response, at least in the case of antigens such as allergens.

D. Cytokines and the Polarization of TH Responses

Different pathogens or protein antigens administered by variable doses, routes, and stimulation protocols appear to favor the induction of different TH subsets (Stevens *et al.*, 1988; Carballido *et al.*, 1992; Pfeiffer *et al.*, 1991; Bretscher *et al.*, 1992). In murine leishmaniasis the same antigen (*Leishmania major*) has the potential to direct in a dose-dependent way

the immune response in opposite directions (Reiner and Locksley, 1992). Furthermore, it was clearly shown that the genetic background of the inbred mouse strain used determined the fate of the host. BALB/c mice mount preferentially an insufficient TH2 type response to *L. major* and cannot eradicate the invading pathogen (Scott, 1989a; Heinzel *et al.*, 1989) whereas other mouse strains eliminate the invader via a cell-mediated immune response of the TH1 type.

A similar polarized response was found in human leishmaniasis (Carvalho *et al.*, 1985; Karp *et al.*, 1993; Sacks *et al.*, 1987) and in the two extreme forms of leprosy. Tuberculoid leprosy, characterized by a typical TH1 response, eliminates the bacterium via cell-mediated DTH reactions in contrast to the lepramatous form of leprosy, characterized by a TH2 response (Yamamura *et al.*, 1991; Salgame *et al.*, 1991). Furthermore there exists a correlation between disease susceptibility and the predominance of a particular TH response in listeriosis (Bancroft *et al.*, 1989; Magee *et al.*, 1988; Tripp *et al.*, 1993), HIV infection (Sher and Coffman, 1992; Maggi *et al.*, 1994; Sher *et al.*, 1992; Clerici and Shearer, 1993, 1994; Clerici *et al.*, 1993), helminthic infections (Sher and Coffman, 1992; Urban *et al.*, 1992; Finkelman *et al.*, 1991; Scott *et al.*, 1989), and also in allergic diseases (Wierenga *et al.*, 1990; Maggi *et al.*, 1991; Rousset *et al.*, 1991b; Romagnani, 1990; Yssel *et al.*, 1992b; Ebner *et al.*, 1993; Baum *et al.*, 1990; Kapsenberg *et al.*, 1992; Kay, 1991; Parronchi *et al.*, 1992; Hamid *et al.*, 1991). Many open questions concerning the nature of these signals inducing the polarized response remain unanswered. The biochemical characteristics, the dose and the administration route of the antigen, the activation and differentiation state of the antigen-presenting cell (APC), and the hormonal state of the responding organisms may be considered as factors determining the individual reaction pattern to a distinct antigen. In a reductionistic approach it is often assumed that all these different conditions can be assessed by measuring the corresponding cytokines produced by APC, B cells, or T cells in the presence or absence of antigen.

1. Interleukin-4

The most convincing data on the role of IL-4 were derived from a physiological antigen-specific system using $\alpha\beta$ TCR transgenic mice (Seder *et al.*, 1992b; Hsieh *et al.*, 1992; Swain *et al.*, 1991; Hsieh *et al.*, 1992; Macatonia *et al.*, 1993; Seder *et al.*, 1994; Tanaka *et al.*, 1993). The administration of IL-4, the key cytokine to induce IgE synthesis, to cultured naive $CD4^+$ T cells from these mice in the presence of various APC induced an *in vitro* TH2 response as assessed by the induction of IL-4 and by the inhibition of IL-2 and IFN-γ synthesis by $CD4^+$ cell clones. The effect of IL-4 in this system seems to be independent of the APC and could not

be replaced by other cytokines. IL-4 requirement for a TH2 "priming" was confirmed in other systems using polyclonal T cell stimuli, such as Con A and anti-CD3 antibody as well as antigens (Swain and Weinberg, 1990; Betz and Fox, 1991; Le Gros *et al.*, 1990). Briefly, IL-4-producing T cells emerged only in the presence of exogeneously added IL-4 and IFN-γ synthesis was inhibited independent of APC. This requirement of IL-4 for an *in vivo* TH2 priming was also demonstrated in the case of *L. major* and *Candida albicans* infections (Sadick *et al.*, 1990; Chatelain *et al.*, 1992; Romani *et al.*, 1992).

Interestingly, IL-4 knockout mice were described to have an impaired TH2 type immune response as assessed by infection with *Nippostrongulus brasiliensis* Kopf *et al.*, 1993). The levels of the TH2 type cytokines IL-5, IL-9, and IL-10, produced by anti-CD3-stimulated $CD4^+$ T cells from *N. brasiliensis* immunized mice, were diminished *in vitro*. IgE synthesis could not be detected and IgG_1 serum titers were minimal. The reduction of the induced IL-5 secretion correlated with the observed decrease in eosinophilia, suggesting that IL-5, usually correlating with eosinophilia (Coffman *et al.*, 1989), can also be produced by cells other than $CD4^+$ T cells. In addition, levels of the TH1 cytokine IFN-γ were not altered in IL-4-deficient mice (in contrast to IL-3-deficient mice), and DTH reactions to infectious choriomeningitis virus appeared normal.

These results provided the first evidence that the TH2 response *in vivo* is critically dependent on the availability of IL-4. Interestingly, other reports showed that IL-4-deficient mice efficiently eliminated plasmodium chabaudi parasites, suggesting that IL-4 per se is not required for parasite elimination (Von der Weid *et al.*, 1994). In contrast to other reports, these IL-4-deficient mice produced IgE. During the late phase of infection mice produced five times less IgE than normal litter mates. However, in another IL-4-deficient mouse strain allergen-induced airway inflammation and bronchial hyperresponsiveness were reduced (Brusselle *et al.*, 1995).

For an allergic response it should be noted that the primary source of IL-4 needed to mount an appropriate allergen-specific TH2 response has not yet been identified. Activated T cells and cells from the mast cell and basophil lineage also depend on prior exposition to IL-4, or previously induced IgE synthesis by B cells, to synthesize IL-4 (Ben-Sasson *et al.*, 1990; Piccinni *et al.*, 1991; Brunner *et al.*, 1993). Alternatively there may exist another set of IL-4-producing T cells that comprise this primary source of IL-4 at the onset of an immune response (Bendelac *et al.*, 1992; Arase *et al.*, 1993).

Additional candidates for providing IL-4 (or yet unidentified signals) after an antigen challenge are Langerhans cells (LC), dentritic cells in the follicles, keratinocytes, or $\gamma\delta$ T cells in the skin (Goodman *et al.*, 1994;

Wang *et al.*, 1994; Ferrick *et al.*, 1995; Chomarat *et al.*, 1994; Ronchese *et al.*, 1994). Most interestingly in the mouse $CD4^+$ IL-4 producing T cells were generated in the absence of IL-4 provided by non-T cells (Schmitz *et al.*, 1994), suggesting that at least some TH cells can express IL-4 in response to an as yet unidentified signal. However, in mice in which only $CD4^-$ cells express IL-4, IgE synthesis was not detected, indicating that for IgE synthesis the $CD4^+$ T cells are the primary if not the only source of signals for initiating an IgE response.

2. *Interferon-γ*

The debate on the role of IFN-γ as a TH polarizing agent is highly controversial (Seder *et al.*, 1992b; Seder and Paul, 1994). Using indirect evidence, e.g., by using neutralizing anti-IFN-γ antibody, it has been suggested that IFN-γ may induce a TH1 phenotype development (Parronchi *et al.*, 1992; Swain *et al.*, 1991; Hsieh *et al.*, 1992; Macatonia *et al.*, 1993; Maggi *et al.*, 1992; Belosevic *et al.*, 1992; Scott, 1991, 1989; Gollob *et al.*, 1993), but direct addition of IFN-γ had no effect. Other reports supported this notion because IFN-γ activated only a small subset of $CD4^+$ T cells (O'Garra and Murphy, 1994). IFN-γ has a variety of paracrine effects including antiviral, antiprotozoal (Paliard *et al.*, 1988; Christmas, 1992; Billiau and Dijkmans, 1990; Bruserud *et al.*, 1993), and immunoregulatory effects such as the up-regulation of MHC class I molecules on monocytes/macrophages, increase of IL-1, IL-8, and platelet-activating factor synthesis, and decrease of TGF-β receptors, but also autocrine effects, because IFN-γ seems to regulate its own production (Billiau and Dijkmans 1990; Sen, 1992; Gusella *et al.*, 1993; Bulut *et al.*, 1993; Espinoza-Delgado *et al.*, 1994; Bosco *et al.*, 1994).

The interpretation of such IFN-γ effects is also hampered by the observations that IL-12 induced IFN-γ synthesis in natural killer (NK) cells independent of T cells (D'Andrea *et al.*, 1992). Nevertheless, this offers a novel pathway of the natural immune response to influence the cellular microenvironment regulating a TH response, as IL-12 is mainly produced by antigen-presenting macrophages and can be induced by intracellular bacteria or viruses.

3. *Interleukin-12*

IL-12 is a typical pleiotropic cytokine that increases antibody-depedendent cellular cytotoxicity (ADCC) and cell-mediated cytotoxicity by NK cells and acts back on macrophages inducing TNF-α and IFN-γ. With respect to T cells, IL-12 was recently shown to induce a typical TH1 response (Manetti *et al.*, 1993; Schmitt *et al.*, 1994). However, this effect can be overcome by the addition of IL-4. Application of recombinant IL-

12 to *L. major*-infected mice preferentially induced a protective IFN-γ-mediated TH1 response as assessed by a decreased parasite burden and IL-4 production in the regional lymph nodes (Sypek *et al.*, 1993; Heinzel *et al.*, 1993). Antigen-specific T cells produced significantly more IFN-γ if cultures were supplemented with IL-12 (Manetti *et al.*, 1993). Unexpectedly IL-12 in the presence of IL-2 also induced IFN-γ production in TH2 clones (Yssel *et al.*, 1994). Thus, the humoral immune response is influenced by IL-12 as it inhibits an IL-4-induced IgE synthesis in PBMNC (Kiniwa *et al.*, 1992; Brunda, 1994). This inhibition of IgE synthesis seems to be T cell dependent and distinct from the effects of IFN-γ or TGF-β.

4. Interleukin-10

IL-10 was first described as a typical product of TH2 clones suppressing cytokine production by TH1 clones in the presence of APC (Fiorentino *et al.*, 1989). This inhibition by IL-10 in the mouse was found to be indirectly mediated due to effects on the accessory function and antigen-presenting capacity of monocytes or macrophages (Fiorentino *et al.*, 1991a). Similarly, IL-10 inhibited monocyte- or macrophage-dependent antigen-stimulated cytokine synthesis by human PBMNC and NK cells (Yssel *et al.*, 1991). In addition, IL-10 has also been shown to inhibit monocyte- or macrophage-dependent antigen-specific proliferation of mouse TH1 clones as well as human TH0, TH1, and TH2 like T cell clones (de Waal Malefyt *et al.*, 1993). IL-10 down-regulates cell-mediated immune responses by suppressing the production of prostaglandin E (PGE_2) and of proinflammatory cytokines such as IL-2, TNF-α, IL-1, IL-6, and IL-8 (de Waal Malefyt *et al.*, 1993; Fiorentino *et al.*, 1991b; Niiro *et al.*, 1994). One important mechanism by which IL-10 inhibits accessory functions of macrophages/APCs for TH1 inhibition is the suppression of MHC class I expression (de Waal Malefyt *et al.*, 1991) and IL-12 production (Murphy *et al.*, 1994; D'Andrea *et al.*, 1993), as well as expression of B7 (Ding *et al.*, 1993; Kubin *et al.*, 1994). IL-10 also markedly influences B cells by inducing IgA synthesis in CD40-activated B cells and promotes naive B cells to secrete IgG_1 and IgG_3 (Briere *et al.*, 1994a,b). Data from IL-10 knockout mice suggest that these mice show a normal lymphocyte development and antibody responses but frequently develop bowel inflammation due to a defective immune response to enteric antigens (Kuhn *et al.*, 1993). IL-10 inhibits an IL-4-induced *in vitro* IgE synthesis in human PBMNC cultures indirectly via monocytes (Punnonen *et al.*, 1993a; Spits and de Waal Malefyt, 1992; Nonoyama *et al.*, 1994; Bober *et al.*, 1994).

5. Interleukin-2

IL-2 together with IFN-γ is a marker for the TH1 type $CD4^+$ T cells. TH cells respond upon activation by expressing IL-2 and the corresponding

IL-2 receptor complex (Morgan *et al.*, 1976; Smith *et al.*, 1980). At this level IL-2 drives the outgrow of antigen-specific T cells by an autocrine mechanism. IL-2 expression known to be cell cycle dependent (Stadler *et al.*, 1982) can act also as a paracrine factor influencing B cells, NK cells (Henney *et al.*, 1981; Tsudo *et al.*, 1987; Trinchieri *et al.*, 1984; Siegel *et al.*, 1987; Biron *et al.*, 1990; Spagnoli *et al.*, 1993; Shibuya *et al.*, 1993; Waldmann *et al.*, 1984; Zubler *et al.*, 1984), macrophages, and neutrophils (Malkovsky *et al.*, 1987; Djeu *et al.*, 1993). IL-2 by itself seems not to be capable of polarizing a TH response, but together with IL-12 or PGE_2, IL-2 drives a TH response in the direction of the TH1 type or TH2 type (Betz and Fox 1991; DeKruyff *et al.*, 1995). The most important observation in this context may be that parts of the trimeric $\alpha\beta\gamma$ IL-2 receptor complex are also used by cytokines other than IL-2. It was shown that the γ chain can be a ligand for IL-4 (Russel *et al.*, 1993), IL-7 (Noguchi *et al.*, 1993; Kondo *et al.*, 1994), IL-9 (Russel *et al.*, 1994), and IL-15 (Grabstein *et al.*, 1994; Giri *et al.*, 1994).

These data explain the obvious lack of severe immunodeficiencies in L-2 knock-out mice (Schorle *et al.*, 1991; Kündig *et al.*, 1993; Sadlack *et al.*, 1993) because the IL-2 can be bypassed by the redundancy of the immune system. A very important finding in view of the connection of our immune system with other systems is the observation that IL-2 may play a role as a neuromodulator (Nistico and De Sarro, 1991; Nistico, 1993; Hanisch *et al.*, 1993) as it can influence oligodendrocytes and microglia cells in the brain (Benveniste and Merrill, 1986; Arzt *et al.*, 1993; Eitan and Schwartz, 1993; Eitan *et al.*, 1994).

6. *Interleukin-5*

IL-5 is, together with IL-4, the typical TH2 cytokine produced by $CD4^+$ T cell clones (Coffman and Carty, 1986; Finkelmann *et al.*, 1988; Mosmann and Coffman, 1989; Coffman *et al.*, 1989). Based on *in vitro* and *in vivo* observations its biological effects seem to be restricted, at least in humans, to the eosinophilic lineage. *In vitro*, IL-5 is chemotactic for eosinophils (Limaye *et al.*, 1990) and *in vivo* eosinophilia as observed during helminth infections (Schweizer *et al.*, 1994) is abrogated by the administration of an anti-IL-5 antibody (Coffman *et al.*, 1989). IL-5 production, however, is not restricted to $CD4^+$ T cells as demonstrated in IL-4 knockout mice infected with *Nippostrongulus brasiliensis* (Kopf *et al.*, 1993). Interestingly, human eosinophils have been described recently to express the complementary molecule to the CD40 structure on B cells, namely the CD40 ligand, known to profoundly influence the development and the switching process of B cells (Gauchat *et al.*, 1995). The question whether IL-5 may

indirectly influence IgE regulation by attracting eosinophils in the late phase of an allergic reaction or in allergic asthma is not solved.

7. *Other Cytokines*

The TH-polarizing capacity of other cytokines known to be involved in the regulation of (*in vitro*) IgE synthesis such as IL-6, IFN-α, TNF-β, TNF-α, IL-1, and TGF-β is highly controversial. IFN-α and TGF-β were shown to preferentially promote a TH1 type immune response (Swain *et al.*, 1991; Romagnani, 1992a). Whether these effects on T cell clones are direct or indirect has to be investigated further because TGF-β and IFN-α are multifunctional with respect to their target cells and also with respect to their immunomodulatory capacities. TGF-β1 knockout mice (Kulkarni and Karlsson, 1993; Shull *et al.*, 1992), for example, develop some weeks after birth a diffuse inflammatory syndrome with the infiltration of mononuclear cells in vital organs. An apparent paradox has arisen in a murine *Leishmania* model. Even though the protective immune response was mediated by TH1 type cells, addition of TGF-β to infected animals worsened the disease (Barral-Netto *et al.*, 1992). In this situation TGF-β was acting by inhibiting IFN-γ mRNA and by increasing IL-4 mRNA synthesis. IL-1 was described to be an important costimulator for the proliferation of TH2 type clones, but not TH1 clones, if activated peritoneal macrophages or B cells from the spleen were used as APC (Chang *et al.*, 1990; Weaver *et al.*, 1988). In contrast to these observations are the results on hepatic nonparenchymal APC that preferentially stimulated TH1 but not TH2 cells (Magilavy *et al.*, 1989; Gajewski *et al.*, 1991).

Taken together, these observations suggest that the proliferation of T cells is under more stringent control and requires different signals than their cytokine production. An illustrative experiment to document the different requirements for signals inducing either cytokine synthesis or a proliferative response has been delivered using an amino acid substituted peptide, Hb 64-76, that induced IL-4 secretion but not proliferation. The addition of IL-1 together with the original peptide induced IL-4 production as well as proliferation to the same degree as the modified peptide without IL-1 (Evavold and Allen, 1991). In human cell cultures the removal of IL-1 before cloning of allergen-specific T cells (*Dermatophagoides pteronyssinus*) lead to a shift from a predominant TH2–TH0 response to a TH0–TH1 response (Romagnani, 1994). The author concluded that the presence of IL-1 in addition to IL-4 was required for the generation of human TH2 clones in the presence of APC.

However, IL-1 and IL-6 not only seem to influence individual lymphocyte populations but also have been reported to display hormonal activities. IL-1 and IL-6 have been shown to act systemically in an organism by

directly influencing pathways of neuroendocrine communication. In divers model systems they can directly activate the hypothalamic–pituitary–adrenal axis (HPA) resulting in the release of glucocorticoids from adrenal gland cells (Blalock, 1984; Dinarello, 1988; Woloski *et al.*, 1985; Smith, 1992). These findings are of importance because IL-1 and IL-6 are the first cytokines produced during an ongoing immune response and may therefore reflect a nonspecific mechanism to inform the nervous system about an invading pathogen (Blalock, 1994), and they may in addition elicit a generalized and very important feedback via the neuroendocrine arm on the immune system.

For atopy, human IL-13 may be as important as IL-4, because it induces *in vitro* IgE and IgG_4 synthesis in a subpopulation of activated human B cells that are activated by T cells, CD40 ligand, or anti-CD40 antibody (McKenzie *et al.*, 1993; Punnonen and de Vries, 1994; Kopf *et al.*, 1993). IL-13 can be produced by virtually all T cell subpopulations including TH0-TH1, and TH2-like clones and $CD8^+$ T cells (Zurawski and de Vries, 1994a,b), indicating that IL-13 does not play the same crucial and TH2-restricted role as IL-4 in the polarization of TH responses. However IL-13 does not seem to act on T cells (Zurawski and de Vries, 1994a,b) but inhibits, like IL-4, the IL-2-induced IFN-γ synthesis by NK cells (Zurawski and de Vries, 1994b), up-regulates the expression of surface IgM and class II expression on monocytes and macrophages, inhibits ADCC, and induces CD23 expression on B cells (McKenzie *et al.*, 1993; Zurawski and de Vries, 1994b; Noelle *et al.*, 1992). To date, the reason for this apparent redundancy between IL-13 and IL-4 is not well understood. Functionally it is clear that IL-4 and IL-13 use an identical receptor component because of the findings that an IL-4 mutant protein inhibited the activity of IL-4 as well as IL-13 (Aversa *et al.*, 1993). IL-13, in comparison to IL-4 and IL-10, seems to be equally potent in inducing humoral immune responses through inhibition of cell-mediated nonspecific natural immune responses. A subpopulation of TCR-stimulated naive human $CD4^+$ $45RO^-$ T cells was recently described to secrete IL-13, IL-5, and IFN-γ, but not IL-4. These cells provide helper functions to B cells as assessed by the induction of IgE synthesis (Brinkmann and Kristofic, 1995).

Taken together, the analysis of the distinct cytokine pattern produced by $CD4^+$ T cells revealed a major role for IL-4 and IFN-γ in various situations. IL-4 and IFN-γ show opposite effects on IL-4-induced IgE synthesis *in vitro*. One hypothesis therefore is that the increased production of specific IgE observed in allergic disorders is due to an aberrant T cell response against environmental antigens, rendering the TH1–TH2 concept in humans very attractive. However, oversimplification should be avoided and several important aspects should be kept in mind.

First, redundancy is characteristic for the immune system in terms of both the usage of receptor subunits by cytokines and the multiplicity of cytokines influencing an ongoing immune response.

Second, cytokines affecting the functional properties of different cell populations are pleiotropic.

Third, cell surface accessory molecules (cofactors), such as CD45, CD23, CD40 ligand, and CD28, that are known to be required to mount an appropriate immune response act in concert with cytokines.

Fourth, T cell populations other than $CD4^+$ T cells, such as $\gamma\delta$ T cells or $CD8^+$ T cells, may equally contribute to the regulation of an immune response, via the production of different cytokine combinations or the expression of different accessory molecules on the surface.

Fifth, the nature of the sessile APC and biochemical or biophysical properties of the antigen (allergen) may have a predetermining character.

Sixth, the immune system is linked with other regulatory systems of the body, and therefore one has to consider that the hormonal state of an individual may also influence directly the fate of an invading antigen. In the following sections we will address the above points in relation to the current TH1–TH2 concept.

E. Cell-Surface Molecules and TH Responses

A panel of surface molecules on T cells as well as on other cells, such as CD23, CD28, CD40 ligand, and the CD45 isoforms, are providing accessory signals to B cells for switching to IgE. The functional role of these molecules at the T cell level is poorly understood. Are these cell contacts also required to instruct a naive T cell to become a TH type effector T cell *in vivo*? *In vitro*, the presence of cytokines, such as IL-4, is sufficient to determine the development of a particular TH phenotype. In contrast, the efficacy of a T cell to turn on the humoral immune response strongly depends on additional contact-mediated signals.

Briefly, the interaction of the CD40 ligand on activated T cells with the CD40 molecule on B cells seems to be crucial for IgE class switching (Spriggs *et al.*, 1992; Banchereau *et al.*, 1994; Horner *et al.*, 1995; Punnonen *et al.*, 1992; Gauchat *et al.*, 1995; Jabara *et al.*, 1990; Korthauer *et al.*, 1993; Fanslow *et al.*, 1992; Gauchat *et al.*, 1992a; Shapira *et al.*, 1992; Garrone *et al.*, 1994; Kroczek *et al.*, 1994; Lederman *et al.*, 1994; Fuleihan *et al.*, 1993; Foy *et al.*, 1993; Burdin *et al.*, 1995). CD40 ligand positive T cells are found in secondary follicles. The CD40 counterstructure is found on thymic epithelial cells, activated monocytes, basophils, and also Langerhans cells (Caux *et al.*, 1994). The CD40 ligand may also act as T cell stimulus (Ramsdell *et al.*, 1994; Armitage *et al.*, 1993b). Another receptor

different from CD40 ligand may be present, as T cells from CD40 ligand-deficient individuals provide B cell help for IgE switching (Life *et al.*, 1994).

Inhibition of LFA-1-dependent aggregation of B cells decreases IgE synthesis (Bjorck and Paulie, 1993) and activation of LFA-1 receptor-mediated signaling is independent of signaling via the CD40 molecule on B cells (Diaz-Sanchez *et al.*, 1994), suggesting a role for LFA-1 and ICAM-1 in B cell activation (Bjorck *et al.*, 1992). Signaling via B cell surface anchored CD23 (low-affinity IgE-receptor, FcεRII) inhibits B cell proliferation and differentiation (Luo *et al.*, 1991; Bonnefoy *et al.*, 1990). An antisense DNA strategy to both forms of CD23 DNA also leads to inhibition of proliferation (Bhatti *et al.*, 1992). CD23 is found on monocytes (Vercelli *et al.*, 1988), on follicular dentritic cells (Rieber *et al.*, 1993), on bone marrow stromal cells (Fourcade *et al.*, 1992), on some T cells (Maekawa *et al.*, 1992; Gagro and Rabatic, 1994), on intestinal epithelial cells (Kaiserlian *et al.*, 1993), on keratinocytes (Becherel *et al.*, 1994), on Langerhans cells (Bieber *et al.*, 1989), and on eosinophils (Jouault *et al.*, 1988). The TH2 type cytokine IL-4 is the strongest inducer of CD23 expression on most cell types (Bonnefoy *et al.*, 1990; Vercelli *et al.*, 1988; Bieber *et al.*, 1989; Katira *et al.*, 1993; Lecron *et al.*, 1991; Herbelin *et al.*, 1994) and the same is true for IFN-γ on Langerhans cells (Bieber *et al.*, 1989). Cleaved and secreted CD23 was described to influence CD21-bearing T cells, monocytes, and B cells (Lecron *et al.*, 1991; Herbelin *et al.*, 1994; Henchoz *et al.*, 1994; Aubry *et al.*, 1992). A natural ligand for CD23 is the CD21 molecule, also claimed to be involved in IgE synthesis (Henchoz *et al.*, 1994; Aubry *et al.*, 1992). A distinct set of anti-CD21 molecules rescued human germinal B cells from apoptosis (Bonnefoy *et al.*, 1993). CD23 expression and secretion is a marker of activated cells. As CD23 and CD21 can be coexpressed on B cells as well as on T cells, one may assume that secreted CD23 acts as an autocrine factor. In autoimmune diseases the level of soluble CD23 was reported to be enhanced (al-Janadi *et al.*, 1994; Yoshikawa *et al.*, 1994; Bansal *et al.*, 1993). It should be mentioned that many of the above activities are questioned by results from CD23 knockout mice. In these mice B cell and T cell development is normal and the IgE response after *N. brasiliensis* infection is not prohibited (Stief *et al.*, 1994).

Signaling via CD28 on T cells inhibited human *in vitro* IgE synthesis (Life *et al.*, 1995) and this inhibition could be bypassed by *B. pertussis* infection (Van der Pauw-Kraan *et al.*, 1995). For comparison, CD28 knockout mice seem to have normal DTH and CTL but reduced humoral immune responses (Shahinian *et al.*, 1993). CD45 isoforms account for more than 10% of the cell membrane proteins in B cells and T cells. A crosslink of CD45 and CD40 on human B cells resulted in the inhibition

of IgE synthesis (Loh *et al.*, 1995). CD14 engagement by anti-CD14 antibody on normal human monocytes terminated T cell proliferation and inhibited IgM and IgG synthesis, as well as IL-4-induced IgE synthesis in human PBMNC cultures (Jabara and Vercelli, 1994).

In summary, surface molecules on T cells provide accessory signals to B cells, thereby amplifying the humoral immune response including IgE synthesis. The role of these molecules during the ontogenetic development of a naive T cell to become a mature T cell is not established in great detail. Interestingly, many of the well-defined surface molecules play a decisive role in the switching event of B cells, to a greater and more selective degree than the concurrent cytokine regulation. The puzzling fact remains why IgE synthesis is more tightly controlled than the other isotypes. It should not be forgotten that serum IgE levels are 1000- to 10,000-times lower than IgG levels.

F. Extrinsic Factors and TH Response

A conservative, but still challenging, concept is the idea that the local environment determines the development of an individual naive T cell to become a mature effector T cell. In other words the anatomic compartmentalization may influence and determine the functional competence of T cells and therefore also the quality of an ongoing immune response. From the TH1–TH2 concept it is difficult to extract more than minimal information about mechanisms controlling the initial step for the recruitment of T cells with a restricted cytokine pattern, but the particularity of the concept may in part be explained by factors other than cytokines. Following exposure to physiological amounts of glucocorticoids, naive T cells produced high levels of IL-4 if cells were cultured under serum-free conditions (Daynes *et al.*, 1990a) suggesting that the absence of IL-4 production by naive T cells in serum-containing media may be an *in vitro* artifact. This notion is supported by findings that dehydroepisterone (DHEA), a steroid with multiple functions on the immune system (Gordon *et al.*, 1986), directly influenced the cytokine pattern of T cells (Daynes *et al.*, 1990b). DHEA is the most abundant steroid in the circulation of humans, but it has to be enzymatically activated to become functional. Macrophages are known to contain this DHEA sulfatase enzyme within their granules (Hennebold and Daynes, 1994). Increased DHEA activity was also found in human keratinocytes and in the placenta (Milewich *et al.*, 1988; Haning *et al.*, 1990). High DHEA sulfatase activity was found in the spleen and also in lymph nodes draining nonmucosal tissues such as the skin, whereas low activity was found in other lymph nodes, such as the Peyer's patches.

Concerning the role of TH2 type cells in allergy, it is thus interesting that T cells from lymph nodes draining mucosal tissues produced high

levels of IL-4 and IL-5 but low levels of IL-2 and IFN-γ if stimulated under serum-free conditions (Daynes *et al.*, 1990a). The observation that locally activated steroids can modulate T cell activation was extended to dihydrotestosterone and also 1,25-dihydroxyvitamin D3 (Araneo *et al.*, 1991; Daynes *et al.*, 1991b). Platelet-derived growth factor (PDGF), another compound found in serum, directly influenced the production of T cell cytokines (Daynes *et al.*, 1991a) even though T cells seem not to bear a PDGF receptor (Nakano *et al.*, 1994). A yet unexplained finding is the preferential induction of a TH1 type response and an impaired humoral response in vitamin A-deficient animals (Cantorna *et al.*, 1994; Wiedermann *et al.*, 1993).

This regulatory compartmentalization concept awaits confirmation, but it is clear that the amount and the duration of cytokine production can be controlled by interlinked systems.

G. CD8⁺ T Cells and the Polarization of TH Responses

CD8⁺ cells are more heterogeneous than hitherto assumed; they contribute to the redundancy of the immune system and may play an active role in the regulation of polarized TH responses, too.

The great majority of human and mouse CD8⁺ T cells seem to behave like TH1 CD4⁺ T cells by producing predominantly IFN-γ and TNF but not IL-4 (Maggi *et al.*, 1988; Fong and Mosmann, 1990). There are convincing data demonstrating different subsets of CD8⁺ T cells in biopsies from patients with leprosy (Salgame *et al.*, 1991). *Mycobacterium leprae*-specific, MHC class I-restricted, and cytotoxic CD8⁺ T cell clones, secreting IFN-γ but not IL-4, were generated from healed lesions of tuberculoid leprosy patients. In contrast, specific, MHC class II-restricted CD8⁺ T cell clones were obtained from patients with lepromatous leprosy. These clones produced IL-4, IL-5, IL-10, and IFN-γ, but not IL-6. These exciting observations are in line with the patterns of the TH1 and TH2 type CD4⁺ T cell in these extreme forms of leprosy (Yamamura *et al.*, 1991; Salgame *et al.*, 1991). The notion that distinct CD8⁺ T cell populations may be discriminated according to their cytokine profile is also supported by recent reports describing the generation of IFN-γ and IL-5 but not IL-4 producing CD8⁺ T cell clones from bronchial biopsies of asthma probands (Romagnani, 1992b). In addition CD8⁺ T cell clones secreting IL-4 and IL-5 but not IFN-γ were derived from skin lesions in Kaposi's sarcoma patients (Romagnani, 1992b). In the presence of IL-4 murine naive CD8⁺ T cells were primed to secrete TH2 type cytokines providing help to B cells (Seder *et al.*, 1992a; Erhard *et al.*, 1993). IL-12 was shown to reciprocally promote the generation of TH1 cytokines (Croft *et al.*, 1994). CD8⁺ T cells *in vitro*, generated without TCR involvement and producing TH2 cytokines upon

IL-4 stimulation, still remained cytotoxic (Le Gros and Erhard, 1994). Additionally a new, noncytolytic $CD8^-$ $CD4^-$ T cell population was observed in a virus-specific TCR transgenic mouse model (Kyburz *et al.*, 1993; Le Gros and Erhard, 1994). The loss of cytotoxicity and the loss of the CD8 marker correlated with the down-regulation of perforin mRNA and CD8 α chain. The question of whether these TH2 type cytotoxic $CD8^+$ T cells or the noncytolytic $CD8^-$ $CD4^-$ T cells are suppressor cells of a TH1 type response remains open.

$CD8^+$ T cells, probably MHC class I-restricted $\gamma\delta$ T cells, producing IFN-γ in the airways were reported to modulate the extent of a TH2 like immune response after allergen challenge in humans (McMenamin and Holt, 1993). A phenotypic $CD8^+$ switch toward IL-5 production may also occur *in vivo* based on the observed eosinophilia in a biphasic superinfection mouse model, where a TH2 type response was induced prior to a challenge with a virus peptide, recognized by the transgenic TCR (Le Gros and Erhard, 1994). Indeed $CD8^+$ T cell clones influence IgE synthesis by providing an additional signal to IL-4 to human B cells (Kemeny and Diaz-Sanchez, 1993; Paganelli *et al.*, 1995). Whether activated TH2-like $CD8^+$ T cell subpopulations induce IgE synthesis directly, via a dual signaling of secreted IL-4 and surface expression of accessory molecules, is not known. However, an inhibitory effect of $CD8^+$ T cells on IgE synthesis would be of great practical interest. Interestingly, $CD8^+$ T cells derived from HIV-infected individuals with hyper-IgE have been shown to provide help to B cells for class switching to IgE, and IgE measurement in serum of HIV-infected persons correlated with the depletion of $CD4^+$ T cells *in vivo* (Paganelli *et al.*, 1995; Lucey *et al.*, 1990).

In a rat model the involvement of $CD8^+$ T cells in the regulation of specific IgE synthesis to allergen has been reported (Thorpe *et al.*, 1988, 1989; Diaz-Sanchez and Kemeny, 1990, 1991). In this system IgE synthesis was induced using castor bean extract in the presence or absence of ricin. Ricin is a toxic lectin that elicits no IgE synthesis by itself but serves as an adjuvant, and functionally inactivates a subpopulation of $CD8^+$ T cells within 1 day after immunization (Diaz-Sanchez and Kemeny, 1991; Kemeny *et al.*, 1994). After immunization with castor bean extract, an adoptive transfer of freshly activated $CD8^+$ T cells inhibited IgE synthesis almost completely in syngeneic mice. A subsequent *in vitro* culture of the recipient's PHA-stimulated spleen cells revealed a dramatically increased capacity to secrete IFN-γ. Ricin application in the absence of $CD8^+$ T cells produced an inhibition too. However, a simple depletion of $CD8^+$ T cells in this model did not yield clear results concerning the role of $CD8^+$ T cells on IgE synthesis.

In conclusion, $CD8^+$ T cells and $CD8^+$ T cell clones can behave like polarized TH2 phenotypes in terms of IL-4, IL-5, and IL-10 synthesis. Thereby they may influence an ongoing humoral immune response, including IgE synthesis. The mechanisms, how these TH2 type $CD8^+$ T cells interact with the appropriate APC, their activation mode, and their recruitment are nevertheless only poorly understood (Fig. 1).

IV. Mast Cells and Basophil Granulocytes: Innate TH2 Type Cells?

The role of tissue mast cells and circulating basophils during the effector phase of an IgE-mediated hypersensitivity reaction is very well established (Helm *et al.*, 1988; Kinet, 1990; Liu *et al.*, 1986; Lowman *et al.*, 1988a; Haydik and Ma, 1988; Sheard *et al.*, 1967; Gauchat *et al.*, 1993; Brunner *et al.*, 1993). Recently it has been shown that lung mast cells and basophils also secrete IL-4 if appropriately stimulated *in vitro* (Ben-Sasson *et al.*, 1990; Piccinni *et al.*, 1991; Brunner *et al.*, 1993; Bradding *et al.*, 1992; Smith *et al.*, 1994; MacGlashan *et al.*, 1994a,b; Tunon de Lara *et al.*, 1994; Bradding *et al.*, 1993). Dual stimulation of human basophils with IL-3 and IgE crosslinking agents, allergen or anti-IgE antibody, induced IL-4 production. In a coculture system, consisting of purified human tonsillar B cells and purified basophils, IgE synthesis was induced in the absence of exogenous IL-4. Obviously in this system, the stimulated basophils provided to the B cells not only IL-4 (Mueller *et al.*, 1994) but also a secondary contact-mediated signal that was inhibited by the addition of a soluble CD40 construct (Gauchat *et al.*, 1993). Interestingly IL-4 production by human basophils was induced by an IgE-independent mechanism *in vitro* (Ochensberger *et al.*, submitted).

It is tempting to speculate that such a phenomenon could eventually represent the hypothetical first step in the cascade leading finally to IgE production, namely the local induction of a "TH2-type microenvironment" by non-T cells, followed by the recruitment and the activation of IL-4-producing specific T cells providing help for B cells to switch to IgE synthesis (Fig. 1).

IgE-independent degranulation of effector cells was also induced by neuropeptides such as substance P and neurokinin A (Fewtrell *et al.*, 1982; Church *et al.*, 1991; Lowman *et al.*, 1988b). Based on these studies it is feasible to explain the occurrence of eosinophilia in nonallergic asthma by a similar mechanism, namely the local induction of a TH2 microenvironment by mast cells and/or basophils, followed by recruitment of TH2 type T cells and the production of IL-4 and IL-5. However, the question of whether mast cells or basophils are able to induce or to shift a particular

TH pattern during an ongoing immune response has not yet been answered *in vivo*.

V. APCs and the Antigen Play a Decisive Role for the Onset of a Particular TH Response

It is not trivial to focus for a while on the role of APCs and especially on allergens, because a large body of literature on the *in vitro* regulation of IgE synthesis neglected the first signal, namely the allergen. There are even reports claiming that the first signal required for B cell activation is IL-4 followed by signals from accessory molecules! Indeed, *in vitro* these signals suffice to induce IgE synthesis but have so far never induced an allergen-specific response (Zürcher *et al.*, 1995). One may argue that specific IgE responses may be difficult to induce because of the rare frequency of allergen-specific B cells. However, the observed IgE responses in the absence of allergen and induced by IL-4 and cosignaling with CD40 always lead to an exaggerated IgE synthesis as it would never be observed *in vivo*. We also assumed that these artificial *in vitro* systems mainly drive naive B cells to switch to IgE (Zürcher *et al.*, 1995).

A. APCs and the TH1–TH2 Concept

The functional redundancy observed in the immune system is also reflected in the capacity of nearly all cell types, including monocytes, macrophages, B cells, T cells, NK cells, cells of the polymorph-nuclear lineage, and other cells such as follicular dentritic cells and Langerhans cells in the skin, to process and to present antigens. The characteristics of the antigen and the activation or differentiation state of the APC profoundly influence an ongoing immune reaction. Antigen presentation and effector functions are not exclusive and can both be accomplished by individual cells. In view of the observed TH polarization of immune responses an obvious catch 22 has to be resolved. Is the APC instructing the naive $CD4^+$ T cell to become a TH1 or TH2 type helper cell or vice versa? Upon activation APCs and T cells produce various cytokines, express different surface molecules, and accentuate or diminish the development of target cells in a pleiotropic manner.

The nature of APCs plays no crucial role for the development of naive T cells into TH2 type cells, if IL-4 is available (Seder *et al.*, 1992b; Hsieh *et al.*, 1992; Swain *et al.*, 1991; Hsieh *et al.*, 1992; Macatonia *et al.*, 1993; Seder and Paul, 1994; Tanaka *et al.*, 1993). IL-4 alone was sufficient in these cases and could not be replaced, stressing the need for an already established TH2 type environment during a secondary immune response. IL-1, mainly produced by monocytes and macrophages, seems to be an

important costimulator for the proliferation of TH2 type clones but not of TH1 clones if activated peritoneal macrophages were used as APC (Chang *et al.*, 1990; Weaver *et al.*, 1988). Hepatitic nonparenchymal cells used as APC preferentially stimulated TH1 but not TH2 cells (Magilavy *et al.*, 1989; Gajewski *et al.*, 1991). Many APC-dependent effects on TH responses in mice were indirectly due to the TH2 type cytokine IL-10 (Fiorentino *et al.*, 1989, 1991a).

The pleiotropic effects of IL-10 include inhibition of antigen-specific proliferation of mouse TH1 clones as well as human TH0, TH1, and TH2 like T cell clones (de Waal Malefyt *et al.*, 1993). The role of the APCs in the early phase of a primary immune response cannot be investigated in humans but is in part understood in animals. IL-12, produced by monocytes, macrophages, mast cells, and B cells, or injected at the onset of a *L. major* infection, initiated in mice a protective, IFN-γ-mediated TH1 response (Sypek *et al.*, 1993; Heinzel *et al.*, 1993). In $\alpha\beta$ TCR transgenic mice dendritic cells and macrophages were shown to be required for establishing a TH1 response. In this system macrophages could be replaced by IL-12 substitution (Macatonia *et al.*, 1993). A panel of mouse splenic macrophage cell lines activated TH subsets and $CD8^+$ T cells as a function of their antigen-presenting activity (McCormack *et al.*, 1992). Additionally, the specific humoral immune response in mice depended on the presence of functional antigen-presenting macrophages and dentritic cells (Berg *et al.*, 1994) and an IgG2a isotype switch by B cells could be initiated by macrophages *in vivo* (Brewer *et al.*, 1994). *Leishmania major*-infected murine macrophages increased T cell polarization into TH2 type cells (Chakkalath and Titus, 1994) and antigen presentation by B cells or macrophages resulted in a different cytokine pattern of TH0 cells (Duncan and Swain, 1994). Interestingly, thymic macrophages were reported to polarize TH1 type cells (Jayaraman *et al.*, 1992).

In the skin, Langerhans cells (LC) bearing MHC class II molecules, and also FcεRI, are known to be professional APC. There is evidence that UV-activated LC or LC used as APC preferentially favor the generation of TH2 type clones, and this feature seems to correlate with the expression of LFA-1 and ICAM-1 (Simon *et al.*, 1990; Cumberbatch *et al.*, 1992; Hauser, 1992; Simon *et al.*, 1993). Interestingly, IL-10 produced *in vivo* by neighboring keratinocytes (Nickoloff *et al.*, 1994; Goodman *et al.*, 1994; Ullrich, 1994) abrogated the antigen-presenting capacity of LC (Enk *et al.*, 1993).

An underestimated cell in the skin is the keratinocyte capable of producing a large range of cytokines, e.g., IL-1 (Luger *et al.*, 1982), IL-3 (Luger *et al.*, 1985), granulocyte-macrophage colony-stimulating factor (Kupper *et al.*, 1988), TGF-β (Akhurst *et al.*, 1988), and IL-10 (Nickoloff *et al.*,

1994; Goodman *et al.*, 1994; Ullrich, 1994). Keratinocytes are capable of generating a polarized TH response via cytokine production, via MHC class IL-TCR-mediated signaling, or eventually via the production of pro-opiomelanocortin (POMC)-derived peptides (Goodman *et al.*, 1994; Viac *et al.*, 1994; Schauer *et al.*, 1994). Interestingly, in the mouse the POMC-derived peptide α-melanocyte-stimulating hormone (α-MSH) inhibited contact hypersensitivity (Shimizu and Streilein, 1994). Besides Langerhans cells, a second population of skin dentritic cells was described in the mouse. These resident $\gamma\delta$ T cells *in vitro* expressed IL-4 mRNA transiently if cultured with IL-2 and restimulated with Con A (Matsue *et al.*, 1993).

Taken together these reports indicate that different APCs may prime naive TH cells to turn into T cells of the TH1 or TH2 type. Thus, most provocatively one might claim that it is not the T cell that is generating the primary TH2 microenvironment. APC as discussed here or basophils/mast cells as discussed earlier might play a more crucial role. TH2 cells are definitely the indicator cells for what has happened before.

B. The Nature of the Allergen and TH Polarization

Biochemical and biophysical properties of antigens and especially of allergens are known to influence the cellular and the humoral immune response. A TH2 polarization, leading to IL-4 and IL-5 production, during helminthic infections is well established (Hagel *et al.*, 1993; Abdel-Azim *et al.*, 1989; Sher and Coffman, 1992; Finkelman *et al.*, 1991; Scott *et al.*, 1989; Schweizer *et al.*, 1994; Coffman *et al.*, 1989), but these findings were not related to distinct physical properties of the parasites. Thus the question remains whether there exist similarities between an anti-helminthic and an allergic response except for the common induction of increased serum IgE levels.

1. Proteolytic Activities of Allergens

The penetration process of a parasites into distinct target cells often requires the local destruction of host tissue. Proteases, produced by the parasites, are known to participate (Sakanari *et al.*, 1989; McKerrow, 1988). Allergens have protease sequence homology, or even protease activity (Stewart *et al.*, 1994; Wongtim *et al.*, 1993). It is known that pollen depends on protease activities to emerge from the large pollen's grain and to penetrate their plant target tissue. Papain, a model molecule for proteolytic allergens, induced asthma and local anaphylaxis in humans (Novey *et al.*, 1979; Bernstein *et al.*, 1984; Mansfield *et al.*, 1985). In mice, papain injection into the foot pad increased IL-4 gene expression transiently in draining lymph nodes and enhanced subsequently IgE and IgG_1 production (Urban *et al.*, 1992). IgE synthesis was considerably decreased if inactive

papain was used. Interestingly, not only allergenic compounds but also mast cells contain as major protein compounds neutral proteases, tryptase, chymase, and carboxypeptidases in their granules (Schwartz *et al.*, 1981; Johnson *et al.*, 1986; Goldstein *et al.*, 1987). Other reports demonstrated that IgE responses can be polarized by chemically modified allergens or by varying antigen concentrations (Baum *et al.*, 1990; Gieni *et al.*, 1993; Marcelletti and Katz, 1992; Hayglass *et al.*, 1991), further supporting the idea that allergens may polarize an immune response. However, it is clear that these data are not very helpful for understanding the genetic predisposition for atopy or the genetic background in inbred mouse strains that are either high or low producers of specific IgE independent of the applied immunization protocol.

2. *Adjuvants and Polarization of TH Responses*

In the early 1920s, it was observed that horses produced significantly more antibody if a weak inflammation was present at the site of antigen injection (Ramon, 1925). These observations were the basis for the development of many different adjuvants designed to yield greater antibody production, especially for vaccination strategies (Warren *et al.*, 1986; Audibert and Lise, 1993; Bomford *et al.*,. 1992). *Bordetella pertussis* as adjuvant was shown to increase IgE synthesis in mice (Revoltella and Ovary, 1969) and is suspected to influence allergic reactions in humans (Pauwels *et al.*, 1983). The mechanisms of this adjuvant effect become now gradually more defined, and it was shown that adjuvants influence antibody synthesis via generation of TH1 or TH2 type T cells, probably indirectly through IL-1-producing accessory cells (Grun and Maurer, 1989; Xu-Amano *et al.*, 1993; Hornquist and Lycke, 1993; Valensi *et al.*, 1994).

A similar adjuvant effect seems to be of particular importance in the etiology of allergic diseases. Diesel exhaust, applied in the airways, led to increased IgE synthesis in mice (Takafuji *et al.*, 1987) and to speculations that there exists a correlation between allergy and the amount of diesel exhaust in the air (Ishizaki *et al.*, 1987). However, such a correlation was not observed in all the countries analyzed (Bonini *et al.*, 1994), indicating that additional parameters must exist to explain the observed increase of allergic diseases in this century.

VI. The Integrative Role of the Nervous System

The nervous system communicates directly or indirectly with the immune system and therefore may also influence the onset and the extent of allergic reactions. Many reductionistic approaches at the molecular and cellular level, as summarized in this chapter, deliver fascinating aspects

about the mechanisms driving the specific adaptive immune system in response to a variety of offending pathogens and noxious allergens.

Parasites and hosts coevoluted in intimate, close contact. The immune system of a host is constantly challenged by the capacities of parasites to develop new inhibitory strategies. It is vital for every invading pathogen to circumvent the defense systems of the host. In the light of the TH1–TH2 concept, it is obvious that individual pathogens directly subverse the cytokine pattern induced during an immune response. Unfortunately, unwanted and misled pathological reactions during or after immune reactions are known to occur. This is particularly clear in the case of allergic reactions to basically inoffensive agents such as pollens.

Sensory nerves represent an alarm system and a first line of defense for the human organism, and a large body of literature indicates that the interplay between nerve cell derived factors and effector cells influenced the course of an acute allergic reaction locally, and eventually also systematically. Whether these interactions are important and crucial during the first contact with an allergen is not understood.

It is also tempting to address the problem from the opposite viewpoint. Considering the original findings from Besedowsky (Besedowsky and Sorkin, 1977), who stressed bidirectional communication between the immune system and the neuroendocrine system via neuroendocrine pathways, one may speculate that neuroendocrine factors influence an ongoing immune response to antigens in general and to allergens in particular. Neuroendocrine–immune interactions have been shown to influence *in vivo* lymphocyte migration, regulation of inflammation, pregnancy and thymus development, and many other hallmarks of an immune response (Immune-neuroendocrine special issue, 1994).

A. Bidirectional Communication Networks of the Body

1. Morphological Evidence

The immune system communicates with the nervous system via soluble mediators produced by the invading immune cells directly in the central nervous system (CNS). Activated immunocompetent cells such as lymphocytes and monocytes can cross the blood–brain barrier and secrete cytokines and other inflammatory mediators that act directly within the CNS, causing damage and leading to behavioral disturbances, anorexia, dementia, and even coma (Benveniste, 1992; Pober and Cotran, 1990; Dinarello and Wolf, 1993). Antigens from the periphery that enter the nervous system via the efferent blood vessels can directly activate in the brain resident microglia cells resulting in IL-1, IL-2, IL-4, IL-6, and TNF-α production (Benveniste, 1992).

The nervous system influences the immune system indirectly via the stimulation of endocrine glands. Neuronal influences modulate the immune response characteristically via the hormonal activation of external glands. Numerous hormones produced and stored in the pituitary gland are controlled by the hypothalamus. Activation of the hypothalamus upon psychological or physical stress provokes the release of such hormones in the blood stream and finally modulates the immune response through the secretion of mediators from the respective target glands. The most convincing example of this type of indirect interaction is the adrenocortical secretion in response to antigenic stimulation (Axelrod and Reisine, 1984; Munck *et al.*, 1984).

In addition the immune system and the nervous system communicate directly via cell contact between nerve axons and the local environment. Close contacts between nerve axons and different cell types including mast cells were described in the respiratory and gastrointestinal tract (Stead *et al.*, 1987) and also in primary and secondary lymphoid organs (Felten *et al.*, 1992).

All these morphological characteristics provide the physical bases for an intensive communication between the immune system, the neuroendocrine system, and the nervous system. Thus, it is feasible to postulate also that the TH1–TH2 concept is part of this general regulatory system.

2. *A Common Language: Shared Receptors and Ligands*

Shared receptors and ligands may be the basis for a common language between the central nervous, endocrine, and immune systems. Namely, it was shown that the immune system and the neuroendocrine system mutually produce neuropeptides, neurotransmitters, hormones, and cytokines. Cells of the immune system seem to produce more than 20 different neuroendocrine peptides and express their corresponding receptors. The first evidence that the immune system produces neuropeptides that are classically associated with neuronal or endocrine tissues was derived from reports on substance P, nerve growth factor, vasoactive intestinal peptide, β-endorphin, and adrenocorticotropic hormone (ACTH) (Carr, 1992; Blalock, 1989; Reichlin, 1993).

3. *Neuroendocrine Factors Influence the Immune System*

Various factors, steroids or peptides, produced or induced by the nervous system directly affect immune functions (Blalock, 1989; Stanisz *et al.*, 1994, 1986; Besedowsky *et al.*, 1986; Ahmed *et al.*, 1989; Gala, 1991; Shibata *et al.*, 1985; Johnson *et al.*, 1992; Gilman *et al.*, 1982; Van den Bergh *et al.*, 1991; Alvarez-Man *et al.*, 1985; Munn and Lum, 1989; Bost *et al.*, 1990; Harbuz *et al.*, 1993) and seem to have an influence on the polarized TH

compartments (Araneo *et al.*, 1989; Daynes *et al.*, 1990b; Rook *et al.*, 1994; Chiappelli *et al.*, 1994).

The role of cytokines on the nervous and endocrine systems has been known since the early 1980s (Blalock, 1984). Interferons were described to be potent inducers of steroid production in adrenal gland cells and IL-1 turned out to be the endogenous pyrogen (Dinarello, 1988). The cytokines IL-1 and IL-6, both potent proinflammatory cytokines, stimulate pituitary cells to release the "stress" hormone ACTH and also β-endorphin (Woloski *et al.*, 1985). However, it is still controversial whether they act at the pituitary or hypothalamic site (Smith, 1992). Much is known about the role of IL-2 on immune functions. Nevertheless some findings indicate that IL-2 also displays hormonal activity as it enhances in pituitary cells gene expression of proopiomelanocortin, the precursor protein of ACTH, α-MSH, and the endorphin family (Brown *et al.*, 1987).

The nervous system acts both locally, via the production of neuropeptides, and systemically, via the activation of the HPA axis. Both mechanisms might be of importance in allergy for generating a particular TH response, leading finally to modified IgE production and, under pathological conditions, to IgE-mediated hypersensitivity.

B. The Hypothalamic–Pituitary–Adrenal Axis and TH-Polarized Diseases

Deficiencies affecting the HPA axis may be the basis for the development of a TH polarization and even of autoimmune diseases such as rheumatic arthritis (Chikanza *et al.*, 1992). Animal models provide possible explanations for these findings. The obese strain of chicken develops spontaneous autoimmune thyroiditis, while other chickens do not (Hu *et al.*, 1993; Schauenstein *et al.*, 1987). Obese strain chickens have several abnormal features in their regulation of the HPA axis, indicating that deficient corticosteroid production by the adrenal gland is an important component for the development of disease. Lewis rats are susceptible to a number of inducible autoimmune diseases, including experimental allergic encephalomyelitis (EAE). They have a deficient HPA axis response to inflammatory stimuli and stress mediators. Lewis rats have markedly reduced plasma ACTH levels and a reduced cortisol response to IL-1 and corticotropin-releasing factor (CRF). They have, in addition, smaller adrenal glands and a larger thymus (Sternberg *et al.*, 1989). There is strong evidence that EAE, induced in Lewis rats and in mice, is a typical TH1-mediated autoimmune disease as EAE can be induced by adoptive transfer of $CD4^+$ TH1 type T cells (Myers *et al.*, 1992; Zhao *et al.*, 1992). Mucosa-derived TH2 clones suppressed EAE, induced with major basic protein or proteolipid protein

(Chen *et al.*, 1994), as did the inhibition of the TH1 response, by pentoxifylline or retinoid treatment (Rott *et al.*, 1993; Racke *et al.*, 1995).

Whether the unbalanced communication betwen the immune system and the neuroendocrine system in Lewis rats or in obese chickens is important or even causative at the onset of a particular TH response in these autoimmune diseases remains speculative and unsolved. However, we discuss these issues here because there is growing evidence that the allergic response is accompanied by autoimmune phenomena such as anti-IgE autoantibodies (rheumatoid-factors of allergy?) (Stadler *et al.*, 1991a,b) or that some reactions to allergens may be a broad cross-reaction to self (profilin) (Valenta *et al.*, 1991).

VII. Psychological Factors and Allergic Diseases

A. General Aspects

The question of whether the emotional state or the individual response to stress plays a role in a person's capacity to deal with diseases including infections, autoimmune diseases, allergies, and even cancer has always been obvious to the layman but has recently also become a focus of interest to immunologists. Thus, recent findings suggesting a bidirectional functional link between the different regulatory systems of the body may in the future allow a more precise and scientific approach to the question. A relationship between allergy and human emotions has been suspected ever since the time of Hippocrates. Psychological factors have been reported to play a role in allergic diseases such as urticaria, hay fever, atopic dermatitis, and asthma (McGovern and Knight, 1967). These case reports and anecdotes do not clarify the controversial issue of whether or not psychological factors associated with allergic diseases are a bystander effect, even though it is widely accepted that there exists a fundamental relationship between the nervous system and the immune system.

B. Conditioning and Allergic Reactions

The most famous classical example was described in 1886. The presentation of an artificial rose to an individual known to be allergic to these stimuli induced immediate, strong asthmatic symptoms (McKenzie, 1886). Deep hypnosis was reported to abrogate allergen-induced as well as tuberculin-induced skin reactions (Black, 1963). These exemplary reports suggest a direct influence of psychological factors on the effector phase of the allergic response, suggesting the participation of learning procedures, that can be regarded as a kind of Pavlovian conditioning (Pavlov, 1922). The described influences of psychological factors, as well as the observed learning phenomena involved in allergic disorders, suggest that a key element, namely mast cell activation, might partly be regulated by the nervous

system via peripheral nerves or via the endocrine system. Indeed, neuropeptides degranulate mast cells without IgE crosslinking (Fewtrell *et al.*, 1982; Church *et al.*, 1991; Lowman *et al.*, 1988b) (see Fig. 1, and the assumption that mast cells might be a kind of innate TH2 cell).

Many studies on conditioned allergic reactions were undertaken in the guinea pig and in the rat. In these studies respiratory distress and bronchoconstriction were induced by conditioned environmental or olfactory stimuli. It was also demonstrated that conditioned learning was as causative for asthmatic attacks (Ottenberg *et al.*, 1958; Justesen *et al.*, 1970), thereby supporting the view that the extent of an allergic reaction may be influenced in part by learning, due to classical conditioning.

A paradigm of mast cell activation by conditioning is a model with "allergic" rats. Rats were sensitized to egg albumin and then trained three times to associate this provocation with a combined stimulus using light and noise. Subsequently rats were exposed to light and noise alone in the absence of egg albumin and mast cell mediator release was measured in the serum. Levels of mast cell protease activity measured in these conditioned and sensitized rats were equal to the levels obtained by antigen provocation (McQueen *et al.*, 1989). Recently, another conditioning phenomenon was reported in a mouse model. Repeated immunizations with keyhole limpet hemocyanin (KLH), paired with a gustatory conditioned stimulus consisting of chocolate milk, produced an increase of anti-KLH antibody titers due to classical conditioning, if conditioned mice were reexposed to the conditioned stimulus together with a minimal dose of KLH (Ader *et al.*, 1993). Taken together these conditioning studies demonstrated that an immediate type reaction can be initiated by neurological factors in the absence of antigen, and that minimal amounts of antigen can be used as an unconditioned stimulus to enhance a humoral immune response. It is tempting to speculate that similar phenomena may occur in most allergic individuals, as they become aware of the announced pollen counts. As we will discuss later it is clear that neuroendocrine factors, broadly classified as stressors, even have an effect on human IgE synthesis and that their relation to the TH1–TH2 concept has to be analyzed.

In agreement are data from conditioning experiments in an allogenic skin graft model in mice (Nickerson *et al.*, 1994), as tolerance and allograft rejections seem also to be dominated by TH subpopulations (Qin *et al.*, 1994; Gorczynski *et al.*, 1982). These findings suggest that signals derived from the neuroendocrine and the immune system can strongly influence cell-mediated and humoral immune responses.

VIII. Neuroendocrine Factors and IgE Regulation

Stress modulates an ongoing immune response, the generation of immunological memory (Moynihan *et al.*, 1990), and classical conditioning seems

to influence endocrine responses (Stanton and Levine, 1988). Thus we intended to study the effect of neuropeptides on human *in vitro* IgE synthesis. The activation of the hypothalamic–pituitary–adrenal axis with psychological or physical stimuli leads, via the production of CRF in the hypothalamus, to the release of POMC-derived peptides, such as ACTH in the pituitary gland. ACTH released in the bloodstream provokes the release of steroids from adrenal gland cells *in vivo* (Fig. 2). CRF, ACTH, and steroids are described to influence various parameters of the immune system (Johnson *et al.*, 1992; Breuninger *et al.*, 1993) including the synthesis of IL-1 and IL-6, which are known to influence *in vitro* IgE synthesis.

A. Glucocorticoids and the Regulation of Human *in Vitro* IgE Synthesis

Glucocorticoids, such as hydrocortisone, stimulate IL-6 production by human mononuclear cells (Breuninger *et al.*, 1993), and natural as well as synthetic glucocorticoids deliver, in addition to IL-4, signal for B cells to switch to IgE synthesis *in vitro* (Sarfati *et al.*, 1989; Wu *et al.*, 1991; Jabara *et al.*, 1991; Nüsslein *et al.*, 1992, 1994, 1993; Jabara *et al.*, 1993; Kimata *et al.*, 1995). Most importantly, levels of serum cortisol seem to correlate with the extent of the late-phase reaction in humans (Herrscher *et al.*,

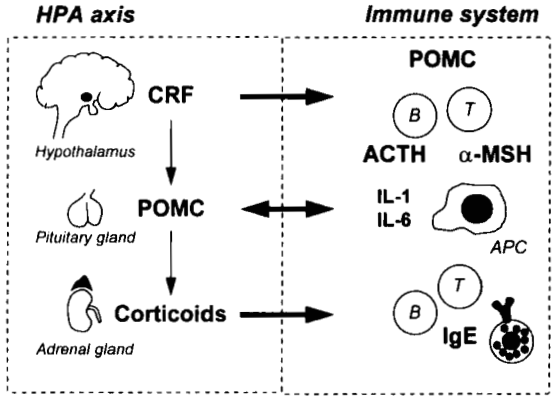


Fig. 2. Connection between the hypothalamus–pituitary–adrenal gland axis and the immune system. Besides corticoids, immune cells produce many of the factors that arise within the HPA axis, and immune cells also dispose of receptors to respond to factors of the HPA axis. It is well documented that neuroendocrine factors and nerve peptides have an influence on Type I allergic reactions, and we recently showed that human IgE synthesis also is influenced by such factors. Interestingly, in an immune microenvironment we have not been able to detect corticoids, but the neuroendocrine factors seem to have similar properties as the corticoids that arise during a HPA axis reaction.

1992). The extent of this IgE-dependent late-phase reaction follows a circadian rhythm as well. These findings also support the hypothetical action of the endocrine system on IL-4 production by activated mast cells or basophils *in vivo* during the late phase.

B. Neuroendocrine Peptides and Human *in Vitro* IgE Synthesis

ACTH and other peptides, involved in signaling via the HPA axis, as well as the glucocorticoids, released from adrenal gland cells, can be regarded as possible systemic modulators of factors involved in immune regulation and also in IgE regulation. POMC is a pituitary-derived precursor protein that is post-translationally cleaved into different biologically active proteins such as ACTH. All these sequential cleavage products are bioactive. Alpha-melanocyte-stimulating hormone, as well as the members of the endorphin family, displays various and different biological functions (De Wied and Jolles, 1982; Heagy *et al.*, 1990; Sibinga and Goldstein, 1988). Therefore all these peptides in addition to ACTH are of interest in IgE regulation, as they are synthesized together with ACTH in the pituitary, in extrapituitary tissues (Autelitano *et al.*, 1989), in cells of the immune system (Blalock, 1994), and also in the testis, in the placenta, and probably in the skin. The amount of neuroendocrine peptides not only is influenced by physical or psychological stimuli, but also depends on a circadian rhythm (Veldhuis *et al.*, 1990). The circadian rhythm *in vivo* can be overridden by chronic stress, by fear, by pain, by forced exercise, by hemorrhage, and by hypoglycemia (Carnes *et al.*, 1992).

We showed that neuropeptides such as CRF, the inducer of ACTH *in vivo*, ACTH itself, and a cleavage product of ACTH, namely α-MSH, increased or decreased class switching to IgE in presence of IL-4 (Aebischer *et al.*, 1994). These data indicate that neuroendocrine peptides that govern via endocrine pathways the HPA axis also influence the extent of an IgE response *in vitro*. In addition, we tested the effect of CRF, ACTH, and α-MSH on IgG, IgM, and IgA synthesis. In contrast to IgE synthesis these neuroendocrine peptides did not influence the other immunoglobulin isotypes in nonstimulated or in IL-4-stimulated mononuclear cell cultures. CRF, ACTH, and α-MSH did not act directly on the level of a switching B cell but indirectly via accessory cells. Monocytes seem to play a key role in the PBMNC culture system because their removal completely abrogated the IL-4-induced IgE synthesis. Furthermore, CRF, ACTH, and α-MSH had no effect on purified B cells as assessed in the CD40 system (Banchereau *et al.*, 1991). These data clearly showed that neuropeptides act not at the level of B cells, but indirectly via accessory cells. This notion is supported by the observation that the effect of neuropeptides on IgE synthesis appears only late during the culture period and

is strikingly dependent on cell density and culture conditions (Aebischer *et al.*, 1995). For example, IgE synthesis in cell cultures performed at various cell densities was never proportional to the number of B cells in the culture wells. CRF, ACTH, and α-MSH influenced a given cellular microenvironment via accessory cells leading finally to a decrease or increase of IgE synthesis *in vitro*.

To focus more precisely on the mechanism responsible for the observed phenomenon we studied the effect of neuropeptides on cytokine production in mononuclear cell cultures. ACTH induced *de novo* synthesis of the proinflammatory cytokines IL-6 and TNF-α. The same effect was observed on IL-6 and TNF-α synthesis if monocytes were stimulated with CRF or α-MSH. However, neuropeptide-induced cytokine production by monocytes was completely inhibited if IL-4 was added to the cell cultures. These data indicate that a sole cytokine such as IL-6 and TNF-α cannot be responsible for the observed ACTH effects as IL-4 remained mandatory in the culture system to induce B cells to switch to IgE. Nevertheless, monocyte activation by neuropeptides leading to IL-6 and TNF-α production may be important in clinical situations where IL-4 is not counteracting the effect of neuropeptides, for example in virus infections that are characteristically defined by massive IFN-γ production by T cells. Additional experiments concerning the influence of neuropeptides on cytokine production in mononuclear cell cultures revealed that IL-4, IL-5, IL-10, IL-12, GM-CSF, and IFN-γ levels are not altered if cells were stimulated with neuropeptides in a system optimized to yield high amounts of IgE. Nevertheless, it is very challenging that a typical TH2 type microenvironment, created by the addition of IL-4 and assessed by the measurement of IgE in culture supernatants, was modified by the addition of neuroendocrine peptides, resulting finally in increase or decrease of IgE synthesis (Fig. 3). Figure 3 summarizes the events as discussed so far. While it seems clear that an established TH polarization is the result of a preceding cytokine regulation, it is also clear that these cytokines cannot be derived from TH cells as they would not exist at the onset of a polarization. On the other hand non-T cells are probably interfering constantly with an existing TH polarization. This is also the most likely route of interference by the neuroendocrine or nervous system.

In addition to these POMC-derived peptides, other neuropeptides produced locally by nerve axons in primary and secondary lymphoid organs could be of importance in the regulation of IgE synthesis. For example Substance P (SP) was described to influence various arms of an immune response including chemotaxis, proliferation, antibody synthesis, and degranulation of mast cells and basophils (Fewtrell *et al.*, 1982; Stanisz *et al.*, 1986; Shibata *et al.*, 1985; Hirai *et al.*, 1988; Lowman *et al.*, 1988a; Payan

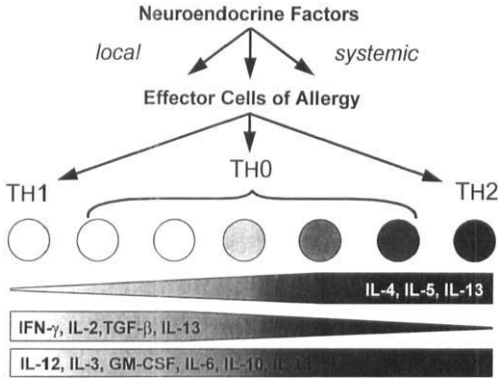


FIG. 3. Neuroendocrine factors shift the TH polarization. The dynamic cytokine expression by TH subpopulations can be influenced by factors of the local or systemic neuroendocrine system by primarily acting on the effector cells of allergy. These cells in turn have an influence on the development of TH cells and can shift a TH2 response toward TH1 or vice versa.

and Goetzl, 1988; Damonneville *et al.*, 1990; Louis and Radermecker, 1990; Kaminer *et al.*, 1991; Church *et al.*, 1991; Cohan *et al.*, 1991; Louis and Radermecker, 1991; Okayama *et al.*, 1994; Sperr *et al.*, 1994). We found that SP, similarly to the systemically acting neuroendocrine peptides, can influence human *in vitro* IgE synthesis. SP in contrast to ACTH also influenced the synthesis of other immunoglobulin classes such as IgM, IgG, and IgA (Aebischer *et al.*, 1995). The relevance of our *in vitro* observations is supported by data obtained in animal models. SP was described to modulate the specific *in vivo* IgE response in monkeys and in rats (Patterson and Harris, 1993; Carucci *et al.*, 1994).

A peculiar observation that fits our *in vitro* observations, and that might also explain the high levels of IgE in helminthic infestations, was recently published (Duvaux-Miret *et al.*, 1992). The parasite *Schistostoma mansoni*, which induces a polyclonal IgE response in mice and humans, also secretes a parasitic ACTH that can be cleaved into α-MSH by an endopeptidase found on cocultured human neutrophils, suggesting that the anti-inflammatory capacity of α-MSH (Korthauer *et al.*, 1993) may contribute an escape mechanism for the host defense and as a bystander effect eventually enhance IgE synthesis. In adult humans biologically active α-MSH is not found systemically *in vivo*, but is assumed to be produced locally in the skin and, in particular, in the placenta of a pregnant woman.

So far we discussed the decisive role of accessory cells for the development of a TH1–TH2 microenvironment and we termed the developing TH cells "indicator" cells for a regulation that took place before. Here we

showed that the decisive role of the accessory cells is further controlled by a superseding network of neuroendocrine factors supplied systemically or produced locally during an immune response.

IX. Concluding Remarks

Immune responses to apparently inoffensive allergens in humans are very heterogeneous. Because IgE antibodies are directly involved in mediating many allergic reactions, the molecular mechanisms regulating IgE synthesis are therefore of evident interest. The TH1–TH2 concept provided a simplified model for a T cell regulation. However, allergies cannot simply be regarded as a consequence of a deviated T cell response dominated by IL-4-producing $CD4^+$ T cells. On the one hand, the TH1–TH2 concept was rapidly adapted by allergists as it replaced many hypothetical factors and concepts at the time, and by immunologists because it made it clear that not every T cell is producing every cytokine at a given time. On the other hand, the TH1–TH2 concept was also too quickly adapted as a phenotypic concept without considering the dynamic aspects of cytokine production. The cytokine network not only is redundant but disposes of many feedback control mechanisms.

It was exactly these feedback mechanisms that made the TH1–TH2 concept so attractive. Until today IL-4 and IFN-γ have been considered the paradigm of mutual and opposite T cell regulation, especially for the regulation of IgE synthesis. Nevertheless, TGF-β is a much stronger inhibitor of human IgE synthesis. The clearest example of the TH1–TH2 regulation, namely IgE synthesis, is again jeopardized by the fact that IL-13, a "redundant" IL-4, is produced by most TH cells.

Furthermore, the TH1–TH2 model provides no real help for the understanding of the specific B cell response that seems to occur only in allergic individuals. It has to be admitted that thanks to human IL-4, and the developing understanding of the TH1–TH2 concept, it became possible to induce a human IgE synthesis *in vitro*. Together with the CD40 cofactor system IL-4 became a potent *in vitro* tool to culture cells that switched with a high rate to IgE. But does such a dramatic switch ever occur *in vivo*? We and many others in the field used the system to study IgE regulation, but nobody was capable of assessing an allergen-specific response, nor was the allergen helpful in inducing specific IgE. Here awaits a great challenge for the TH1–TH2 concept!

It is known that an individual response to antigen can also be influenced by psychological factors. The most trivial example is the differential IL-2 or IL-4 production by splenocytes from either individually or group-housed mice known to produce a predominantly TH1 or TH2 response (Karp *et*

al., 1994). Our findings also support the general view that an allergic response mediated by IgE can be modulated by factors influencing the communication between the immune system and the nervous system via the HPA axis. Therefore the total and specific IgE response may be influenced in a similar manner as has been suggested for autoimmune diseases, namely by chronic stress that interferes with the normal circadian rhythm of the neuropeptides.

References

Abdel-Azim, A., Rafick, M. M., el-Tahawy, M., and Fikry, A. A. (1989). Total and specific immunoglobulin E in schistosomiasis. *J. Egypt. Soc. Parasitol.* **19,** 497–506.

Ader, R., Kelly, K., Moynihan, J. A., Grota, L. J., and Cohen, N. (1993). Conditioned enhancement of antibody production using antigen as the unconditioned stimulus. *Brain Behav. Immun.* **7,** 334–343.

Aebischer, I., Stämpfli, M. R., Zürcher, A., Miescher, S., Urwyler, A., Frey, B., Luger, T., White, R. R., and Stadler, B. M. (1994). Neuropeptides are potent modulators of human in vitro immunoglobulin E synthesis. *Eur. J. Immunol.* **24,** 1908–1913.

Aebischer, I., Stämpfli, M. R., Miescher, S., Horn, M., Zürcher, A. W., and Stadler, B. M. (1995). Neuropeptides accentuate interleukin-4 induced human immunoglobuline E synthesis in vitro. *J. Exp. Dermat.*, in press.

Ahmed, S. A., Dauphinée, M. J., Monotoya, A. I., and Tala, N. (1989). Estrogen induces normal murine $CD5^+$ B cells to produce autoantibodies. *J. Immunol.* **142,** 2647–2653.

Akhurst, R. J., Fee, F., and Balmain, A. (1988). Localized production of TGF-β mRNA in tumor promoter-stimulated mouse epidermis. *Nature* **331,** 363–365.

al-Janadi, M., al-Wabel, A., and Raziuddin, S. (1994). Soluble CD23 and interleukin-4 levels in autoimmune chronic active hepatitis and systemic lupus erythematosus. *Clin. Immunol. Immunopathol.* **71,** 33–37.

Alvarez-Man, M., Kehrl, J.-H., and Fanci, A. S. (1985). A potential role for adrenocorticotropin in regulating human B lymphocyte functions. *J. Immunol.* **135,** 3823–3826.

Andersson, U., Andersson, J., Lindfors, A., Wagner, K., Möller, G., and Heusser, C. H. (1990). Simultaneous production of interleukin-2, interleukin-4 and interferon-γ by activated human blood lymphocytes. *Eur. J. Immunol.* **20,** 1591–1596.

Anfosso, F., Soler, M., Mallea, M., and Charpin, J. (1977). Isolation and characterization in vitro of an allergen from plane-tree (Platanus acerifolia) pollen. *Int. Arch. Allergy Appl. Immunol.* **54,** 481–486.

Araneo, B. A., Dowell, T., Moon, H. B., and Daynes, R. A. (1989). Regulation of murine lymphokine production in vivo. Ultraviolet radiation exposure depresses IL-2 and enhances IL-4 production by T cells through an IL-1 independent mechanism. *J. Immunol.* **143,** 1737–1744.

Araneo, B. A., Dowell, T., Terui, T., Diegel, M., and Daynes, R. A. (1991). Dihydrotestosterone exerts a depressive influence on the production of IL-4, IL-5 and g-IFN, but not IL-2 by activated murine cells. *Blood* **78,** 688–699.

Arase, H., Arase, N., Nakagawa, K., Good, R. A., and Onoe, K. (1993). NK1.1+CD4+CD8- thymocytes with specific lymphocyte secretion. *Eur. J. Immunol.* **23,** 307–310.

Armitage, R. J., Macduff, B. M., Spriggs, M. K., and Fanslow, W. C. (1993a). Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. *J. Immunol.* **150,** 3671–3680.

Armitage, R. J., Tough, T. W., Macduff, B. M., Fanslow, W. C., Spriggs, M. K., Ramsdell, F., Alderson, M. R. (1993b). CD40 ligand is a T cell growth factor. *Eur. J. Immunol.* **23,** 2326–2331.

Arzt, E., Buric, R., Stelzer, G., Stalla, J., Sauer, J., Renner, U., and Stalla, G. K. (1993). Interleukin involvement in anterior pituitary cell growth regulation: Effects of IL-2 and IL-6. *Endocrinology* **132,** 459–467.

Aubry, J.-P., Pochon, S., Graber, P., Jansen, K. U., and Bonnefoy, J.-Y. (1992). CD21 is a ligand for CD23 and regulates IgE production. *Nature* **358,** 505–507.

Audibert, F. M., and Lise, L. D. (1993). Adjuvants, current status, clinical perspectives and future prospects. *Trends Pharmacol. Sci.* **14,** 174–178.

Autelitano, D. J., Lundblad, J. R., Blum, M., and Roberts, J. L. (1989). Hormonal regulation of POMC gene expression. *Annu. Rev. Physiol.* **51,** 715–726.

Aversa, G., Punnonen, J., Cocks, B. G., de Waal Malefyt, R., Vega, F. Jr., Zurawski, S. M., Zurawski, G., de Vries, J. E. (1993). An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG_4) and IgE synthesis and B cell proliferation: Support for a common component shared by IL-4 and IL-13 receptors. *J. Exp. Med.* **178,** 2213–2218.

Axelrod, J., and Reisine, T. D. (1984). Stress hormones: Their interaction and regulation. *Science* **224,** 452–459.

Banchereau, J., de Paoli, P., Valle, A., Garcia, E., and Rousset, F. (1991). Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science* **251,** 70–72.

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F., and Saeland, S. (1994). The CD40 antigen and its ligand. *Annu. Rev. Immunol.* **12,** 881–922.

Bancroft, G. J., Sheehan, K. C. F., Schreiber, R. D., and Unanue, E. R. (1989). Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in SCID mice. *J. Immunol.* **143,** 127–130.

Bansal, A. S., Ollier, W., Marsh, M. N., Pumphrey, R. S., and Wilson, P. B. (1993). Variations in serum sCD23 in conditions with either enhanced humoral or cell-mediated immunity. *Immunology* **79,** 285–289.

Barral-Netto, M., Barral, A., Brownell, C. E., Skeiky, Y. A., Ellingsworth, L. R., Twardzik, D. R., and Reed, S. G. (1992). Transforming growth factor-beta in leishmanial infection: A parasite escape mechanism. *Science* **257,** 545–548.

Baum, C. G., Szabo, P., Siskind, G. W., Becker, C. G., Firpo, A., Clarick, C. J., and Francus, T. (1990). Cellular control of IgE induction by a polyphenyl-rich compound. *J. Immunol.* **145,** 779–784.

Becherel, P. A., Mossalayi, M. D., Ouaaz, F., Le Goff, L., Dugas, B., Paul-Eugene, N., Frances, C., Chosidow, O., Kilchherr, E., and Guillosson, J. J. (1994). Involvement of cyclic AMP and nitric oxide in immunoglobulin E-dependent activation of Fc epsilon RII/CD23+ normal human keratinocytes. *J. Clin. Invest.* **93,** 2275–2279.

Belosevic, M., Finbloom, D. S., Meide, P. H. V. D., Slayter, M. V., and Nacy, C. A. (1992). Administration of monoclonal anti-IFN-γ antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. *J. Immunol.* **143,** 266–274.

Ben-Sasson, S. Z., Le Gros, G., Conrad, D. H., Finkelman, F. D., and Paul, W. E. (1990). Cross-linking Fc receptors stimulates splenic non-B, non-T cells to secrete interleukin 4 and other cytokines. *Proc. Natl. Acad. Sci. USA* **87,** 1421–1425.

Bendelac, A., Matzinger, P., Seder, A., Paul, W. E., and Schwartz, R. H. (1992). Activation events during thymic selection. *J. Exp. Med.* **174,** 731–742.

Benveniste, E. N. (1992). Cytokines: Influence on glial cell gene expression and function. *Clin. Immunol.* **52,** 106–153.

Benveniste, E. N., and Merrill, J. E. (1986). Stimulation of oligodendroglial proliferation and maturation by interleukin-2. *Nature* **321,** 610–613.

Berg, S. F., Mjaaland, S., and Fossum, S. (1994). Comparing macrophages and dendritic leukocytes as antigen-presenting cells for humoral responses in vivo by antigen targeting. *Eur. J. Immunol.* **24,** 1262–1268.

Bernstein, D. J., Gallegher, J. S., Grad, M., and Bernstein, I. L. (1984). Local ocular anaphylaxis to papain enzyme contained in a contact lens cleaning solution. *J. Allergy Clin. Immunol.* **74,** 258–263.

Besedowsky, H., and Sorkin, E. (1977). Network of immune-neuroendocrine interactions. *Clin. Exp. Immunol.* **27,** 1–12.

Besedowsky, H., del Rey, A. E., Sorkin, E., and Dinarello, C. A. (1986). Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* **233,** 652–654.

Betz, M., and Fox, B. S. (1991). Prostaglandin E2 inhibits production of Th 1 lymphokines but not of Th2 lymphokines. *J. Immunol.* **146,** 108–113.

Bhatti, L., Behle, K., and Stevens, R. H. (1992). Inhibition of B cell proliferation by antisense DNA to both alpha and beta forms of Fc epsilon R II. *Cell. Immunol.* **144,** 117–130.

Bieber, T., Rieger, A., Neuchrist, C., Prinz, J. C., Rieber, E. P., Boltz-Nitulescu, G., Scheiner, O., Kraft, D., Ring, J., and Stingl, G. (1989). Induction of Fc epsilon R2/CD23 on human epidermal Langerhans cells by human recombinant interleukin 4 and gamma interferon. *J. Exp. Med.* **170,** 309–314.

Bieber, T. (1994). Fc epsilon RI on human Langerhans cells: A receptor in search of new functions. *Immunol. Today* **15,** 52–53.

Billiau, A., and Dijkmans, R. (1990). Interferon-gamma: Mechanism of action and therapeutic potential. *Biochem. Pharmacol.* **40,** 1433–1439.

Biron, C. A., Young, H. A., and Kasaian, M. T. (1990). Interleukin 2-induced proliferation of murine natural killer cells in vivo. *J. Exp. Med.* **171,** 173–188.

Bjorck, P., Paulie, S., and Axelsson, B. (1992). Interleukin-4-mediated aggregation of anti-IgM-stimulated human B cells: Inhibition of aggregation but enhancement of proliferation by antibodies to LFA-1. *Immunology* **75,** 122–128.

Bjorck, P., and Paulie, S. (1993). Inhibition of LFA-1-dependent human B-cell aggregation induced by CD40 antibodies and interleukin-4 leads to decreased IgE synthesis. *Immunology* **78,** 218–225.

Black, S. (1963). Shift in dose-response curve of Prausnitz-Küstner reaction by direct suggestion under hypnosis. *Br. Med. J.* **1,** 990–992.

Blalock, J. E. (1984). Relationship between neuroendocrine hormones and lymphokines. *Lymphokines* **9,** 1–12.

Blalock, J. E. (1989). A molecular basis for bidirectional communication between the immune and neuroendocrine systems. *Physiol. Rev.* **69,** 1–32.

Blalock, J. E. (1994). The immune system: Our sixth sense. *The Immunologist* **2,** 8–15.

Bober, L. A., Grace, M. J., Pugliese-Sivo, C., Waters, T. A., Sullivan, L. M., and Narula, S. K. (1994). Human IL-10 reduces the number of IL-4-induced IgE B cells in cultures of atopic mononuclear cells. *Int. Arch. Allergy Immunol.* **105,** 26–31.

Bomford, R., Stapleton, M., Winsor, S., McKnight, A., and Andronova, T. (1992). The control of the antibody isotype response to recombinant human immunodeficiency virus gp120 antigen by adjuvants. *AIDS Res. Hum. Retroviruses* **8,** 1765–1771.

Bonini, S., Magrini, L., Rotiroti, G., Ronchetti, M. P., and Onorati, P. (1994). Genetic and environmental factors in the changing incidence of allergy. *Allergy* **49,** 6–14.

Bonnefoy, J.-Y., Shields, J., and Mermod, J. J. (1990). Inhibition of human interleukin 4-induced IgE synthesis by a subset of anti-CD23/Fc epsilon RII monoclonal antibodies. *Eur. J. Immunol.* **20,** 139–144.

Bonnefoy, J.-Y., Henchoz, S., Hardie, D., Holder, M. J., and Gordon, J. (1993). A subset of anti-CD21 antibodies promote the rescue of germinal center B cells from apoptosis. *Eur. J. Immunol.* **23,** 969–972.

Bosco, M. C., Espinoza-Delgado, I., Schwabe, M., Gusella, G. L., Longo, D. L., Sugamura, K., and Varesio, L. (1994). Regulation by interleukin-2 (IL-2) and interferon gamma of IL-2 receptor gamma chain gene expression in human monocytes. *Blood* **83,** 2995–3002.

Bost, K. L., Clarke, B. L., Xu, J. C., Kiyuno, H., Mc Ghee, J. R., and Pascual, D. (1990). Modulation of IgM secretion and H chain mRNA expression in CH12.LX.C4.5F5 B cells by adrenocorticotropic hormone. *J. Immunol.* **145,** 4326–4331.

Bradding, P., Feather, I. H., Howarth, P. H., Mueller, R., Roberts, J. A., Britten, K., Bews, J. P., Hunt, T. C., Okayama, Y., Heusser, C. H., Bullock, G. R., Church, M. K., and Holgate, S. T. (1992). Interleukin 4 is localized to and released by human mast cells. *J. Exp. Med.* **176,** 1381–1386.

Bradding, P., Feather, I. H., Wilson, S., Bardin, P. G., Heusser, C. H., Holgate, S. T., and Howarth, P. H. (1993). Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J. Immunol.* **151,** 3853–3865.

Brantschen, S., Gauchat, J.-F., de Weck, A. L., and Stadler, B. M. (1989). Regulatory effect of recombinant IL-3 and IL-4 on cytokine gene expression of bone marrow and peripheral blood lymphocytes. *Eur. J. Immunol.* **19,** 2017–2023.

Bretscher, B. A., Wu, G., Menon, N., and Ohmann, H. (1992). Establishment of stable cell-mediated immunity that makes susceptible mice resistant to Leishmania major. *Science* **257,** 539–542.

Breuninger, L. M., Dempsey, W. K., Uhl, J., and Murasko, D. M. (1993). Hydrocortisone regulation of interleukin-6 protein production by a purified population of human peripheral blood monocytes. *Clin. Immunol. Immunpathol.* **69,** 205–214.

Brewer, J. M., Richmond, J., and Alexander, J. (1994). The demonstration of an essential role for macrophages in the in vivo generation of IgG2a antibodies. *Clin. Exp. Immunol.* **97,** 164–171.

Brière, F., Bridon, J. M., Chevet, D., Souillet, G., Bienvenu, F., Guret, C., Martinez-Valdez, H., and Banchereau, J. (1994a). Interleukin 10 induces B lymphocytes from IgA-deficient patients to secrete IgA. *J. Clin. Invest.* **94,** 97–104.

Brière, F., Servet-Delprat, C., Bridon, J. M., Saint-Remy, J. M., and Banchereau, J. (1994b). Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG_1 and IgG_3. *J. Exp. Med.* **179,** 757–762.

Brinkmann, V., and Kristofic, C. (1995). TCR-stimulated naive human CD4+ 45RO− T cells develop into effector cells that secrete IL-13, IL-5, and IFN-gamma, but no IL-4, and help efficient IgE production by B cells. *J. Immunol.* **154,** 3078–3087.

Brown, S. L., Smith, L. R., and Blalock, J. E. (1987). Interleukin 1 and interleukin 2 enhance proopiomelanocortin gene expression in pituitary cells. *J. Immunol.* **139,** 3181–3183.

Brunda, M. J. (1994). Interleukin-12. *J. Leukoc. Biol.* **55,** 280–288.

Brunner, T., Heusser, C. H., and Dahinden, C. A. (1993). Human peripheral blood basophils primed with IL-3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* **177,** 605–612.

Bruserud, O., Hamann, W., Patel, S., Ehninger, G., Schmidt, H., and Pawelec, G. (1993). IFN-gamma and TNF-alpha secretion by CD4+ and CD8+ TCR alpha beta + T-cell clones derived early after allogeneic bone marrow transplantation. *Eur. J. Haematol.* **51,** 73–79.

Brusselle, G., Kips, J., Joos, G., Bluethmann, H., and Pauwels, R. (1995). Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell. Mol. Biol.* **12,** 254–259.

Bulut, V., Severn, A., and Liew, F. Y. (1993). Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP. *Biochem. Biophys. Res. Commun.* **195,** 1134–1138.

Burdin, N., Van Kooten, C., Galibert, L., Abrams, J. S., Wijdenes, J., Banchereau, J., and Rousset, F. (1995). Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. *J. Immunol.* **154,** 2533–2544.

Busse, W. W. (1989). The relationship between viral infections and onset of allergic diseases and asthma. *Clin. Exp. Allergy* **19,** 1–9.

Cantorna, M. T., Nashold, F. E., and Hayes, C. E. (1994). In vitamin A deficiency multiple mechanisms establish a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function. *J. Immunol.* **152,** 1515–1522.

Carballido, J. M., Carballido-Perrig, N., Terres, G., Heusser, C. H., and Blaser, K. (1992). Bee venom phospholipase A2 specific T cell clones from human allergic and non-allergic individuals, cytokine patterns change in response to the antigen concentration. *Eur. J. Immunol.* **22,** 1357–1363.

Carnes, M., Brownfield, M., Lent, S. J., Nichols, K., and Schuler, L. (1992). Pulsatile ACTH and cortisol in goats: Effects of insulin-induced hypoglycemia and dexamethasone. *Neuroendocrinol.*, **55,** 97–104.

Carr, D. J., Radulescu, R. T., deCosta, B. R., Rice, K. C., and Blalock, J. E. (1990). Differential effect of opioids on immunoglobulin production by lymphocytes isolated from Peyer's patches and spleen. *Life Sci.* **47,** 1059–1069.

Carr, J. J. D. (1992). Neuroendocrine peptide receptors on cells of the immunes system. *Chem. Immunol.* **52,** 84–105.

Carucci, J. A., Herrick, C. A., and Durkin, H. G. (1994). Neuropeptide-mediated regulation of hapten-specific IgE responses inmice. II. Mechanisms of substance P-mediated isotype-specific suppression of BPO-specific IgE antibody-forming cell responses induced in vitro. *J. Neuroimmunol.* **49,** 89–95.

Carvalho, E. M., Badaro, R., Reed, S. G., Jones, T. C., and Johnson, W. D. (1985). Absence of gamma interferon and interleukin-2 production during active visceral leishmaniasis. *J. Clin. Invest.* **76,** 2066–2069.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* **180,** 1263–1272.

Chakkalath, H. R., and Titus, R. G. (1994). Leishmania major-parasitized macrophages augment Th2-type T cell activation. *J. Immunol.* **153,** 4378–4387.

Chang, T. L., Shea, C. M., Urioste, S., Thompson, R. C., Boom, W. H., and Abbas, A. K. (1990). Heterogeneity of helper/inducer T lymphocytes. III. Responses of IL-2- and IL-4-producing (Th1 and Th2) clones to antigens presented by different accessory cells. *J. Immunol.* **145,** 2803–2808.

Chatelain, R., Varkila, K., and Coffman, R. L. (1992). IL-4 induces a Th2 response in Leishmania major-infected mice. *J. Immunol.* **148,** 1182–1187.

Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A., and Weiner, H. L. (1994). Regulatory T cell clones induced by oral tolerance, suppression of autoimmune encephalomyelitis. *Science* **265,** 1237–1240.

Chiappelli, F., Manfrini, E., Franceschi, C., Cossarizza, A., and Black, K. L. (1994). Steroid regulation of cytokines. Relevance for TH1-to-TH2 shift? *Ann. N. Y. Acad. Sci.* **746,** 204–215.

Chikanza, I. C., Petrou, P., Kingsley, G., Chrousos, G., and Panayi, G. S. (1992). Defective hypothalamic response to immune and inflammatory stimuli in patients with rheumatoid arthritis. *Arthritis Rheum.* **35,** 1281–1288.

Chomarat, P., Kjeldsen-Kragh, J., Quayle, A. J., Natvig, J. B., and Miossec, P. (1994). Different cytokine production profiles of gamma delta T cell clones: Relation to inflammatory arthritis. *Eur. J. Immunol.* **24,** 2087–2091.

Christmas, S. E. (1992). Cytokine production by T lymphocytes bearing the gamma-delta T cell antigen receptor. *Chem. Immunol.* **53,** 32–46.

Chruch, M. K., el-Lati, S., and Caulfield, J. P. (1991). Neuropeptide-induced secretion from human skin mast cells. *Int. Arch. Allergy Appl. Immunol.* **94,** 310–318.

Claassen, J. L., Levine, A. D., and Buckley, R. H. (1990a). Recombinant human IL-4 induces IgE and IgG synthesis by normal and atopic donor mononuclear cells. Similar dose response, time course, requirement for T cells, and effect of pokeweed mitogen. *J. Immunol.* **144,** 2123–2130.

Classen, J. L., Levine, A. D., Buckley, R. H. (1990b). A cell culture system that enhances mononuclear cell IgE synthesis induced by recombinant human interleukin-4. *J. Immunol. Methods* **126,** 213–222.

Claman, H. N., Chaperon, E. A., and Triplett, R. F. (1966). Thymus-marrow cell combinations. Synergism in antibody production. *Proc. Soc. Exp. Biol. Med.* **122,** 1167–1171.

Clerici, M., and Shearer, G. M. (1993). A Th1-Th2 switch is a critical step in the etiology of HIV infection. *Immunol. Today* **14,** 107–111.

Clerici, M., Hakin, F. T., Venzon, D. J., Batt, S., Hendrix, C. W., Wynn, T. A., and Shearer, G. M. (1993). Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. *J. Clin. Invest.* **91,** 759–765.

Clerici, M., and Shearer, G. M. (1994). The Th1-Th2 hypothesis of HIV infection: New insights. *Immunol. Today* **15,** 575–581.

Coffman, R., and Carty, J. (1986). A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J. Immunol.* **136,** 949–954.

Coffman, R. L., Seymour, B. W. P., Hudak, S., Jackson, J., and Rennick, D. (1989). Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* **245,** 308–310.

Coffman, R. L., Lebman, D. A., and Rothman, P. (1993). Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* **54,** 229–270.

Cohan, V. L., MacGlashan, D. W. Jr., Warner, J. A., Lichtenstein, L. M., and Proud, D. (1991). Mechanisms of mediator release from human skin mast cells upon stimulation by the bradykinin analog, [DArg0-Hyp3-DPhe7] bradykinin. *Biochem. Pharmacol.* **41,** 293–300.

Croft, M., Carter, S., Swain, S., and Dutton, R. W. (1994). Generation of polarized antigen-specific CD8 effector populations: Reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J. Exp. Med.* **180,** 1715–1728.

Cumberbatch, M., Gould, S. J., Peters, S. W., Basketter, D. A., Dearman, R. J., and Kimber, I. (1992). Langerhans cells, antigen presentation, and the diversity of responses to chemical allergens. *J. Invest. Dermatol.* **99,** 107S-108S.

Damonneville, M., Monte, D., Auriault, C., and Capron, A. (1990). The neuropeptide substance P stimulates the effector functions of platelets. *Clin. Exp. Immunol.* **81,** 346–351.

D'Andrea, A. M., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., Wolf, S. F., and Trinchieri, G. (1992). Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* **176,** 1387–1398.

D'Andrea, A. D., Aste-Amezaga, M., Valainte, N. M., Ma, X., Kubin, M., and Trinchieri, G. (1993). Interleukin-10 inhibits lymphocyte IFN-γ-production by suppressing natural killer cell stimulatory factor/interleukin-12 synthesis in accessory cells. *J. Exp. Med.* **178,** 1041–1048.

Daynes, R. A., and Araneo, B. A. (1989). Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin-2 and interleukin-4. *Eur. J. Immunol.* **19,** 2319–2325.

Daynes, R. A., Araneo, B. A., Dowell, T. A., Huang, K., and Dudley, D. (1990a). Regulation of murine lymphokine production in vivo. III. The lymphoid tissue microenvironment exerts regulatory influences over helper T-cell function. *J. Exp. Med.* **171,** 979–996.

Daynes, R. A., Dudley, D. J., and Araneo, B. A. (1990b). Regulation of murine lymphokine production in vivo. II. Dehydroepiandrosterone is a natural enhancer of interleukin-2 synthesis by helper T cells. *Eur. J. Immunol.* **20,** 793–802.

Daynes, R. A., Dowell, T., and Araneo, B. A. (1991a). Platelet-derived growth factor is a potent biologic response modifier of T cells. *J. Exp. Med.* **174,** 1323–1333.

Daynes, R. A., Meike, A. W., and Araneo, B. A. (1991b). Locally active steroid hormones may facilitate compartementalization of immunity by regulating the types of lymphokines produced by helper T cells. *Res. Immunol.* **142,** 40–44.

Daynes, R. A., and Araneo, B. A. (1992). Programming of lymphocyte response to activation, Extrinsic factors, provided microenvironmentally, confer flexibility and compartimentalization to T-cell function. *Chem. Immunol.* **54,** 1–20.

DeKruyff, R. H., Turner, T., Abrams, J. S., Palladino, M. A., and Umetsu, D. T. (1989). Induction of human IgE synthesis by CD4+ T cell clones. Requirement for interleukin 4 and low molecular weight B cell growth factor. *J. Exp. Med.* **170,** 1477–1493.

DeKruyff, R. H., Fang, Y., Wolf, S. F., and Umetsu, D. T. (1995). IL-12 inhibits IL-4 synthesis in keyhole limpet hemocyanin-primed CD4+ T cells through an effect on antigen-presenting cells. *J. Immunol.* **154,** 2578–2587.

Delespesse, G., Sarfati, M., and Heusser, C. (1989). IgE synthesis. *Curr. Op. Immunol.* **2,** 506–512.

de Vries, J. E., de Waal Malefyt, R., Yssel, H., Roncarolo, M. G., and Spits, H. (1991). Do human TH1 and TH2 CD4+ clones exist? CD4+ T-cell subsets, differentiation and function. *Res. Immunol.* **142,** 59–63.

de Vries, J. E., Punnonen, J., Cocks, B. G., de Waal Malefyt, R., and Aversa, G. (1993). Regulation of the human IgE response by IL-4 and IL-13. *Res. Immunol.* **144,** 597–601.

de Waal Malefyt, R., Haanen, J., Yssel, H., Roncarolo, M.-G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Spits, H., and de Vries, J. E. (1991). IL-10 and v-IL-10 strongly reduce antigen specific human T cell responses by diminishing the antigen presenting capacity of monocytes via down-regulation of class I MHC expression. *J. Exp. Med.* **174,** 915–924.

de Waal Malefyt, R., Yssel, H., and de Vries, J. E. (1993). Direct effects of IL-10 on subsets of human CD4+ T cell clones and resting T cells. Specific inhibition of IL-2 production and proliferation. *J. Immunol.* **150,** 4754–4765.

De Wied, D., and Jolles, J. (1982). Neuropeptides derived from proopiomelanocortin: Behavioral, physiological and neurochemical effects. *Physiol. Rev.* **62,** 976–1059.

Diaz-Sanchez, D., and Kemeny, D. M. (1990). The sensitivity of rat CD8+ and CD4+ T cells to ricin in vivo and in vitro and their relationship to IgE regulation. *Immunology* **69,** 71–77.

Diaz-Sanchez, D., and Kemeny, D. M. (1991). Generation of a long-lived IgE response in high and low responder strains of rat by co-administration of ricin and antigen. *Immunology* **72,** 297–303.

Diaz-Sanchez, D., Chegini, s., Zhang, K., and Saxon, A. (1994). CD58 (LFA-3) stimulation provides a signal for human isotype switching and IgE production distinct from CD40. *J. Immunol.* **153,** 10–20.

Dinarello, C. A. (1988). Biology of interleukin 1. *FASEB J.* **2,** 108–115.

Dinarello, C. A., and Wolf. (1993). The role of interleukin-1 in disease. *N. Engl. J. Med.* **328,** 106–113.

Ding, L., Linsley, P. S., Huang, L. Y., Germain, R. N., and Shevatch, E. M. (1993). IL-12 inhibits macrophage co-stimulatory activity by selectively inhibiting the upregulation of B7 expression. *J. Immunol.* **151,** 1224–1234.

Djeu, J. Y., Liu, J. H., Wei, S., Rui, H., Pearson, C. A., Leonard, W. J., and Blanchard, D. K. (1993). Function associated with IL-2 receptor-beta on human neutrophils. Mechanism of activation of antifungal activity against Candida albicans by IL-2. *J. Immunol.* **150,** 960–970.

Dugas, B., Renauld, J. C., Pene, J., Bonnefoy, J. Y., Petit-Frère, C., Braquet, P., Bousquet, J., Van Snick, J., and Mencia-Huerta, J. M. (1993). Interleukin-9 potentiates the interleukin-4 induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. *Eur. J. Immunol.* **23,** 1687–1692.

Duncan, D. D., and Swain, S. L. (1994). Role of antigen-presenting cells in the polarized development of helper T cell subsets, evidence for differential cytokine production by Th0 cells in response to antigen presentation by B cells and macrophages. *Eur. J. Immunol.* **24,** 2506–2514.

Duvaux-Miret, O., Stefano, G. B., Smith, E. M., Dissous, C., and Capron, A. (1992). Immunosuppression in the definitive and intermediate hosts of the human parasite Schistosoma mansoni by release of immunoactive neuropeptides. *Proc. Natl. Acad. Sci. USA* **89,** 778–781.

Ebner, C., Szepfalusi, Z., Ferreira, F., Jilek, A., Valenta, R., Parronchi, P., Maggi, E., Romagnani, S., Scheiner, O., and Kraft, D. (1993). Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J. Immunol.* **150,** 1047–1054.

Eitan, S., and Schwartz, M. (1993). A transglutaminase that converts interleukin-2 into a factor cytotoxic to oligodendrocytes. *Science* **261,** 106–108.

Eitan, S., Solomon, A., Lavie, V., Yoles, E., Hirschberg, D. L., Belkin, M., and Schwartz, M. (1994). Recovery of visual response of injured adult rat optic nerves treated with transglutaminase. *Science* **264,** 1764–1768.

Enk, A. H., Angeloni, V. L., Udey, M. C., and Katz, S. I. (1993). Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. *J. Immunol.* **151,** 2390–2398.

Erhard, F., Wild, M. T., Garcia-Sanz, J. A., and le Gros, G. (1993). Switch of CD8 T cells to noncytolytic CD8-CD4 cells that make Th2 cytokines and help B cells. *Science* **260,** 1802–1805.

Espinoza-Delgado, I., Bosco, M. C., Musso, T., Mood, K., Ruscetti, F. W., Longo, D. L., and Varesio, L. (1994). Inhibitory cytokine circuits involving transforming growth factor-beta, interferon-gamma, and interleukin-2 in human monocyte activation. *Blood* **83,** 3332–3338.

Evavold, B. D., and Allen, P. M. (1991). Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* **252,** 1308–1310.

Evavold, B. D., Williams, S. G., Hsu, B. L., Buus, S., and Allen, P. M. (1992). Complete dissection of the Hb(64-76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. *J. Immunol.* **148,** 347–353.

Fanslow, W. C., Anderson, D. M., Grabstein, K. H., Clark, E. A., Cosman, D., and Armitage, R. J. (1992). Soluble forms of CD40 inhibit biologic responses of human B cells. *J. Immunol.* **149,** 655–660.

Felten, S. Y., Felten, D. L., Bellinger, D. L., and Olschowka, J. A. (1992). Noradrenergic and peptidergic innervation of lymphoid organs. *Chem. Immunol.* **52,** 25–48.

Ferrick, D. A., Schrenzel, M. D., Mulvania, T., Hsieh, B., Ferlin, W. G., and Lepper, H. (1995). Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* **373,** 255–257.

Fewtrell, C. M., Foreman, J. C., Jordan, C. C., Oehme, P., Renner, H., and Stewart, J. M. (1982). The effects of substance P on histamine and 5-hydroxy-tryptamine release in the rat. *J. Physiol.* **330,** 393–411.

Finkelman, F. D., Katona, I. M., Urban, J. F., Jr., Holmes, J., and Ohara, J. (1988). IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* **141,** 2335–2341.

Finkelman, F. D., Holmes, J., Katona, I. M., Urban, J. F., Beckmann, M. P., Park, L. S., Schooley, K. A., Coffman, R. L., Mosmann, T. R., and Paul, W. E. (1990). Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* **8,** 303–333.

Finkelman, F. D., Pearce, E. J., Urban, J. F., and Sher, A. (1991). Regulation and biological function of helminth-induced cytokine responses. *Immunoparasitol. Today* **12,** A62–A66.

Fiorentino, D. F., Bond, M. W., and Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170,** 2081–2095.

Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W., and O'Garra, A. (1991a). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* **146,** 3444–3451.

Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. H., Howard, M., and O'Gara, A. (1991b). IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147,** 3815–3922.

Firestein, G. S., Roeder, W. D., Laxer, J. A., *et al.* (1989). A new murine CD4+ T cell subset with an unrestricted cytokine profile. *J. Immunol.* **143,** 518–525.

Fong, T. A., and Mosmann, T. R. (1990). Alloreactive murine CD8+ T cell clones secrete the Th1 pattern of cytokines. *J. Immunol.* **144,** 1744–1752.

Fourcade, C., Arock, M., Ktorza, S., Ouaaz, F., Merle-Beral, H., Mentz, F., Kilchherr, E., Debre, P., and Mossalayi, M. D. (1992). Expression of CD23 by human bone marrow stromal cells. *Eur. Cytokine Netw.* **3,** 539–543.

Foy, T. M., Shepherd, D. M., Durie, F. H., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J. Exp. Med.* **178,** 1567–1575.

Fraser, E. M., Christie, J. F., and Kennedy, M. W. (1993). Heterogeneity amongst infected children in IgE antibody repertoire to the antigens of the parasitic nematode Ascaris. *Int. Arch. Allergy Immunol.* **100,** 283–286.

Frick, O. L., German, D. F., and Mills, J. (1979). Development of allergy in children: Association with virus infections. *J. Allergy Clin. Immunol.* 1979. **63,** 228–241.

Fuleihan, R., Ramesh, N., and Geha, R. S. (1993). Role of CD40–CD40-ligand interaction in Ig-isotype switching. *Curr. Op. Immunol.* **5,** 963–967.

Gagro, A., and Rabatic, S. (1994). Allergen-induced CD23 on CD4+ T lymphocytes and CD21 on B lymphocytes in patients with allergic asthma, evidence and regulation. *Eur. J. Immunol.* **24,** 1109–1114.

Gajewski, T. F., and Fitch, F. W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* **140,** 4245–4252.

Gajewski, T. F., Pinnas, M. M., Wong, T., and Fitch, F. W. (1991). Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J. Immunol.* **146,** 1750–1758.

Gala, R. R. (1991). Prolactin and growth hormone in the regulation of the immune system. *Proc. Soc. Exp. Biol. Med.* **198,** 513–527.

Garrone, P., Galibert, L., Rousset, F., Fu, S. M., and Banchereau, J. (1994). Regulatory effects of prostaglandin E2 on the growth and differentiation of human B lymphocytes activated through their CD40 antigen. *J. Immunol.* **152,** 4282–4290.

Gauchat, D., Gauchat, J.-F., Bettens, F., de Weck, A. L., and Stadler, B. M. (1990). Cytokine gene expression in atopics: Effect of IL-4 on IL-1β and IL-6 mRNA levels. *Eur. Cytokine Net.* **1,** 85–90.

Gauchat, J.-F., Walker, C., de Weck, A. L., and Stadler, B. M. (1988). Stimulation-dependent lymphokine mRNA levels in human mononuclear cells. *Eur. J. Immunol.* **18,** 1441–1446.

Gauchat, J.-F., Gauchat, D., de Weck, A. L., and Stadler, B. M. (1989). Cytokine mRNA levels in antigen-stimulated peripheral blood mononuclear cells. *Eur. J. Immunol.* **19,** 1079–1085.

Gauchat, J.-F., Gauchat, D., Gang, Q., Mandallaz, M., and Stadler, B. M. (1991). Detection of cytokine mRNA in polyclonally-, antigen- or allergen-stimulated mononuclear cells. *Immunol. Rev.* **119,** 147–161.

Gauchat, J.-F., Aversa, G., Gascan, H., and de Vries, J. E. (1992a). Modulation of IL-4 induced germline epsilon RNA synthesis in human B cells by tumor necrosis factor-alpha, anti-CD40 monoclonal antibodies or transforming growth factor-beta correlates with levels of IgE production. *Int. Immunol.* **4,** 397–406.

Gauchat, J.-F., Gascan, H., de Waal Malefyt, R., and de Vries, J. E. (1992b). Regulation of germ-line epsilon transcription and induction of epsilon switching in cloned EBV-transformed and malignant human B cell lines by cytokines and CD4+ T cells. *J. Immunol.* **148,** 2291–2299.

Gauchat, J.-F., Henchoz, S., Mazzei, G., Aubry, J.-P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J., Kishi, K., Butterfield, J., Dahinden, C., and Bonnefoy, J.-Y. (1993). Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* **365,** 340–343.

Gauchat, J.-F., Henchoz, S., Fattah, D., Mazzei, G., Aubry, J.-P., Jomotte, T., Dash, L., Page, K., Solari, R., Aldebert, D., Capron, M., Dahinden, C., and Bonnefoy, J.-Y. (1995). CD40 ligand is functionally expressed on human eosinophils. *Eur. J. Immunol.* **25,** 863–865.

Gieni, R. S., Yang, X., and Hayglass, K. T. (1993). Allergen-specific modulation of cytokine synthesis patterns and IgE responses in vivo with chemically modified allergen. *J. Immunol.* **150,** 302–310.

Gilman, S. C., Schwartz, J. M., Milner, R. J., Bloom, F. E., and Feldman, J. D. (1982). β-Endorphin enhances lymphocyte proliferative responses. *Proc. Natl. Acad. Sci. USA* **79,** 4226–4230.

Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994). Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* **13,** 2822–2830.

Glasebrook, A. L., and Fitch, F. W. (1980). Alloreactive cloned T cell lines. I. Interactions between cloned amplifier and cytolytic T cell lines. *J. Exp. Med.* **151,** 876–895.

Goetzl, E. J. (1980). Mediators of immediate hypersensitivity derived from arachidonic acid. *N. Engl. J. Med.* **303,** 822–825.

Goldstein, s., Kaempfer, M., Proud, C. E., Schwartz, L. B., Irani, A. A., and Wintroub, B. U. (1987). Detection and characterization of a human mast cell carboxypeptidase. *J. Immunol.* **139,** 2724–2729.

Gollob, K. J., Nagelkerken, L., and Coffman, R. L. (1993). Endogenous retroviral superantigen presentation by B cells induces the development of type 1 CD4+ T helper lymphocytes. *Eur. J. Immunol.* **23,** 2565–2571.

Goodman, R. E., Nestle, F., Naidu, Y. M., Green, J. M., Thompson, C. B., Nickoloff, B. J., and Turka, L. A. (1994). Keratinocyte-derived T cell costimulation induces preferential production of IL-2 and IL-4 but not IFN-gamma. *J. Immunol.* **152,** 5189–5198.

Gorczynski, R. M., Macrae, A. S., and Kennedy, M. (1982). Conditioned immune response associated with allogeneic skin grafts in mice. *J. Immunol.* **129,** 704–709.

Gordon, G. B., Shantz, L. M., and Talahy, P. (1986). Modulation of growth differentiation and carcinogenesis by dehydroepiandrosterone. *Adv. Enzyme Reg.* **26,** 355–382.

Grabstein, K. H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M. A., Ahdieh, M., Johnson, L., Alderson, M. R., Watson, J. D., Anderson, D. M., and Giri, J. G. (1994). Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* **264,** 965–968.

Grogg, D., Hahn, S., and Erb, P. (1992). CD4+ T cell-mediated killing of major histocompatibility complex class II-positive antigen-presenting cells (APC). III. CD4+ cytotoxic T cells induce apoptosis of APC. *Eur. J. Immunol.* **22,** 267–272.

Grun, J. L., and Maurer, P. H. (1989). Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles, the role of endogenous interleukin 1 in proliferative responses. *Cell. Immunol.* **121,** 134–145.

Gusella, G. L., Musso, T., Bosco, M. C., Espinoza-Delgado, I., Matsushima, K., and Varesio, L. (1993). IL-2 up-regulates but IFN-gamma suppresses IL-8 expression in human monocytes. *J. Immunol.* **151,** 2725–2732.

Haanen, J. B. G., de Waal Malefyt, R., Res, P. C. M. Kraakman, E. M., Ottenhof, T. H. M., De Vries, J. E., and Spits, H. (1991). Selection of a human Th1-like T cell subset by mycobacteria. *J. Exp. Med.* **174,** 583–592.

Hagel, I., Lynch, N. R., Perez, M., Di Prisco, M. C., Lopez, R., and Rojas, E. (1993). Modulation of the allergic reactivity of slum children by helminthic infection. *Parasite Immunol.* **15,** 311–315.

Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A. J., Corrigan, C. J., Bradley, B., Durham, S. R., Collins, J. V., Jeffery, P. K., Quint, D. J., and Kay, A. B. (1991). Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* **87,** 1541–1546.

Haning, R. V. Jr., Hackett, R. J., Boothroid, R. I., and Canick, J. A. (1990). Steroid sulphatase activity in the human ovarian corpus luteum, stroma, and follicle: Comparison to activity in other tissues and the placenta. *J. Steroid Biochem.* **36,** 175–179.

Hanisch, U. K., Seto, D., and Quirion, R. (1993). Modulation of hippocampal acetylcholine release: A potent central action of interleukin-2. *J. Neurosci.* **13,** 3368–3374.

Harbuz, M. S., Chalmares, J., De Souza, L., and Lightman, S. L. (1993). Stress-induced activation of CRF and c-fos mRNAs in the paraventricular nucleus are not affected by serotonin depletion. *Brain Res.* **609,** 167–173.

Hauser, C. (1992). The interaction between Langerhans cells and CD4+ T cells. *J. Dermatol.* **19,** 722–725.

Haydik, I. B., and Ma, W. S. (1988). Basophil histamine release. Assays and interpretation. *Clin. Rev. Allergy* **6,** 141–162.

Hayglass, K. T., Gieni, R. S., and Stefura, W. P. (1991). Long-lived reciprocal regulation of antigen-specific IgE and IgG2a responses in mice treated with glutaraldehyde-polymerized ovalbumin. *Immunology* **73,** 407–414.

Heagy, W., Laurance, M., Cohen, E., and Finberg, R. (1990). Neurohormones regulate T cell function. *J. Exp. Med.* **171,** 1625–1633.

Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L., and Locksley, R. M. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or

progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* **169,** 59–72.

Heinzel, F. P., Schoenhaut, D. S., Rerko, R. M., Rosser, L. E., and Gately, M. K. (1993). Recombinant interleukin 12 cures mice infected with Leishmania major. *J. Exp. Med.* **177,** 1505–1509.

Helm, B., Marsh, P., Vercelli, D., Padlan, E., Gould, H., and Geha, R. (1988). The mast cell binding site on human immunoglobulin E. *Nature* **331,** 180–183.

Henchoz, S., Gauchat, J. F., Aubry, J. P., Graber, P., Pochon, S., and Bonnefoy, J. Y. (1994). Stimulation of human IgE production by a subset of anti-CD21 monoclonal antibodies, requirement of a co-signal to modulate epsilon transcripts. *Immunology* **81,** 285–290.

Hennebold, J. D., and Daynes, R. A. (1994). Regulation of macrophage dehydroepiandrosterone sulfate metabolism by inflammatory cytokines. *Endocrinology* **135,** 67–75.

Henney, C. S., Kuribayashi, K., Kern, D. E., and Gillis, S. (1981). Interleukin-2 augments natural killer cell activity. *Nature* **291,** 335–338.

Herbelin, A., Elhadad, S., Ouaaz, F., de Groote, D., and Descamps-Latscha, B. (1994). Soluble CD23 potentiates interleukin-1-induced secretion of interleukin-6 and interleukin-1 receptor antagonist by human monocytes. *Eur. J. Immunol.* **24,** 1869–1873.

Herrod, H. G. (1989). Interleukins in immunologic and allergic diseases. *Ann. Allergy* **63,** 269–272.

Herrscher, R. F., Kasper, C., and Sullivan, T. J. (1992). Endogenous cortisol regulates immunoglobulin E-dependent late phase reactions. *J. Clin. Invest.* **90,** 596–603.

Hide, M., Francis, D. M., Grattan, C. E., Hakimi, J., Kochan, J. P., and Greaves, M. W. (1993). Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N. Engl. J. Med.* **328,** 1599–1604.

Hirai, K., Morita, Y., Misaki, Y., Ohta, K., Takaishi, T., Suzuki, S., Motoyoshi, K., and Miyamoto, T. (1988). Modulation of human basophil histamine release by hemopoietic growth factors. *J. Immunol.* **141,** 3958–3964.

Horner, A. A., Jabara, H., Ramesh, N., and Geha, R. S. (1995). gamma/delta T lymphocytes express CD40 ligand and induce isotype switching in B lymphocytes. *J. Exp. Med.* **181,** 1239–1244.

Hornquist, E., and Lycke, N. (1993). Cholera toxin adjuvant greatly promotes antigen priming of T cells. *Eur. J. Immunol.* **23,** 2136–2143.

Hsieh, C. S., Heimberger, A. B., Gold, J. S., O'Garra, A., and Murphy, K. M. (1992). Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* **89,** 6065–6069.

Hu, Y., Dietrich, H., Herold, M., Heinrich, P. C., and Wick, G. (1993). Disturbed immunoendocrine communication via the hypothalamo-pituitary-adrenal axis in autoimmune disease. *Int. Arch. Allergy Immunol.* **102,** 232–241.

Immune-neuroendocrine special issue (1994). *Immunol. Today* **15,** 503–552.

Ishizaka, K., Ishizaka, T., and Hornbrook, M. M. (1966). Physiochemical properties of reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J. Immunol.* **97,** 75–85.

Ishizaka, K. (1976). Cellular events in the IgE antibody response. *Adv. Immunol.* **231,** 1–75.

Ishizaki, T., Koizumi, K., Ikemori, R., Ishiyama, Y., and Kushibiki, E. (1987). Studies of prevalence of Japanese ceder pollinosis among the residents in a densely cultivated area. *Ann. Allergy* **58,** 265–270.

Jabara, H. H., Fu, S. M., Geha, R. S., and Vercelli, D. (1990). CD40 and IgE, synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* **172,** 1861–1864.

Jabara, H. H., Ahern, D. J., Vercelli, D., and Geha, R. S. (1991). Hydrocortisone and IL-4 induce IgE isotype switching in human B cells. *J. Immunol.* **147,** 1557–1560.

Jabara, H. H., Loh, R., Ramesh, N., Vercelli, D., and Geha, R. S. (1993). Sequential switching from mu to epsilon via gamma 4 in human B cells stimulated with IL-4 and hydrocortisone. *J. Immunol.* **151,** 4528–4533.

Jabara, H. H., and Vercelli, D. (1994). Engagement of CD14 on monocytes inhibits the synthesis of human Igs, including IgE. *J. Immunol.* **153,** 972–978.

Jayaraman, S., Luo, Y., and Dorf, M. E. (1992). Tolerance induction in T helper (Th1) cells by thymic macrophages. *J. Immunol.* **148,** 2672–2681.

Johansson, S. G. O. (1967). Raised levels of a new immunoglobulin class (IgND) in asthma. *Lancet* **I,** 951.

Johansson, S. G. O., Mellbin, T., and Vahlquist, B. (1968). Immunoglobulin levels in Ethiopian preschool children with special reference to high concentrations of immunoglobulin E (IgND). *Lancet* **i,** 1118–1121.

Johansson, S. G. O. (1983). The clinical significance of IgE. Clinical Immunology update. *In* "Reviews for Physicians" (E. C. Franklin, Ed.), p. 123. Churchill Livingstone, Robert Stevenson House, Edinburgh.

Johnson, H. M., Smith, E. M., Torres, B. A., and Blalock, J. E. (1992). Neuroendocrine peptide hormone regulation of immunity. *Chem. Immunol.* **52,** 49–83.

Johnson, L. A., Moon, K. E., and Eisenberg, M. (1986). *Anal. Biochem.* **155,** 358–364.

Jones, R. E., Finkelman, F. D., Hester, R. B., and Kayes, S. G. (1994). Toxocara canis, failure to find IgE receptors (Fc epsilon R) on eosinophils from infected mice suggests that murine eosinophils do not kill helminth larvae by an IgE-dependent mechanism. *Exp. Parasitol.* **78,** 64–75.

Jouault, T., Capron, M., Balloul, J. M., Ameisen, J. C., and Capron, A. (1988). Quantitative and qualitative analysis of the Fc receptor for IgE (Fc epsilon RII) on human eosinophils. *Eur. J. Immunol.* **18,** 237–241.

Justesen, D. R., Braun, E. W., Garrison, R. G., and Pendleton, R. B. (1970). Pharmacological differentiation of allergic and classically conditioned asthma in the guinea pig. *Science* **170,** 864–866.

Kaiserlian, D., Lachaux, A., Grosjean, I., Graber, P., and Bonnefoy, J. Y. (1993). Intestinal epithelial cells express the CD23/Fc epsilon RII molecule, enhanced expression in enteropathies. *Immunology* **80,** 90–95.

Kaminer, M. S., Lavker, R. M., Walsh, L. J., Whitaker, D., Zweiman, B., and Murphy, G. F. (1991). Extracellular localization of human connective tissue mast cell granule contents. *J. Invest. Dermatol.* **96,** 857–863.

Kapsenberg, M. L., Jansen, H. M., Bos, J. D., and Wierenga, E. A. (1992). Role of type 1 and type 2 T helper cells in allergic diseases. *Curr. Op. Immunol.* **4,** 788–793.

Karp, C. L., el-Safi, S. H., Wynn, T. A., Satti, M. M., Kordofani, A. M., Hashim, F. A., Hag-Ali, M., Neva, F. A., Nutman, T. B., and Sacks, D. L. (1993). In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma. *J. Clin. Invest.* **91,** 1644–1648.

Karp, J. D., Cohen, N., and Moynihan, J. A. (1994). Quantitative differences in interleukin-2 and interleukin-4 production by antigen-stimulated splenocytes from individually- and group-housed mice. *Life Sci.* **55,** 789–795.

Katira, A., Knox, K. A., Finney, M., Mitchell, R. H., Wakelam, M., and Gordon, J. (1993). Inhibition by glucocorticoid and staurosporine of IL-4-dependent CD23 production in B lymphocytes is reversed on engaging CD40. *Clin. Exp. Immunol.* **92,** 347–352.

Kay, A. B. (1991). T lymphocytes and their products in atopic allergy and asthma. *Int. Arch. Allergy Appl. Immunol.* **94,** 189–193.

Kemeny, D. M., and Diaz-Sanchez, D. (1993). The role of CD8+ T cells in the regulation of IgE. *Clin. Exp. Allergy* **23,** 466–470.

Kemeny, D. M., Noble, A., Holmes, B. J., and Diaz-Sanchez, D. (1994). Immune regulation: A new role for the CD8+ T cell. *Immunol. Today* **15,** 107–110.

Kennedy, M. W., Tomlinson, L. A., Fraser, E. M., and Christie, J. F. (1990). The specificity of the antibody response to internal antigens of Ascaris: Heterogeneity in infected humans, and MHC (H-2) control of the repertoire in mice. *Clin. Exp. Immunol.* **80,** 219–224.

Kimata, H., Yoshida, A., Ishioka, C., and Mikawa, H. (1992a). Differential effect of vasoactive intestinal peptide, somatostatin, and substance P on human IgE and IgG subclass production. *Cell. Immunol.* **144,** 429–442.

Kimata, H., Yoshida, A., Ishioka, C., Lindley, I., and Mikawa, H. (1992b). Interleukin 8 (IL-8) selectively inhibits immunoglobulin E production induced by IL-4 in human B cells. *J. Exp. Med.* **176,** 1227–1231.

Kimata, H., Lindley, I., and Furusho, K. (1995). Effect of hydrocortisone on spontaneous IgE and IgG_4 production in atopic patients. *J. Immunol.* **154,** 3557–3566.

Kinet, J. P. (1990). The high affinity receptor for immunoglobulin E. *Curr. Op. Immunol.* **2,** 499–505.

King, C. L., and Nutman, T. B. (1993). IgE and IgG subclass regulation by IL-4 and IFN-gamma in human helminth infections. Assessment by B cell precursor frequencies. *J. Immunol.* **151,** 458–465.

Kiniwa, M., Gately, M., Gubler, U., Chizzonite, R., Fargeas, C., and Delespesse, G. (1992). Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J. Clin. Invest.* **90,** 262–266.

Knoller, I., Bujanowski-Weber, J., Brings, B., and König, W. (1989). Influence of IL-2 and IL-4 on the IgE synthesis and the IgE-binding factor (sCD23) production by human lymphocytes in vitro. *Immunology* **66,** 368–375.

Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S., and Sugamura, K. (1994). Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science* **263,** 1453–1454.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. L., Bluethmann, H., and Köhler, G. (1993). Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* **362,** 245–248.

Korthauer, U., Graf, D., Mages, H. W., Briere, F., Padayachee, M., Malcolm, S., Ugazio, A. G., Notarangelo, L. D., Levinsky, R. J., and Kroczek, R. A. (1993). Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* **361,** 539–541.

Kroczek, R. A., Graf, D., Brugnoni, D., Giliani, S., Korthuer, U., Ugazio, A., Senger, G., Mages, H. W., Villa, A., and Notarangelo, L. D. (1994). Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)." *Immunol. Rev.* **138,** 39–59.

Kubin, M., Kamoun, M., and Trinchieri, G. (1994). Interleukin-12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T-cells. *J. Exp. Med.* **180,** 211–222.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Müller, W. (1993). Interleukin-10 deficient mice develop chronic enterocolitis. *Cell* **75,** 263–274.

Kulkarni, A. B., and Karlsson, S. (1993). Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am. J. Pathol.* **143,** 3–9.

Kündig, T. M., Schorle, H., Bachmann, M. F., Hengartner, H., Zinkernagel, R. M., and Horak, I. (1993). Immune responses in interleukin-2-deficient mice. *Science* **262,** 1059–1061.

Kupper, T. S., Lee, F., Coleman, D., Chodakewitz, J., Flood, P., and Horowitz, M. (1988). Keratinocyte derived T-cell growth factor (KTGF) is identical to granulocyte macrophage colony stimulating factor (GM-CSF). *J. Invest. Dermatol.* **91,** 185–188.

Kusnecov, A. W., Husband, A. J., King, M. G., and Smith, R. (1989). Modulation of mitogen-induced spleen cell proliferation and the antibody-forming cell response by beta-endorphin in vivo. *Peptides* **10,** 473–479.

Kyburz, D., Aichel, P., Speiser, D. E., Hengartner, H., Zinkernagel, R. M., and Pircher, H. (1993). T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol.* **23,** 1956–1962.

Lecron, J. C., Morel, F., Tanzer, J., Gombert, J., and Goube de Laforest, P. (1991). Soluble CD23 displays T-cell growth enhancing activity. *Immunology* **74,** 561–563.

Lederman, S., Yellin, M. J., Cleary, A. M., Pernis, A., Inghirami, G., Cohn, L. E., Covey, L. R., Lee, J. J., Rothman, P., and Chess, L. (1994). T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death. *J. Immunol.* **152,** 2163–2171.

Le Gros, G., Ben-Sasson, S., Seder, R., Finkelman, F. D., and Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4 producing cells. *J. Exp. Med.* **172,** 921–929.

Le Gros, G., and Erhard, F. (1994). Non-cytotoxic, IL-4, IL-5, IL-10 producing CD8+ T cells: Their activation and effector functions. *Curr. Op. Immunol.* **6,** 453–457.

Lehrer, S. B., Barbandi, F., Taylor, J. P., and Salvaggio, J. E. (1984). Tobacco smoke "sensitivity" -is there an immunological basis? *J. Allergy Clin. Immunol.* **73,** 240–245.

Life, P., Gauchat, J. F., Schnuriger, V., Estoppey, S., Mazzei, G., Durandy, A., Fischer, A., and Bonnefoy, J.-Y. (1994). T cell clones from an X-linked hyper-immunoglobulin (IgM) patient induce IgE synthesis in vitro despite expression of nonfunctional CD40 ligand. *J. Exp. Med.* **180,** 1775–1784.

Life, P. Aubry, J. P., Estoppey, S., Schnuriger, V., and Bonnefoy, J. Y. (1995). CD28 functions as an adhesion molecule and is involved in the regulation of human IgE synthesis. *Eur. J. Immunol.* **25,** 333–339.

Limaye, A. P., Abrams, J. S., Silver, J. E., Ottesen, E. A., and Nutman, T. B. (1990). Regulation of parasite-induced eosinophilia: Selectively increased interleukin 5 production in helminth-infected patients. *J. Exp. Med.* **172,** 399–402.

Lipton, J. M., and Clark, W. G. (1986). Neurotransmitters in temperature control. *Annu. Rev. Physiol.* **48,** 613–623.

Liu, M. C., Proud, D., Lichtenstein, L. M., MacGlashan, D. W., Schleimer, R. P., Adkinson, N. F., Karey-Sobotka, A., Schulman, E. S., and Plant, M. (1986). Human lung macrophage derived histamine releasing activity is due to IgE-dependent factors. *J. Immunol.* **136,** 2588–2595.

Loh, R. K. S., Jabara, H. H., Ren, C. L., and Fu, S. M. (1995). Role of protein tyrosine kinases and phosphatases in isotype switching, crosslinking CD45 to CD40 inhibits IgE isotype switching in human B cells. *Immunol. Lett.* **45,** 99.

Louis, R. E., and Radermecker, M. F. (1990). Substance P-induced histamine release from human basophils, skin and lung fragments, effect of nedocromil sodium and theophylline. *Int. Arch. Allergy Appl. Immunol.* **92,** 329–333.

Louis, R. E., and Radermecker, M. F. (1991). Cutaneous and basophilic sensitivity to substance P and gastrin in non-atopic versus atopic subjects. *Allergy* **46,** 30–34.

Lowman, M. A., Rees, P. H., Benyon, R. C., and Church, M. K. (1988a). Human mast cell heterogeneity: Histamine release from mast cells dispersed from skin, lung, adenoids, tonsils and intestinal mucosa in response to IgE-dependent and non-immunological stimuli. *J. Allergy Clin. Immunol.* **81,** 590–597.

Lowman, M. A., Benyon, R. C., and Church, M. K. (1988b). Characterization of neuropeptide-induced histamine release from human dispersed skin mast cells. *Br. J. Pharmacol.* **95,** 121–130.

Lucey, D. R., Zajac, R. A., Melcher, G. P., Butzin, C. A., and Boswell, R. N. (1990). Serum IgE levels in 622 persons with human immunodeficiency virus infection, IgE elevation with marked depletion of CD4+ T-cells. *AIDS Res. Hum. Retroviruses* **6,** 427–429.

Luger, T. A., Stadler, B. M., Luger, B. J., Mathieson, M., Mage, J. A., Schmidt, J. A., and Oppenheim, J. J. (1982). Murine epidermal cell-derived thymocyte activating factor resembles murine IL-1. *J. Immunol.* **128,** 2147–2152.

Luger, T. A., Wirth, U., and Köck, A. (1985). Epidermal cells synthesize a cytokine with interleukin 3-like properties. *J. Immunol.* **134,** 915–919.

Luo, H. Y., Hofstetter, H., Banchereau, J., and Delespesse, G. (1991). Cross-linking of CD23 antigen by its natural ligand (IgE) or by anti-CD23 antibody prevents B lymphocyte proliferation and differentiation. *J. Immunol.* **146,** 2122–2129.

Macatonia, S. E., Hsieh, C. S., Murphy, K. M., and O'Garra, A. (1993). Dendritic cells and macrophages are required for Th1 development of CD4+ T cells from alpha beta TCR transgenic mice, IL-12 substitution for macrophages to stimulate IFN-gamma production is IFN-gamma-dependent. *Int. Immunol.* **5,** 1119–1128.

MacGlashan, D. Jr., White, J. M., Huang, S. K., Ono, S. J., Schroeder, J. T., and Lichtenstein, L. M. (1994a). Secretion of IL-4 from human basophils. The relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J. Immunol.* **152,** 3006–3016.

MacGlashan, D. W. Jr., Bochner, B. S., and Warner, J. A. (1994b). Graded changes in the response of individual human basophils to stimulation: Distributional behavior of early activation events. *J. Leukoc. Biol.* **55,** 13–23.

Machold, K. P., Carson, D. A., and Lotz, M. (1993). Transforming growth factor-beta (TGF beta) inhibition of Epstein-Barr virus (EBV)- and interleukin-4 (IL-4)-induced immunoglobulin production in human B lymphocytes. *J. Clin. Immunol.* **13,** 219–227.

Maekawa, N., Kawabe, T., Sugie, K., Kawakami, T., Iwata, S., Uchida, A., and Yodoi, J. (1992). Induction of Fc epsilon RII/CD23 on PHA-activated human peripheral blood T lymphocytes and the association of Fyn tyrosine kinase with Fc epsilon RII/CD23. *Res. Immunol.* **143,** 422–425.

Magee, D. M., and Wing, E. J. (1988). Cloned L3T4+ T lymphocytes protect mice against Listeria monocytogenes by secreting IFN-γ. *J. Immunol.* **141,** 3203–3207.

Maggi, E., Del Prete, G., Macchia, D., Parronchi, P., Tiri, A., Chretien, I., Ricci, M., Romagnani, S. (1988). Profiles of lymphokine activities and helper function for IgE in human T cell clones. *Eur. J. Immunol.* **18,** 1045–1050.

Maggi, E., Del Prete, G. F., Parronchi, P., Tiri, A., Macchia, D., Biswas, P., Simonelli, C., Ricci, M., and Romagnani, S. (1989). Role for T cells, IL-2 and IL-6 in the IL-4-dependent in vitro human IgE synthesis. *Immunology* **68,** 300–306.

Maggi, E., Biswas, P., Del Prete, G., Parronchi, P., Macchia, D., Simonelli, C., Emmi, L., De Carli, M., Tiri, A., Ricci, M., and Romagnani, S. (1991). Accumulation of TH2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. *J. Immunol.* **146,** 1169–1174.

Maggi, E., Parronchi, P., Manetti, R., Simonelli, C., Piccinni, M.-P., Rugiu, F. S., de Carli, M., Ricci, M., and Romagnani, S. (1992). Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol.* **148,** 2142–2147.

Maggi, E., Mazzetti, M., Ravina, A., Annunziato, F., de Carli, M., Piccinni, M. P., Manetti, R., Carbonari, M., Pesce, A. M., del Prete, G., and Romagnani, S. (1994). Ability of HIV

to promote a TH1 to TH0shift and to replicate preferentially in TH2 and TH0 cells. *Science* **265,** 244–248.

Magilavy, D. B., Fitch, F. W., and Gajewski, T. F. (1989). Murine hepatic accessory cells support the proliferation of Th1 but not Th2 helper T lymphocyte clones. *J. Exp. Med.* **170,** 985–990.

Malkovsky, M., Loveland, B., North, M., Asherson, G. L., Gao, L., Ward, P., and Fiers, W. (1987). Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. *Nature* **325,** 262–265.

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G., and Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* **177,** 1199–1204.

Mansfield, L. E., Ting, S., Haverly, R. W., and Yoo, T. J. (1985). The incidence and clinical implications of hypersensitivity to papain in an allergic population, confirmed by blinded oral challenge. *Ann. Allergy* **55,** 541–543.

Marcelletti, J. F., and Katz, D. H. (1992). Antigen concentration determines helper T cell subset participation in IgE antibody responses. *Cell. Immunol.* **143,** 405–419.

Marsh, D., Belin, L., Bruce, A., Lichtenstein, L., and Hussain, R. (1981). Rapidly released allergens from short ragweed pollen. I. Kinetics of release of known allergens in relation to biologic activity. *J. Allergy Clin. Immunol.* **67,** 206–216.

Marsh, D. G., Meyers, D. A., and Friedhoff, L. R. (1982). HLA-Dw2: A genetic marker for human immune response to short ragweed pollen allergen Ra5 II. Response after ragweed immunotherapy. *J. Exp. Med.* **155,** 1452–1463.

Matsue, H., Cruz, P. D., Bergstresser, P. R., and Takashima, A. (1993). Profiles of cytokine mRNA expressed by dendritic epidermal T cells in mice. *J. Invest. Dermatol.* **101,** 537–542.

McCormack, J. M., Moore, S. C., Gatewood, J. W., and Walker, W. S. (1992). Mouse splenic macrophage cell lines with different antigen-presenting activities for CD4+ helper T cell subsets and allogeneic CD8+ T cells. *Cell. Immunol.* **145,** 359–371.

McGovern, J. P., and Knight, J. A. (1967). Allergy and human emotions. Charles C. Thomas, Springfield, IL.

McKenzie, J. N. (1886). The production of the so-called "rose cold" by means of an artificial rose. *Am. J. Med. Sci.* **91,** 45–57.

McKenzie, A. N., Culpepper, J. A., de Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., and Menon, S. de Vries, J. E., Banchereau, J., and Zurawski, G. (1993). Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc. Natl. Acad. Sci. USA* **90,** 3735–3739.

McKerrow, J. H. (1988). Parasite proteases. *Exp. Parasitol.* **68,** 111–115.

McMenamin, C., and Holt, P. G. (1993). The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J. Exp. Med.* **178,** 889–899.

McQueen, G. M., Marshall, J., Perdue, M., Siegel, S., and Bienenstock, J. (1989). Pavlovian conditioning of rat mucosal mast cells to secrete rat mast cell protease II. *Science* **243,** 83–85.

Miescher, S., Vogel, M., Stämpfli, M. R., Wasserbauer, E., Kricek, F., Vorburger, S., and Stadler, B. M. (1994). Domain-specific anti-IgE antibodies interfere with IgE binding to Fc epsilon RII. *Int. Arch. Allergy Immunol.* **105,** 75–82.

Milewich, L., Shaw, C. B., and Sontheimer, R. D. (1988). Steroid metabolism by epidermal keratinocytes. *Ann. N.Y. Acad. Sci.* **548,** 66–89.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P., and Caput, D. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* **362,** 248–250.

Mitchell, G. F., and Miller, J. F. A. P. (1968). Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128,** 821–837.

Molfino, N. A., Wright, S. C., Katz, I., Tarlo, S., Silverman, F., McClean, P. A., Szalai, J. P., Raizenne, M., Slutzky, A. S., and Zamel, N. (1991). Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet* **338,** 199–203.

Molina, I. J., and Huber, B. T. (1991). Regulation of macrophage activation markers by IL-4 and IFN-gamma is subpopulation-specific. *Cell. Immunol.* **134,** 241–248.

Möller, C., and Elsayed, S. (1990). Seasonal variation of the conjunctival provocation test, total and specific IgE in children with birch pollen allergy. *Int. Arch. Allergy Appl. Immunol.* **92,** 306–308.

Morgan, D. A., Ruscetti, F. W., and Gallo, R. (1976). Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* **193,** 1007–1008.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348–2357.

Mosmann, T. R., and Coffman, R. L. (1989). Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* **46,** 111–147.

Moynihan, J. A., Schachtman, T. R., Grota, L. J., Cohen, N., and Ader, R. (1990). The effects of stress on the development of immunological memory following low dose antigen priming in mice. *Brain Behav. Immunol.* **4,** 1–12.

Mueller, R., Heusser, C. H., Rihs, S., Brunner, T., Bullock, G. R., and Dahinden, C. A. (1994). Immunolocalization of intracellular interleukin-4 in normal human peripheral blood basophils. *Eur. J. Immunol.* **24,** 2935–2940.

Munck, A., Guyre, P. M., and Holbrook, N. J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrinol. Rev.* **5,** 25–44.

Munn, N. A., and Lum, L. G. (1989). Immunoregulatory effects of alpha-endorphin, beta-endorphin, methionine-enkephalin, and adrenocorticotropic hormone on anti- tetanus toxoid antibody synthesis by human lymphocytes. *Clin. Immunol. Immunopathol.* **52,** 376–385.

Murphy, E. E., Terres, G, Macatonia, S. E., Hsieh, C. S., Mattson, J., Lanier, L., Wysocka, M., Trinchieri, G., Murphy, K., and O'Gara, A. (1994). B7 and interleukin-12 cooperate for proliferation and IFN-γ production by mouse Th1 clones that are unresponsive to B7 co-stimulation. *J. Exp. Med.* **180,** 223–231.

Myers, K. J., Sprent, J., Dougherty, J. P., and Ron, Y. (1992). Synergy between encephalitogenic T cells and myelin basic protein-specific antibodies in the induction of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **41,** 1–8.

Nakano, H., Ohno, H., and Saito, T. (1994). Activation of phospholipase C gamma 1 through transfected platelet-derived growth factor receptor enhances interleukin 2 production upon antigen stimulation in a T-cell line. *Mol. Cell. Biol.* **14,** 1213–1219.

Nanda, N. K., Sercarz, E. E., Hsu, D. H., and Kronenberg, M. (1994). A unique pattern of lymphokine synthesis is a characteristic of certain antigen-specific suppressor T cell clones. *Int. Immunol.* **6,** 731–737.

Nickerson, P., Steurer, W., Steiger, J., Zheng, X., Steele, A., and Strom, T. B. (1994). Cytokines and the Th1/Th2 paradigm in transplantation. *Curr. Op. Immunol.* **6,** 757–764.

Nickoloff, B. J., Fivenson, D. P., Kunkel, S. L., Strieter, R. M., and Turka, L. A. (1994). Keratinocyte interleukin-10 expression is upregulated in tape-stripped skin, poison ivy dermatitis, and Sezary syndrome, but not in psoriatic plaques. *Clin. Immunol. Immunopathol.* **73,** 63–68.

Niiro, H., Otsuka, T., Kuga, S., Nemoto, Y., Abe, M., Hara, N., Nakano, T., Ogo, T., and Niho, Y. (1994). IL-10 inhibits prostaglandin E2 production by lipopolysaccharide-stimulated monocytes. *Int. Immunol.* **6,** 661–664.

Nistico, G., and De Sarro, G. (1991). Is interleukin 2 a neuromodulator in the brain? *Trends Neurosci.* **14,** 146–150.

Nistico, G. (1993). Communications among central nervous system, neuroendocrine and immune systems: Interleukin-2. *Prog. Neurobiol.* **40,** 463–475.

Noelle, R. J., Roy, M., Shepherd, D. M., Stamenkovic, I., Ledbetter, J. A., and Aruffo, A. (1992). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA* **89,** 6550–6554.

Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., and Leonard, W. J. (1993). Interleukin-2 receptor gamma chain: A functional component of the interleukin-7 receptor. *Science* **262,** 1877–1880.

Nonoyama, S., Farrington, M. L., and Ochs, H. D. (1994). Effect of IL-2 on immunoglobulin production by anti-CD40-activated human B cells: Synergistic effect with IL-10 and antagonistic effect with IL-4. *Clin. Immunol. Immunopathol.* **72,** 373–379.

Novey, H. S., Marchioli, L. E., Sokol, W. N., and Wells, I. D. (1979). Papain-induced asthma, physiological and immunological features. *J. Allergy Clin. Immunol.* **63,** 98.

Nüsslein, H. G., Trag, T., Winter, M., Dietz, A., and Kalden, J. R. (1992). The role of T cells and the effect of hydrocortisone on interleukin-4-induced IgE synthesis by non-T cells. *Clin. Exp. Immunol.* **90,** 286–292.

Nüsslein, H. G., Dietz, A., Burger, R., Trag, T., Kalden, J. R., and Gramatzki, M. (1993). Chronic lymphocytic leukemia cells induce non-T cells to produce IgE in the presence of interleukin-4. *J. Clin. Immunol.* **13,** 397–405.

Nüsslein, H. G., Weber, G., and Kalden, J. R. (1994). Synthetic glucocorticoids potentiate IgE synthesis. Influence of steroid and nonsteroid hormones on human in vitro IgE secretion. *Allergy* **49,** 365–370.

Ochensberger, B., Rihs, S., Brunner, T., and Dahinden, C. IgE-independent IL-4 expression and induction of a late phase of leukotriene C4 formation in human blood basophils. *Blood* **86,** 4039–4049.

Oettgen, H. C., Martin, T. R., Wynshaw Boris, A., Deng, C., Drazen, J. M., and Leder, P. (1994). Active anaphylaxis in IgE-deficient mice. *Nature* **370,** 367–370.

O'Garra, A., and Murphy, K. (1994). Role of cytokines in determining T-lymphocyte function. *Curr. Op. Immunol.* **6,** 458–466.

Ogawa, M., McIntire, D. R., Ishizaka, K., Ishizaka, T., Terry, W. D., and Waldmann, T. A. (1971). Biological properties of E myeloma proteins. *Am. J. Med.* **51,** 193–199.

Okayama, Y., el-Lati, S. G., Leiferman, K. M., and Church, M. K. (1994). Eosinophil granule proteins inhibit substance P-induced histamine release from human skin mast cells. *J. Allergy Clin. Immunol.* **93,** 900–909.

Opp, M. R., Obal, F. Jr., and Krueger, J. M. (1988). Effects of alpha-MSH on sleep, behavior, and brain temperature: interactions with IL 1. *Am. J. Physiol.* **255,** 914–922.

Ottenberg, P., Stein, M., Lewis, J., and Hamilton, C. (1958). Learned asthma in the guinea big. *Psychosom. Med.* **20,** 395–401.

Paganelli, R., Scala, E., and Ansotegui, I. J., Ausiello, C. M., Halapi, E., Fanales-Belasio, E., D'Offizi, G., Mezzaroma, I., Pandolfi, F., Fiorilli, M., Cassone, A., and Aiuti, F. (1995). CD8+ T lymphocytes provide helper activity for IgE synthesis in human immunodeficiency virus-infected patients with hyper-IgE. *J. Exp. Med.* **181,** 423–428.

Paliard, X., de Waal Malefyt, R., Yssel, H., Blanchard, D., Chrétien, I., Abrams, J., De Vries, J. E., and Spits, H. (1988). Simultaneous production of IL-2, IL-4 and IFN-gamma by activated human CD4+ and CD8+ T-cell clones. *J. Immunol.* **141,** 849–855.

Parish, C. R. (1972). The relationship between humoral and cell-mediated immunity. *Transplant Rev.* **13,** 35–36.

Parronchi, P., Macchia, D., Piccini, M. P., Biswas, P., Simonelli, C., Maggi, E., Ricci, M., Ansari, A. A., and Romagnani, S. (1991). Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production. *Proc. Natl. Acad. Sci. USA* **88,** 4538–4542.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Piccinni, M. P., Macchia, D., Maggi, E., Del Prete, G. F., Ricci, M., and Romagnani, S. (1992). Aberrant interleukin (IL)-4 and IL-5 production in vitro by CD4+ helper T cells from atopic subjects. *Eur. J. Immunol.* **22,** 1615–1620.

Patterson, R., and Harris, K. E. (1993). Substance P and IgE-mediated allergy. I. Transient increase in airway responsiveness to allergen in primates. *Allergy Proc.* **14,** 45–51.

Pauwels, R., van der Straten, M., Platteay, B., and Bazin, H. (1983). The non-specific enhancement of allergy. I. In vivo effects of Bordetella pertussis vaccine on IgE synthesis. *Allergy* **38,** 239.

Pavlov, I. P. (1922). Lectures on Conditioned Reflexes (W. H. Gantt, Ed.). Liveright, New York.

Payan, D. G., and Goetzl, E. J. (1988). Neuropeptide regulation of immediate and delayed hypersensitivity. *Int. J. Neurosci.* **38,** 211–221.

Pene, J., Rousset, F., Briere, F., Chretien, I., Bonnefoy, J. Y., Spits, H., Yokota, T., Arai, N., Arai, K., Banchereau, J., and de Vries, J. E. (1988). IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc. Natl. Acad. Sci. USA* **85,** 6880–6884.

Pfeiffer, C., Murray, J., Madri, J., and Bottomly, K. (1991). Selective activation of Th1- and Th2-like cells in vivo response to human collagen IV. *Immunol. Rev.* **123,** 65–84.

Piccinni, M. P., Macchia, D., Parronchi, P., Giudizi, M.-G., Bani, D., Alterini, R., Grossi, A., Ricci, M., Maggi, E., and Romagnani, S. (1991). Human bone marrow non-B, non-T cells produce interleukin-4 in response to cross-linkage of Fce and Fcg receptors. *Proc. Natl. Acad. Sci. USA* **88,** 8656–8660.

Pinon, J. M., Toubas, D., Marx, C., Mougeot, G., Bonnin, A., Bonhomme, A., Villaume, M., Foudrinier, F., and Lepan, H. (1990). Detection of specific immunoglobulin E in patients with toxoplasmosis. *J. Clin. Microbiol.* **28,** 1739–1743.

Pober, J. S., and Cotran, R. S. (1990). Cytokines and endothelial cell biology. *Physiol. Rev.* **70,** 427–451.

Presta, L. G., Lahr, S. J., Shields, R. L., Porter, J. P., Gorman, C. M., Fendly, B. M., and Jardieu, P. M. (1993). Humanization of an antibody directed against IgE. *J. Immunol.* **151,** 2623–2632.

Prystowsky, M. B., Ely, J. M., Beller, D. I., Eisenberg, L., Goldman, J., Goldman, M., Goldwasser, E., Ihle, J., Quintans, J., Remold, H., Vogel, S., and Fitch, F. W. (1982). Alloreactive cloned T cell lines. VI. Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocytes. *J. Immunol.* **129,** 2337–2344.

Punnonen, J., Aversa, G. G., Vandekerckhove, B., Roncarolo, M. G., and de Vries, J. E. (1992). Induction of isotype switching and Ig production by CD5+ and CD10+ human fetal B cells. *J. Immunol.* **148,** 3398–3404.

Punnonen, J., de Waal Malefyt, R., van Vlasselaer, P., Gauchat, J. F., and de Vries, J. E. (1993a). IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. *J. Immunol.* **151,** 1280–1289.

Punnonen, J., Punnonen, K., Jansen, C. T., and Kalimo, K. (1993b). Interferon (IFN)-alpha, IFN-gamma, interleukin (IL)-2, and arachidonic acid metabolites modulate IL-4-induced IgE synthesis similarly in healthy persons and in atopic dermatitis patients. *Allergy* **48,** 189–195.

Punnonen, J., and de Vries, J. E. (1994). IL-13 induces proliferation, Ig isotype switching, and Ig synthesis by immature human fetal B cells. *J. Immunol.* **152,** 1094–1102.

Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J., and Waldmann, H. (1994). "Infectious" transplantation tolerance. *Science* **259,** 974–977.

Qiu, G., Gauchat, J.-F., Wirthmüller, U., de Weck, A. L., and Stadler, B. M. (1988). Lymphokine production by human peripheral blood lymphocytes. Analysis by in situ hybridization. *Lymphokine Res.* **7,** 385–392.

Qiu, G., Vogel, M., Gauchat, J.-F., Mandallaz, M., de Weck, A. L., and Stadler, B. M. (1990). Human IgE mRNA expression by peripheral blood lymphocytes stimulated with Interleukin 4 and pokeweed mitogen. *Eur. J. Immunol.* **20,** 2191–2199.

Racke, M. K., Burnett, D., Pak, S. H., Albert, P. S., Cannella, B., Raine, C. S., McFarlin, D. E., and Scot, D. E. (1995). Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. *J. Immunol.* **154,** 450–458.

Ramon, G. (1925). Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de sérum antidiphtérique. *Bull. Soc. Centr. Med. Vet.* **101,** 227.

Ramsdell, F., Seaman, M. S., Clifford, K. N., and Fanslow, W. C. (1994). CD40 ligand acts as a costimulatory signal for neonatal thymic gamma delta T cells. *J. Immunol.* **152,** 2190–2197.

Reichlin, S. (1993). Neuroendocrine-immune interactions. *N. Engl. J. Med.* **10,** 1246–1253.

Reiner, S. L., and Locksley, R. M. (1992). Lessons from Leishmania: A model for investigations of CD4+ subset differentiation. *Infect. Agents Dis.* **1,** 33–42.

Revoltella, R., and Ovary, Z. (1969). Reaginic antibody production in different mouse strains. *Immunology* **17,** 45–54.

Rieber, E. P., Rank, G., Kohler, I., and Krauss, S. (1993). Membrane expression of Fc epsilon RII/CD23 and release of soluble CD23 by follicular dendritic cells. *Adv. Exp. Med. Biol.* **329,** 393–398.

Ring, J., and Landthaler, M. (1989). Hyper-IgE syndromes. *Curr. Probl. Dermatol.* **18,** 79–88.

Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R., and Kay, A. B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* **326,** 298–304.

Rocken, M., Saurat, J. H., and Hauser, C. (1992). A common precursor for CD4+ T cells producing IL-2 or IL-4. *J. Immunol.* **148,** 1031–1036.

Romagnani, S. (1990). Regulation and deregulation of human IgE synthesis. *Immunol. Today* **11,** 316–321.

Romagnani, S. (1991). Human TH1 and TH2 subsets, doubt no more. *Immunol. Today* **12,** 256–257.

Romagnani, S. (1992a) Induction of TH1 and TH2 responses: A key role for the 'natural' immune response? *Immunol Today* **13,** 379–381.

Romagnani, S. (1992b). Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. *Int. Arch. Allergy Immunol.* **98,** 279–285.

Romagnani, S. (1994). Lymphokine production by human T cells in disease states. *Annu. Rev. Immunol.* **12,** 227–253.

Romani, L., Mencacci, A., Grohmann, M. S., Mosci, P., Puccetti, P., and Bistoni, F. (1992). CD4+ subset expression in murine candidiasis. Th responses correlate directly with genetically determined susceptibility or vaccine-induced resistance. *J. Immunol.* **150,** 925–931.

Ronchese, F., Hausmann, B., and Le Gros, G. (1994). Interferon-gamma- and interleukin-4-producing T cells can be primed on dendritic cells in vivo and do not require the presence of B cells. *Eur. J. Immunol.* **24,** 1148–1154.

Rook, G. A. W., Hernandez-Pando, R., and Lightman, S. L. (1994). Hormones, peripherally activated prohormones and regulation of the TH1/TH2 balance. *Immunol. Today* **15,** 301–303.

Rott, O., Cash, E., and Fleischer, B. (1993). Phosphodiesterase inhibitor pentoxifylline, a selective suppressor of T helper type 1— but not type 2—associated lymphokine production, prevents induction of experimental autoimmune encephalomyelitis in Lewis rats. *Eur. J. Immunol.* **23,** 1745–1751.

Rousset, F., Garcia, E., and Banchereau, J. (1991a). Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* **173,** 705–710.

Rousset, F., Robert, J., Andary, M., Bonnin, J. P., Souillet, G., Chretien, I., Briere, F., Pene, J., and de Vries, J. E. (1991b). Shifts in interleukin-4 and interferon-gamma production by T cells of patients with elevated serum IgE levels and the modulatory effects of these lymphokines on spontaneous IgE synthesis. *J. Allergy Clin. Immunol.* **87,** 58–69.

Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and Leonard, W. J. (1993). Interleukin-2 receptor gamma chain: A functional component of the interleukin-4 receptor. *Science* **262,** 1880–1883.

Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Goldmann, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994). Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: Implications for XSCID and XCID. *Science* **266,** 1042–1045.

Saban, R., Haak-Frendscho, M., Zine, M., Ridgway, J., Gorman, C., Presta, L. G., Bjorling, D., Saban, M., and Jardieu, P. (1994). Human FcERI-IgG and humanized anti-IgE monoclonal antibody MaE11 block passive sensitization of human and rhesus monkey lung. *J. Allergy Clin. Immunol.* **94,** 836–843.

Sacks, D. L., Lal, S. L., Shrivastava, S. N., Blackwell, J., and Neva, F. A. (1987). An analysis of T cell responsiveness in Indian Kalaazar. *J. Immunol.* **138,** 908–913.

Sadick, M. D., Heinzel, F. P., Holaday, B. J., Pu, R. T., Dawkins, R. S., and Locksley, R. M. (1990). Cure of leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T-cell-dependent, interferon gamma-independent mechanism. *J. Exp. Med.* **171,** 115–127.

Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C., and Horak, I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75,** 253–261.

Sakanari, J. A., Staunton, C. E., Eakin, A. E., Craik, C. S., and McKerrow, J. H. (1989). Serine proteases from nematode and protozoan parasites: Isolation of sequence homologs using generic molecular probes. *Proc. Natl. Acad. Sci. USA* **86,** 4863–4867.

Salgame, P., Abrams, J. S., Clayberger, C., Goldstein, H., Convit, J., Modlin, R. J., and Bloom, B. R. (1991). Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254,** 279–282.

Sarfati, M., Luo, H., and Delespesse, G. (1989). IgE synthesis by chronic lymphocytic leukemia cells. *J. Exp. Med.* **170,** 1775–1780.

Schauenstein, K., Fassler, R., Dietrich, H., Schwarz, S., Kromer, G., and Wick, G. (1987). Disturbed immune-endocrine communication in autoimmune disease. Lack of corticosterone response to immune signals in obese strain chickens with spontaneous autoimmune thyroiditis. *J. Immunol.* **139,** 1830–1833.

Schauer, E., Trautinger, F., Kock, A., Schwarz, A., Bhardwaj, R., Simon, M., Ansel, J. C., Schwarz, T., and Luger, T. A. (1994). Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J. Clin. Invest.* **93,** 2258–2562.

Schmitt, E., Hoehn, P., Germann, T., and Rude, E. (1994). Differential effects of interleukin-12 on the development of naive mouse CD4+ T cells. *Eur. J. Immunol.* **24,** 343–347.

Schmitz, J., Thiel, A., Kuhn, R., Rajewsky, K., Muller, W., Assenmacher, M., and Radbruch, A. (1994). Induction of interleukin 4 (IL-4) expression in T helper (Th) cells is not dependent on IL-4 from non-Th cells. *J. Exp. Med.* **179,** 1349–1353.

Schorle, H., Holtschke, T., Hunig, T., Schimpl, A., and Horak, I. (1991). Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* **352,** 621–624.

Schwartz, L. B., Lewis, R. A., and Austen, K. F. (1981). Tryptase from human pulmonary mast cells, purification and characterization. *J. Biol. Chem.* **256,** 11939–11943.

Schweizer, R. C., Welmers, B. A., Raaijmakers, J. A., Zanen, P., Lammers, J. W., and Koenderman, L. (1994). RANTES- and interleukin-8-induced responses in normal human eosinophils: Effects of priming with interleukin-5. *Blood* **83,** 3697–3704.

Scott, P. (1989). The role of TH1 and TH2 cells in experimental cutaneous leishmaniasis. *Exp. Parasitol.* **68,** 369–372.

Scott, P., Pearce, E., Cheever, A. W., Coffman, R. L., and Sher, A. (1989). Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. *Immunol. Rev.* **112,** 161–182.

Scott, P. (1991). IFN-gamma modulates the early development of TH1 and TH2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol.* **147,** 3149–3155.

Seder, R. A., Boulay, J. L., Finkelman, F., Barbier, S., Ben-Sasson, S. Z., Le Gros, G., and Paul, W. E. (1992a). CD8+ T cells can be primed in vitro to produce IL-4. *J. Immunol.* **148,** 1652–1656.

Seder, R. A., Paul, W. E., Davis, M. M., and Fazekas de St. Groth, B. (1992b). The presence of interleukin-4 during in vitro priming determines the lymphocyte producing potential of CD4+ T cells from T cell receptor transgenic mice. *J. Exp. Med.* **176,** 1091–1098.

Seder, R. A., and Paul, W. E. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu. Rev. Immunol.* **12,** 635–673.

Sen, G. C. (1992). The interferon system. A bird's eye view of its biochemistry. *J. Biol. Chem.* **267,** 5017–5020.

Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261,** 609–612.

Shakib, F., and Smith, S. J. (1994). In vitro basophil histamine-releasing activity of circulating IgG_1 and IgG_4 autoanti-IgE antibodies from asthma patients and the demonstration that anti-IgE modulates allergen-induced basophil activation. *Clin. Exp. Allergy* **24,** 270–275.

Shapira, S. K., Vercelli, D., Jabara, H. H., Fu, S. M., and Geha, R. S. (1992). Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. *J. Exp. Med.* **175,** 289–292.

Sheard, P., Killingback, P. G., and Blair, A. M. (1967). Antigen-induced release of histamine and SRS-A from human lung passively sensitized with reaginic serum. *Nature* **216,** 283–284.

Sher, A. R., and Coffman, R. L. (1992). Regulation of immunity to parasites by T cells and T cell derived cytokines. *Annu. Rev. Immunol.* **10,** 385–409.

Sher, A., Gazzinelli, R. T., Oswald, I. P., Clerici, M., Kullberg, M., Pearce, E. J., Berzofsky, J. A., Mosmann, T. R, James, S. L., and Morse, H. C. (1992). Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol. Rev.* **127,** 183–204.

Shibata, H., Mio, M., and Tasaka, K. (1985). Analysis of the mechanism of histamine release induced by substance P. *Biochem. Biophys. Acta* **846,** 1–7.

Shibuya, A., Kojima, H., Shibuya, K., Nagayoshi, K., Nagasawa, T., and Nakauchi, H. (1993). Enrichment of interleukin-2-responsive natural killer progenitors in human bone marrow. *Blood* **81,** 1819–1826.

Shimizu, T., and Streilein, J. W. (1994). Influence of alpha-melanocyte stimulating hormone on induction of contact hypersensitivity and tolerance. *J. Dermatol. Sci.* **8,** 187–193.

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., and Doetschmann, T. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359,** 693–699.

Sibinga, N. E. S., and Goldstein, A. (1988). Opioid peptides and opioid receptors in cells of the immune system. *Ann. Rev. Immunol.* **6,** 219–249.

Siegel, J. P., Sharon, M., Smith, P. L., and Leonard, W. J. (1987). The IL-2 receptor beta chain (p70): Role in mediating signals for LAK, NK, and proliferative activities. *Science* **238,** 75–78.

Simon, J. C., Cruz, P. D., Bergstresser, P. R., and Tigelaar, R. E. (1990). Low dose ultraviolet B-irradiated Langerhans cells preferentially activate CD4+ cells of the T helper 2 subset. *J. Immunol.* **145,** 2087–2091.

Simon, J. C., Girolomoni, G., Edelbaum, D., Bergstresser, P. R., and Cruz, P. D. (1993). ICAM-1 and LFA-1 on mouse epidermal Langerhans cells and spleen dendritic cells identify disparate requirements for activation of KLH-specific CD4+ Th1 and Th2 clones. *Exp. Dermatol.* **2,** 133–138.

Smith, E. M. (1992). Hormonal activities of cytokines. *Chem. Immunol.* **52,** 154–169.

Smith, R. T., Norcross, M., Maino, V., and Konaka, Y. (1980). Helper mechanisms in T-cell activation. *Immunol. Rev.* **51,** 193–214.

Smith, T. J., Ducharme, L. A., and Weis, J. H. (1994). Preferential expression of interleukin-12 or interleukin-4 by murine bone marrow mast cells derived in mast cell growth factor or interleukin-3. *SO:Eur. J. Immunol.* **24,** 822–826.

Spagnoli, G. C., Juretic, A., Schultz, A., Thater, E., Dellabona, P., Filgueira, L., Horig, H., Zuber, M., Garotta, G., and Heberer, M. (1993). On the relative roles of interleukin-2 and interleukin-10 in the generation of lymphokine-activated killer cell activity. *Cell. Immunol.* **146,** 391–405.

Sperr, W. R., Bankl, H. C., Mundigler, G., Klappacher, G., Grossschmidt, K., Agis, H., Simon, P., Laufer, P., Imhof, M., and Radaszkiewicz, T. (1994). The human cardiac mast cell, localization, isolation, phenotype, and functional characterization. *Blood* **84,** 3876–3884.

Spiegelberg, H. L., Beck, L., Stevenson, D. D., and Ishioka, G. Y. (1994). Recognition of T cell epitopes and lymphokine secretion by rye grass allergen Lolium perenne I-specific human T cell clones. *J. Immunol.* **152,** 4706–4711.

Spits, H., and de Waal Malefyt, R. (1992). Functional characterization of human IL-10. *Int. Arch. Allergy Immunol.* **99,** 8–15.

Spriggs, M. K., Armitage, R. J., Strockbine, L., Clifford, K. N., Macduff, B. M., Sato, T. A., Maliszewski, C. R., and Fanslow, W. C. (1992). Recombinant human CD40 ligand

stimulates B cell proliferation and immunoglobulin E secretion. *J. Exp. Med.* **176,** 1543–1550.

Stadler, B. M., Dougherty, S. F., Farrar, J. J., and Oppenheim, J. J. (1982). Relationship of cell cycle to recovery of IL-2 activity from human mononuclear cells, human and mouse T cell lines. *J. Immunol.* **127,** 1936–1940.

Stadler, B. M., Gang, Q. Vogel, M., Jarolim, E., Miescher, S., Aebischer, I., and de Weck, A. L. (1991a). IgG anti-IgE autoantibodies in immunomodulation. *Int. Arch. Allergy. Appl. Immunol.* **94,** 83–86.

Stadler, B. M., Jarolim, E., Vogel, M., Miescher, S., Aebischer, I., and Gang, Q. (1991b). The role of anti-IgE autoantibodies in allergic Inflammation. In "Clinical Impact of the Monitoring of the Allergic Inflammation" (P. Matson, S. Ahlstedt, P. Venge, and J. Thorell, Eds.), pp. 59–70. Academic Press, London.

Stadler, B. M., Stämpfli, M. R., Miescher, S., Furukawa, K., and Vogel, M. (1993). Biological activities of anti-IgE antibodies. *Int. Arch. Allergy Immunol.* **102,** 121–126.

Stämpfli, M. R., Miescher, S., Aebischer, I., Zürcher, A. W., and Stadler, B. M. (1994). Inhibition of human IgE synthesis by anti-IgE antibodies requires divalent recognition. *Eur. J. Immunol.* **24,** 2161–2167.

Stanisz, A. M., Befus, D., and Bienenstock, J. (1986). Differential effects of vasoactive intestinal peptide, substance P and somatostatin on immunoglobulin synthesis and proliferation by lymphocytes from Peyer's patches, mesenteric lymph nodes, and spleen. *J. Immunol.* **136,** 152.

Stanisz, A. M., Kataeva, G., and Bienenstock, J. (1994). Hormones and local immunity. *Int. Arch. Allergy Immunol.* **103,** 217–222.

Stanton, M. E., and Levine, S. (1988). Pavlovian conditioning of endocrine response. In "Experimental Foundations of Behavioral Medicine. Conditioning Approaches" (R. Ader, R. Weinger, A. Baum, Eds.), pp. 25–46. Lawrence Erlbaum, Hillside, NY.

Stead, R. H., Tomioka, M., Quinonez, G., Simon, G. T., Felten, S. Y., and Bienenstock, J. E. (1987). Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. *Proc. Natl. Acad. Sci. USA* **84,** 2975–2979.

Sternberg, E. M., Young, W. S., Bernardini, R., Calogero, A. E., Chrousos, G. P., Gold, P. W., and Wilder, R. L. (1989). A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. *Proc. Natl. Acad. Sci. USA* **86,** 4771–4775.

Stevens, W. J., Feldmeier, H., Bridts, C. H., and Daffalla, A. A. (1983). IgG and IgE circulating immune complexes, total serum IgE and parasite related IgE in patients with Mono- or mixed infection with Schistostoma mansoni and/or S. haematobium. Influence of therapy. *Clin. Exp. Immunol.* **52,** 142–152.

Stevens, T. L., Bossie, A., Sanders, V. M., Fernandez-Botran, R., Coffman, R. L., Mosmann, T. R., and Vitetta, E. S. (1988). Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* **334,** 255–258.

Stewart, G. A., Kollinger, M. R., King, C. M., and Thompson, P. J. (1994). A comparative study of three serine proteases from Dermatophagoides pteronyssinus and D. farinae. *Allergy* **49,** 553–560.

Stief, A., Texido, G., Sansig, G., Eibel, H., Le Gros, G., and van der Putten, H. (1994). Mice deficient in CD23 reveal its modulatory role in IgE production but no role in T and B cell development. *J. Immunol.* **152,** 3378–3390.

Stout, R. D., and Bottomly, K. (1989). Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. *J. Immunol.* **142,** 760–765.

Street, N. E., Schumacher, J. H., Fong, T. A. T., Bass, H., Fiorentino, D. F., Leverah, J. A., and Mosmann, T. R. (1990). Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of TH1 and TH2 cells. *J. Immunol.* **144,** 1629–1639.

Suoniemi, I., Björkstén, F., and Haahthela, T. (1987). Dependence of immediate hypersensitivity in the adolescent period on factors encountered in infancy. *Allergy* **36,** 263–268.

Swain, S. L., McKenzie, D. T., Weinberg, A. D., and Hancock, W. (1988). Characterization of T helper 1 and 2 cell subsets in normal mice. Helper T cells responsible for IL-4 and IL-5 production are present as precursors that require priming before they develop into lymphokine-secreting cells. *J. Immunol.* **141,** 3445–3455.

Swain, S. L., and Weinberg, A. D. (1990). IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* **145,** 3796–3806.

Swain, S. L., Bradley, L. M., Croft, M., Tonkonogy, S., Atkins, G., Weinberg, A. D., Duncan, D. D., Hedrick, S. M., Dutton, R. W., and Huston, G. (1991). Helper T-cell subsets: Phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* **123,** 115–144.

Sypek, J. P., Chung, C. L., Mayor, S. E., Subramanyam, J. M., Goldman, S. J., Sieburth, D. S., Wolf, S. F., and Schaub, R. G. (1993). Resolution of cutaneous leishmaniasis: Interleukin 12 initiates a protective T helper type 1 immune response. *J. Exp. Med.* **177,** 1797–1802.

Takafuji, S., Suzuki, S., Koitumi, K., Tadokoro, T., Miyamoto, T., Ikemori, R., and Muranaka, M. (1987). Diesel-exhaust particulates inoculated the intranasal route have an adjuvant activity for IgE production in mice. *J. Allergy Clin. Immunol.* **79,** 639–645.

Tanaka, T., Hu-Li, J., Seder, R. A., Fazekas de St. Groth, B., and Paul, W. E. (1993). Interleukin-4 suppresses interleukin-2 and interferon g production by naive T cells stimulated by accessory cells-dependent engagement. *Proc. Natl. Acad. Sci. USA* **90,** 5914–5918.

Tao, X., and Stout, R. D. (1993). T cell-mediated cognate signaling of nitric oxide production by macrophages. Requirements for macrophage activation by plasma membranes isolated from T cells. *Eur. J. Immunol.* **23,** 2916–2921.

Thorpe, S. C., Kemeny, D. M., Panzani, R., McGurl, B., and Lord, M. (1988). Allergy to castor bean. II. Identification of the major allergens in castor bean seeds. *J. Allergy Clin. Immunol.* **82,** 67–72.

Thorpe, S. C., Murdoch, R. D., and Kemeny, D. (1989). The effect of the castor bean toxin, ricin, on rat IgE and IgG responses. *Immunology* **68,** 307–311.

Trinchieri, G., Matsumoto, M., Kobayashi, M., Clark, S. C., Seehra, J., London, L., and Perussia, B. (1984). Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* **160,** 1147–1169.

Tripp, C. S., Wolf, S. E., and Unaune, E. R. (1993). Interleukin-12 and tumor necrosis factor a are co-stimulators of interferon-γ production by natural killer cells in severe combined immunodeficiency mice with Listeriasis, and interleukin-10 is a physiological antagonist. *Proc. Natl. Acad. Sci. USA* **90,** 3725–3729.

Tsicopoulos, A., Hamid, Q., Varney, V., Ying, S., Moqbel, R., Durham, S. R., and Kay, A. B. (1992). Preferential messenger RNA expression of Th1-type cells (IFN-gamma+, IL-2+) in classical delayed-type (tuberculin) hypersensitivity reactions in human skin. *J. Immunol.* **148,** 2058–2061.

Tsudo, M., Goldman, C. K., Bongiovanni, K. F., Chan, W. C., Winton, E. F., Yagita, M., Grimm, E. A., Waldmann, T. A. (1987). The p75 peptide is the receptor for interleukin 2 expressed on large granular lymphocytes and is responsible for the interleukin 2 activation of these cells. *Proc. Natl. Acad. Sci. USA* **84,** 5394–5398.

Tunon de Lara, J. M., Okayama, Y., McEuen, A. R., Heusser, C. H., Church, M. K., and Walls, A. F. (1994). Release and inactivation of interleukin-4 by mast cells. *Ann. N. Y. Acad. Sci.* **725,** 50–58.

Ullrich, S. E. (1994). Mechanism involved in the systemic suppression of antigen-presenting cell function by UV irradiation. Keratinocyte-derived IL-10 modulates antigen-presenting cell function of splenic adherent cells. *J. Immunol.* **152,** 3410–3416.

Umetsu, D. T., Jabara, H. H., DeKruyff, R. H., Abbas, A. K., Abrams, J. S., and Geha, R. S. (1988). Functional heterogeneity among human inducer T cell clones. *J. Immunol.* **140,** 4211–4216.

Urban, J. F., Madden, K. B., Svetic, A., Cheever, A., Trotta, P. P., Gause, W. C., Katona, I. M., and Finkelman, F. D. (1992). The importance of Th2 cytokines in protective immunity to nematodes. *Immunol. Rev.* **127,** 205–220.

Valensi, J. P., Carlson, J. R., and Van Nest, G. A. (1994). Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J. Immunol.* **153,** 4029–4039.

Valenta, R., Duchene, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1991). Identification of profilin as a novel pollen allergen, IgE autoreactivity in sensitized individuals. *Science* **253,** 557–560.

Van den Bergh, P., Rozing, J., and Nagelkerken, L. (1991). Two opposite modes of action of beta-endorphin in vivo. *Immunology* **72,** 537–544.

Van der Pouw-Kraan, C. T., Rensink, H. J., Rappuoli, R., and Aarden, L. A. (1995). Co-stimulation of T cells via CD28 inhibits human IgE production, reversal by pertussis toxin. *Clin. Exp. Immunol.* **99,** 473–478.

Van Woudenberg, A. D., Metzelaar, M. J., van der Kleij, A. A., de Wied, D., Burbach, J. P., and Wiegant, V. M. (1993). Analysis of proopiomelanocortin (POMC) messenger ribonucleic acid and POMC-derived peptides in human peripheral blood mononuclear cells: no evidence for a lymphocyte-derived POMC system. *Endocrinology* **133,** 1922–1933.

Veldhuis, J. D., Iranmanesh, A., Johnson, M. L., and Lizarralde, G. (1990). Twenty-four-hour rhythms in plasma concentrations of adenohypophyseal hormones are generated by distinct amplitude and/or frequency modulation of underlying pituitary secretory bursts. *J. Clin. Endocrinol. Metab.* **71,** 1616–1623.

Vercelli, D., Jabara, H. H., Lee, B. W., Woodland, N., Geha, R. S., and Leung, D. Y. (1988). Human recombinant interleukin 4 induces Fc epsilon R2/CD23 on normal human monocytes. *J. Exp. Med.* **167,** 1406–1416.

Vercelli, D., Jabara, H. H., Arai, K., and Geha, R. S. (1989). Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J. Exp. Med.* **169,** 1295–1307.

Vercelli, D., Jabara, H. H., Lauener, R. P., and Geha, R. S. (1990). IL-4 inhibits the synthesis of IFN-gamma and induces the synthesis of IgE in human mixed lymphocyte cultures. *J. Immunol.* **144,** 570–573.

Viac, J., Gueniche, A., Gatto, H., Lizard, G., and Schmitt, D. (1994). Interleukin-4 and interferon-gamma interactions in the induction of intercellular adhesion molecule-1 and major histocompatibility complex class II antigen expression of normal human keratinocytes. *Exp. Dermatol.* **3,** 72–77.

Vogel, M., Miescher, S., Biaggi, C., and Stadler, B. M. (1994). Human anti-IgE antibodies by repertoire cloning. *Eur. J. Immunol.* **24,** 1200–1207.

Von der Weid, T., Kopf, M., Kohler, G., and Langhorne, J. (1994). The immune response to Plasmodium chabaudi malaria in interleukin-4 deficient mice. *Eur. J. Immunol.* **24,** 2285–2293.

Waldmann, T. A., Goldman, C. K., Robb, R. J., Depper, J. M., Leonard, W. J., Sharrow, S. O., Bongiovanni, K. F., Korsmeyer, S. J., Greene, W. C. (1984). Expression of interleukin 2 receptors on activated human B cells. *J. Exp. Med.* **160,** 1450–1466.

Wang, Z. E., Zeiner, S. L., Zheng, S., Dalton, D. K., and Locksley, R. M. (1994). CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. *J. Exp. Med.* **179,** 1367–1371.

Warren, H. S., Vogel, F. R., and Chedid, L. L. (1986). Current status of immunological adjuvants. *Annu. Rev. Immunol.* **4,** 369–388.

Weaver, C. T., Hawrylowicz, C. M., and Unanue, E. R. (1988). T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. USA* **85,** 8181–8185.

Weinberg, A. D., English, M. E., and Swain, S. (1990). Distinct regulation of lymphokine production is found in fresh versus in vitro primed murine helper cells. *J. Immunol.* **144,** 1800–1806.

Wiedermann, U., Hanson, L. A., Kahu, H., and Dahlgren, U. I. (1993). Aberrant T-cell function in vitro and impaired T-cell dependent antibody response in vivo in vitamin A-deficient rats. *Immunology* **80,** 581–586.

Wierenga, E. A., Snoek, M., de Groot, C., Chrétien, I., Bos, J. D., Jansen, H. M., and Kapsenberg, M. L. (1990). Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes. *J. Immunol.* **144,** 4651–4656.

Wierenga, E. A., Backx, B., Snoek, M., Koenderman, L., and Kapsenberg, M. L. (1993). Relative contributions of human types 1 and 2 T-helper cell-derived eosinophilotrophic cytokines to development of eosinophilia. *Blood* **82,** 1471–1479.

Woloski, B. M., Smith, E. M., Meyer, W. J., Fuller, G. M., and Blalock, J. E. (1985). Corticotropin-releasing activity of monokines. *Science* **230,** 1035–1037.

Wongtim, S., Lehrer, S. B., Salvaggio, J. E., and Horner, W. E. (1993). Protease activity in cockroach and basidiomycete allergen extracts. *Allergy Proc.* **14,** 263–268.

Wu, C. Y., Sarfati, M., Heusser, C., Fournier, S., Rubio-Trujillo, M., Peleman, R., and Delespesse, G. (1991). Glucocorticoids increase the synthesis of immunoglobulin E by interleukin 4-stimulated human lymphocytes. *J. Clin. Invest.* **87,** 870–877.

Xu-Amano, J., Kiyono, H., Jackson, R. J., Staats, H. F., Fujihashi, K., Burrows, P. D., Elson, C. O., Pillai, S., and McGhee, J. R. (1993). Helper T cell subsets for immunoglobulin A responses, oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J. Exp. Med.* **178,** 1309–1320.

Yamamura, M., Uyemura, K., Deans, R. J., Weinberg, K., Rea, T. H., Bloom, B. R., and Modlin, R. L. (1991). Defining protective responses to pathogens, cytokine profile in leprosy lesions. *Science* **254,** 277–279.

Yang, X. D., De Weck, A. L., and Stadler, B. M. (1988). Induction of human in vitro IgE synthesis via stimulation by anti-CD3 antibody. *Eur. J. Immunol.* **18,** 467–471.

Yoshikawa, T., Nanba, T., Kato, H., Hori, K., Inamoto, T., Kumagai, S., and Yodoi, J. (1994). Soluble Fc epsilon RII/CD23 in patients with autoimmune diseases and Epstein-Barr virus-related disorders, analysis by ELISA for soluble Fc epsilon RII/CD23. *Immunomethods* **4,** 65–71.

Yssel, H., Shanafelt, M. C., Soderberg, C., Schneider, P. V., Anzola, J., and Peltz, G. (1992a). B. burgdorferi activates a TH1-like T cell subset in Lyme arthritis. *J. Exp. Med.* **174,** 593–601.

Yssel, H., Johnson, K. E., Schneider, P. V., Wideman, J., Terr, A., Kastelein, R., and de Vries, J. E. (1992b). T cell activation inducing epitopes of the house dust mite allergen Der p. 1. Proliferation and lymphokine production patterns by Der p I-specific CD4+ T cell clones. *J. Immunol.* **148,** 738–745.

Yssel, H., Fasler, S., de Vries, J. E., and de Waal Malefyt, R. (1994). IL-12 transiently induces IFN-gamma transcription and protein synthesis in human CD4+ allergen-specific Th2 T cell clones. *Int. Immunol.* **6,** 1091–1096.

Zhao, M. L., Xia, J. Q., and Fritz, R. B. (1992). Experimental allergic encephalomyelitis in susceptible and resistant strains of mice after adoptive transfer of T cells activated by antibodies to the T cell receptor complex. *J. Neuroimmunol.* **40,** 31–39.

Zubler, R. H., Lowenthal, J. W., Erard, F., Hashimoto, N., Devos, R., and MacDonald, H. R. (1984). Activated B cells express receptors for, and proliferate in response to, pure interleukin 2. *J. Exp. Med.* **160,** 1170–1183.

Zurawski, G., and de Vries, J. E. (1994a). Interleukin 13 elicits a subset of the activities of its close relative interleukin 4. *Stem Cells* **12,** 169–174.

Zurawski, G., and de Vries, J. E. (1994b). Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today* **15,** 19–26.

Zürcher, A. W., Lang, A., Aebischer, I., Miescher, S., and Stadler, B. M. (1995). IgE producing hybridomas established after B cell culture in the CD40 system. *Immunol. Lett.* **46,** 49–57.

INDEX

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

R

S

V

X

Z

CONTENTS OF RECENT VOLUMES

Volume 57

Volume 58

Volume 59

Volume 60