Conferences and Reviews

The Second Century of the Antibody
Molecular Perspectives in Regulation, Pathophysiology, and Therapeutic Applications

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This is an edited summary of an Interdepartmental Conference arranged by the Department of Medicine of the UCLA School of Medicine, Los Angeles, California. The Director of Conferences is William M. Pardridge, MD, Professor of Medicine.

The modern age of immunology began in 1890 with the discovery of antibodies as a major component of protective immunity. The 2nd century of the antibody begins with a focus on the molecular physiology and pathophysiology of immunoglobulin production. Numerous human variable-region antibody genes have been identified through advances in molecular cloning and anti-variable-region monoclonal antibodies. Some of these variable-region genes are now known to be involved in specific stages of B-lymphocyte differentiation and immune development. This connection has yielded new insights into the pathogenesis of immune dyscrasias and lymphoid neoplasia; common variable immunodeficiency and cryoglobulinemia are highlighted here. The molecular regulation of immunoglobulin expression suggests new targets for pathogenesis and clinical intervention. Finally, genetically engineered antibodies offer novel opportunities for diagnostic and therapeutic applications.

(Braun J, Saxon A, Wall R, Morrison SL: The second century of the antibody—Molecular perspectives in regulation, pathophysiology, and therapeutic applications. West J Med 1992 Aug; 157:158-168)

JONATHAN BRAUN, MD, PhD*: One hundred years ago, it was well known that a resistance to infections could be elicited by specific immunity. The mechanism of this immunity was unknown, however. During the decade of the 1880s, Loeffler, Roux, and Yersin showed that the diseases diphtheria and tetanus were caused by exotoxins. These diseases were at the time leading causes of death in children, and the findings set off intense efforts by members of Robert Koch's laboratory to find out how immunity to these toxins occurred.

In 1890, two laboratory researchers, Emil Behring and Shidasabura Kitasato, together discovered that the immunity was due to a cell-free serum component that was specific to the toxin used for immunization and that, when injected into a nonimmune recipient, neutralized the toxin's lethality. These findings were reported in the December 1890 issue of *Deutsche Medizinische Wochenschrift*[1] and electrified the world's scientific community. A year later on Christmas night, Behring used this strategy of passive immunization with antitoxin serum to treat a child with diphtheria.

In this conference, we shall emphasize current research focusing on the molecular and developmental regulation of antibodies and its relation to issues in immune pathogenesis and therapeutics.

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Variable-Region Genes

Variable-Region Genes in B-Cell Development

The capacity of persons to produce an almost limitless range of antibody specificities is a key feature of humoral immunity. Its molecular basis was a mystery until 1977 when Tonegawa and colleagues recognized that each active antibody gene was assembled from several gene segment families (V, D, J, and C) by a novel DNA rearrangement process.[2] Diversity is created from the large number of family members (in the case of the V gene family, 100 or more), independent assortment and combination of segments, and imperfect segment joining. All told, these factors permit the generation of more than 10^8 antibody species.[2]

An important practical result of this work was the introduction of recombinant probes for antibody use, which provide an unambiguous identification of B-lymphocyte clones and direct characterization of their genetic relatedness. An early insight from such analysis occurred in studies of B-cell development. It is well known that antibody diversity dramatically increases during fetal development and its cellular counterpart in the pre-B-cell stage. The work of Malynn and associates, however, showed that this increase is not merely quantitative but involves changing patterns of V-gene usage.[3] At the earliest stages, only a few V genes are accessible for rearrangement into active antibody genes. The resulting fetal

This work was supported by grants CA16858 (Dr Morrison), GM40185 (Dr Wall), AI15332, AI15251, and CA23175 (Dr Saxon), and National Institutes of Health Program Project CA12800 (Drs Wall, Saxon, and Braun), IM550 (Dr Morrison) from the American Cancer Society, the Jonsson Comprehensive Cancer Center and the UCLA Clinical Research Center of the UC Taskforce on AIDS (Dr Braun), and Centocor (Dr Morrison). Dr Braun is a Scholar of the Leukemia Society of America and recipient of a Research Scholars Award from RJR-Nabisco.

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ABBREVIATIONS USED IN TEXT

cAMP = cyclic adenosine monophosphate
CVI = common variable immunodeficiency
HIV = human immunodeficiency syndrome
IFN-γ = interferon gamma
Ig = immunoglobulin
IL = interleukin
LPS = lipopolysaccharides
PMA = 12-*O*-tetradecanoylphorbol 13-acetate
TGF-β = transforming growth factor β

antibody repertoire thus contains predominantly these unusual antibodies whose specificity and function, although apparently important for immune ontogeny, remain a mystery. The V_H5 and V_H6 gene families are the earliest detected in human fetal development,[4] followed by certain members of the V_H1 and V_H3.[5,6] Some of these V_H genes are notable for their predominance in certain categories of B-cell neoplasia (Table 1). With maturation a much fuller range of V genes is used, so that the abundance of V genes in the mature B-cell population is roughly proportional to their representation in the immunoglobulin loci.[3] An important role is played by T lymphocytes in this developmental switch in V-gene use, possibly through conventional immune selection.[3,7] In this regard, there is much interest in establishing whether such selection involves exogenous versus "self" antigens, which might bear on the genesis of certain classes of autoantibodies.

A second major expansion and diversification of the antibody repertoire occurs during childhood.[8,9] Recent work in our laboratory has shown that the abundance of B cells in lymphoid germinal centers—representing a precursor stage in the development of immunologic memory—is increased in young children (2 to 4 years) but adjusts to adult levels by preadolescence (8 to 14 years).[10] This evolving abundance is associated with a qualitative shift in the clonal pattern of B cells; for example, the use of certain V_H1 genes is delayed until preadolescence. It may be notable that this corresponds to a shift in serum immunoglobulin (Ig) A and a response to *Haemophilus influenzae* infection to adult levels.[8,11] Other V genes are significantly used only by B cells in the blood and lymphoid mantle zones, most likely representing the mature memory population. These V genes, including V_H3_L (a subgroup of the V_H3 family) and humVκ328 (a member of the VκIIIb light-chain family), can thus be used as clonal markers for this stage of B-cell maturation (Figure 1[10]; and J. Braun, MD, PhD, and Y. Valles-Ayoub, MD, PhD, unpublished data, September 1991).

The V_H3_L marker has been informative regarding the pathogenesis of two immunodeficiency diseases. Common variable immunodeficiency (CVI) has been related by various in vitro assays to an intrinsic block in the B cell differentiative potential (discussed later). A collaborative study with Andrew Saxon, MD, showed that patients with this disease are typically deficient in V_H3_L B cells, a finding consistent with their deficit in mature blood or mantle zone cells.[12]

In a separate study of the human immunodeficiency virus (HIV), infection was prompted by the early occurrence of paradoxic B-cell hyperplasia and humoral immune deficiency. Humoral immune deficiency is seen as a poor response to polysaccharides in vivo,[13] pokeweed mitogen in vitro,[14] a peculiar "bare follicle" histologic appearance of lymph node,[15] and a decline in Leu-8 B-cell expression.[16] We noted that all these features could be attributed to a deficit in the mantle zone B-cell subpopulation. In a study of 20 HIV-positive persons, a selective and profound deficit of V_H3_L B

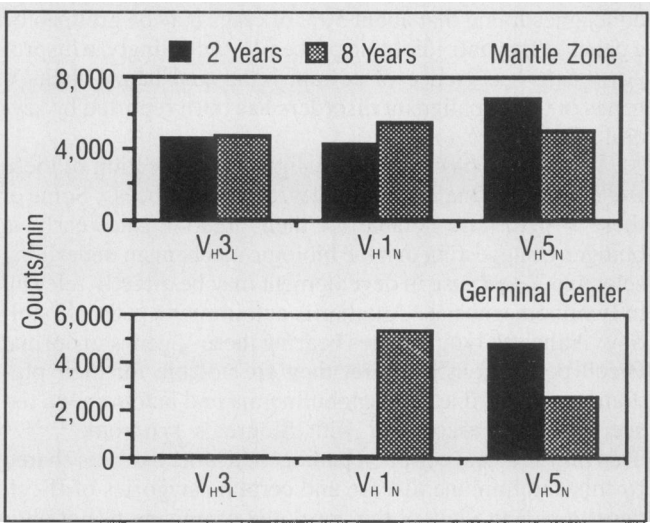

Figure 1.—The effect of age and developmental subpopulation (mantle zone versus germinal center) is shown on the abundance of B cells bearing V gene subfamilies. The representation of V_H1_N and V_H3_L is restricted by these measures to the mantle zone (mature-memory) stage; V_H5_N exemplifies an unrestricted V gene subfamily (from Valles-Ayoub et al[10]; adapted with permission from *Blood*).

TABLE 1.—*Distinct Development or Pathophysiology of B Cells Using Certain V Genes*

Gene	Description	Abundance, %	Reference
Vκ325 . .	CLL	25	Kipps et al, 1990[17]; Kipps et al, 1988[20]
	Waldenström's macroglobulinemia	33	Kipps et al, 1990[17]; Kipps et al, 1988[20]
	Mixed cryoglobulinemia	35	Kipps et al, 1990[17]; Kipps et al, 1988[20]
	Sjögren's syndrome	~15	Kipps et al, 1989[22]
V_H51P1. .	Fetal	--	Schroeder et al, 1987[5]
	CLL	20	Kipps et al, 1989[19]
	Well-differentiated non-Hodgkin's lymphoma	10	Kipps et al, 1989[19]
V_H5.	Earliest fetal	--	Cuisinier et al, 1989[4]
	CLL	20	Humphries et al, 1988[18]
V_H6.	Earliest fetal	--	Cuisinier et al, 1989[4]
	Most proximal	--	Mayer et al, 1990[21]
	Anti-DNA	--	Mayer et al, 1990[21]

CLL = chronic lymphocytic leukemia

cells was found.[17] These findings suggest that the B-cell immune dysfunctions in these two diseases may involve a developmental block at the germinal center stage. In the case of CVI, the block is probably intrinsic, whereas in HIV infection it presumably reflects a deficit in the $CD4^+$ T-cell role needed for the transition of B cells from the germinal center to quiescent memory cell stage. This point is further discussed in the section by Saxon.

B-Cell Neoplasia and Autoimmunity

Like normal lymphocytes, malignant B cells typically express or secrete a clonal antibody. A combination of molecular cloning and monoclonal antibody techniques has shown that B cells bearing certain antibodies are disproportionately represented in lymphoid malignant neoplasia (Table 1). Studies by Miller and co-workers of follicular non-Hodgkin's lymphomas have shown that at least a third of 150 independent lymphomas can be grouped into recurrent clonal families detected by shared idiotypes (unique antigenic determinants on the clonal antibody).[18] In the narrower group of $CD5^+$ B-cell malignancy—chronic lymphocytic leukemia, small cell lymphocytic lymphoma—Kipps and colleagues found that about 50% of cases may be grouped by a panel of five anti-idiotypic probes.[19] Accordingly, a disproportionate occurrence of certain light- and heavy-chain V genes in these malignant disorders has been reported by several groups.[20-23]

What is the basis for the malignant predilection of these B-cell clones? One is the fetal development process: Some of these V genes are notable for their predominance early in ontogeny, suggesting that the biologic mechanism underlying selective V-gene use in development may be directly relevant to lymphomagenesis. Another is autoimmune pathophysiology: Although lymphocytes bearing these V genes in normal B-cell populations are rare, they are notable for their predominance in mixed cryoglobulinemia and autoimmune tissue infiltrates associated with Sjögren's syndrome.[21,22,24] This may indicate common pathogenetic mechanisms shared by this autoimmune disease and certain categories of B-cell neoplasia.[23] In view of the novel and promising use of anti-idiotypic monoclonal antibodies for the treatment of B-cell lymphomas, it is conceivable that anti-idiotypic therapy may also be useful in autoimmune diseases for which a restricted clonal population of pathogenetic B or T lymphocytes are involved.

Common Variable Immunodeficiency—A Maturational Arrest in Germinal Center B-Cell Development

ANDREW SAXON, MD*: Common variable immunodeficiency is a syndrome wherein various pathophysiologic states lead to the inability to produce both normal quantitative and qualitative antibody responses.[25,26] Although it may occur in infancy, it generally is noted at puberty or later, and in most patients there is no evidence for dominant or recessive inheritance.[27] Common variable immunodeficiency has represented the expression of several acquired defects in humoral immunity. Our analysis of the phenotypic, functional, and molecular aspects of the B cells from patients with the disorder, however, suggests that in most patients it may represent a relatively uniform defect in B-cell development.

The hallmark of CVI is the presence of hypogammaglobulinemia that generally affects all the major immunoglobulin effector isotypes—IgM, IgG, and IgA—involved in protecting from infection. Not surprisingly, patients almost uniformly present with complaints of bacterial infection, generally of the respiratory system (sinusitis, bronchitis, or pneumonia).[28] As in other immunodeficiency states, the characteristics of the infections in these immunocompromised patients are their increased frequency, increased severity, the failure to resolve with appropriate therapy, and the presence of unusual organisms. Common variable immunodeficiency manifests two other features that are less obviously related to the humoral immunodeficiency: lymphoid hyperplasia, particularly of the gastrointestinal tract, with an increased frequency of lymphoproliferative disorders,[29,30] and the occurrence of various autoimmune phenomena, including rheumatoid-like arthritis, idiopathic thrombocytopenia, atrophic gastritis with pernicious anemia, secretory diarrhea, parotitis, Guillain-Barré syndrome, and sicca complex.[31-33]

B cells (defined as cells bearing membrane Ig) are present in normal numbers in the circulation and the tissues of two thirds of patients with the disorder and in reduced numbers in the remaining third.[27] This is in contrast to X-linked agammaglobulinemia, which involves an essentially complete deficit in membrane Ig-bearing B cells due to a developmental block at the pre–B-cell stage. Instead, the problem in CVI is the failure of these B cells to mature into Ig-secreting cells in vivo. In most patients, this is reflected in vitro by the inability of their B cells to secrete Ig even when provided with appropriate stimuli for terminal differentiation.[34-36] Although diverse immune mechanisms have been proposed as the underlying cause of this deficit, it is this failure to develop high-rate Ig-secreting cells that directly accounts for the profound deficiency of serum immunoglobulins and resultant recurrent bacterial infections.[28]

Functional and Molecular Features of Common Variable Immunodeficiency

Resting B cells must pass through the stages of activation, proliferation, and then differentiation to become high-rate Ig-secreting cells.[37,38] Thus, resting B cells need an initial activation step, after which they enter the G1 phase of the cell cycle, increasing their expression of DR antigens and displaying receptors for proliferative signals—that is, B-cell growth factors such as interleukin (IL) 4. In the presence of these growth factors, the activated B cells can undergo several rounds of cell division. Finally, under distinctive differentiation drives, the B cells enter a maturational phase in which immunoglobulin is synthesized and secreted. These stages of development can be analyzed in vitro by assaying the response to defined activation, proliferation, and differentiation signals.[37-39]

B cells from 15 patients with CVI were analyzed with respect to this in vitro developmental pathway to determine the nature of their functional arrest (Figure 2).[36] Cells from two patients failed to respond at the activation stage and from one at the proliferative stage (in addition to patients 108 and 112—the two patients with depressed activation responses). This is in agreement with studies by Chien and associates, who found activation unimpaired in B cells from patients with CVI.[40] All the patients' B cells failed to differentiate in the normal range, that is, an induced IgG and IgM response. Four patients did show partial responses to these differentia-

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tion drives (patients 109, 113, 125, and 143), which consisted primarily of enhanced IgM production.

This inability of patients' B cells to respond normally to differentiation signals suggested that altered responses to late-acting cytokines may play a role in the pathophysiology of this immune disorder. Although many cytokines influence the development of human B cells,[41,42] IL-6 or B-cell stimulatory factor 2 is particularly relevant for terminal B-cell differentiation. Interleukin 6 has been shown to drive the differentiation of B cells into high-rate Ig-secreting cells, to induce proliferation of Epstein-Barr virus–transformed cell lines and hybridoma cells in vitro, and to function as an autocrine growth factor for human myeloma cells.[43-46] In addition, IL-6 induces systemic effects similar to those of IL-1 and tumor necrosis factor. Interleukin 6 may even provide a potent stimulus for inducing autoimmunity.[47]

Because of the potential role of IL-6 in B-cell differentiation and growth as well as autoimmune phenomena, we tested the ability of CVI B cells to respond to IL-6. Blood B cells, however (as opposed to tonsil B cells), show minimal differentiation response to IL-6. Therefore, we took advantage of a series of human-human B-cell hybridomas that we had constructed (in collaboration with Randolph Wall, PhD) with either normal or CVI B cells.[48] These hybridomas manifest the functional phenotype of the immunodeficiency's expression of low levels of Ig μ messenger RNA with skewing toward the membrane form. When normal hybridoma B cells producing low levels of IgM were stimulated with IL-6, they showed an increase in IgM production consistent with a differentiation effect, whereas the hybridomas from the persons with CVI failed to do so (Figure 3). This deficit could be overcome by direct activation at the nuclear level using retinoids.[49,50] This indicates that the B cell in these patients does not carry an intrinsic defect in the nuclear read-out of the differentiation signal but in the events directly controlling responsiveness to immunophysiologic stimuli, such as IL-6. On a practical level, the successful restoration of B-cell differentiation by retinoids suggests their potential value as a therapeutic tool in common variable immunodeficiency.

Because of these findings, we investigated whether patients with this disorder had elevated serum levels of IL-6 resulting from their inability to differentiate in response to IL-6.[51] Serum IL-6 levels were elevated 3-fold to 18-fold above the normal range in 13 of 17 patients with CVI. In contrast, serum IL-6 levels were normal in patients with other B-cell immunodeficiencies (selective IgA deficiency and X-linked agammaglobulinemia). Interleukin-6 levels are controlled by a serum binding factor and the rate of IL-6 synthesis. We found that the serum binding factor activity was normal in patients with CVI. Spontaneous IL-6 produc-

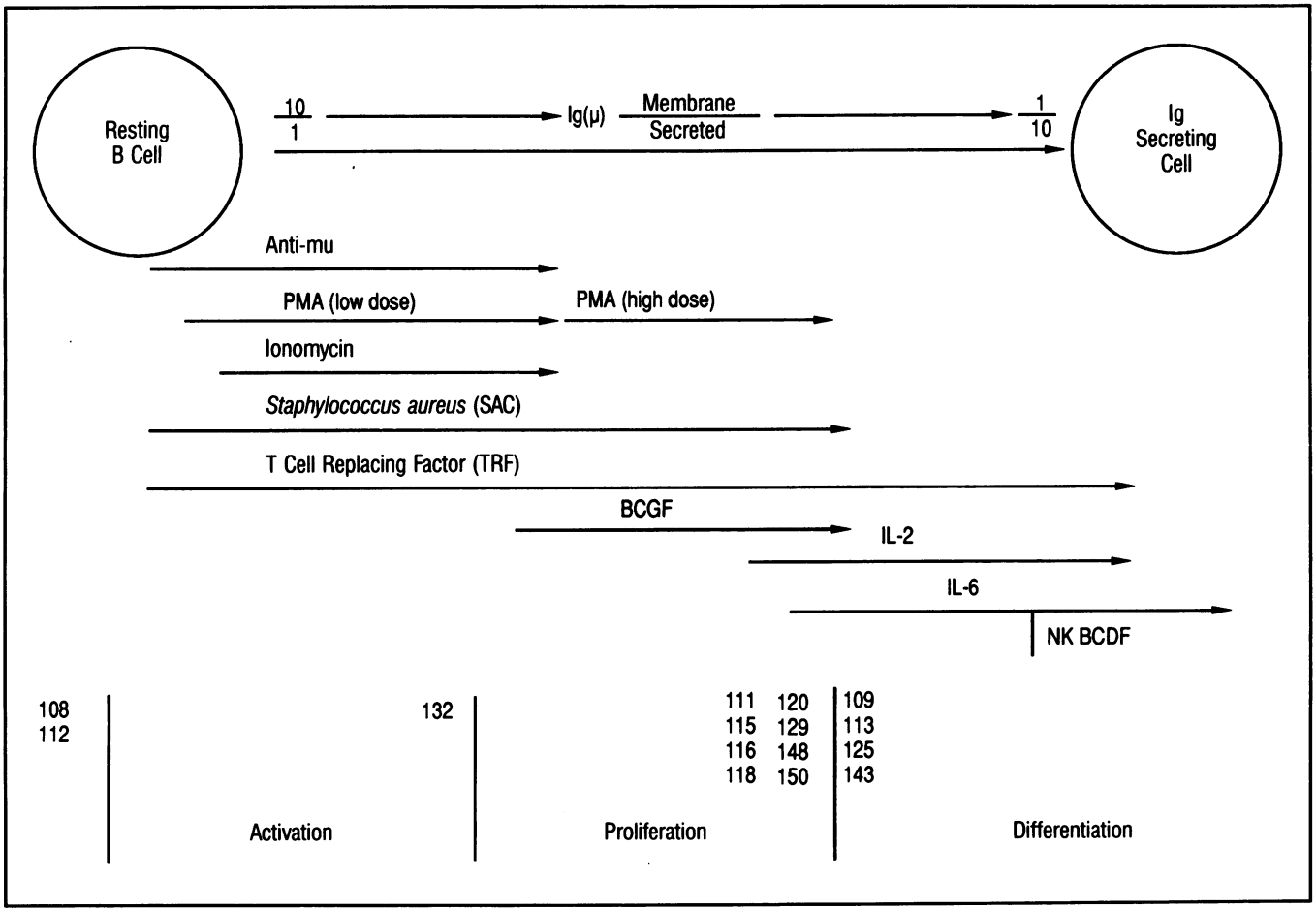

Figure 2.—A schematic is shown of B-cell development from resting B cells through activation, proliferation, and differentiation. The increased amount of messenger RNA for secreted versus membrane immunoglobulin (Ig) is represented in the upper section. Stimuli used in vitro for the different stages of development are shown in the middle section. On the bottom, the point where each patient's cells were unresponsive is represented by the UCLA immunodeficient patient's number. Most B cells failed to differentiate despite undergoing activation and proliferation normally (from Saxon et al[36]; adapted with permission from *Journal of Allergy and Clinical Immunology*). BCGF = B-cell growth factor, IL = interleukin, mu = IgM heavy chain, NK BCDF = NK cell-derived B-cell differentiation factor, PMA = 12-*O*-tetradecanoylphorbol 13-acetate

tion, however, by peripheral blood mononuclear cells from four patients with the immunodeficiency was significantly greater than by peripheral blood mononuclear cells from normal volunteers and a patient with hyper-IgM syndrome. Stimulated IL-6 production (by lipopolysaccharide) raised absolute IL-6 levels in normal persons to the unstimulated level in patients with CVI, suggesting that IL-6 production in these patients is endogenously at maximal levels. Although the endogenous stimulus of elevated IL-6 production in patients with CVI remains to be identified, these IL-6 levels may account for the clinical manifestations of lymphoproliferation and the autoimmune phenomena associated with this disease.

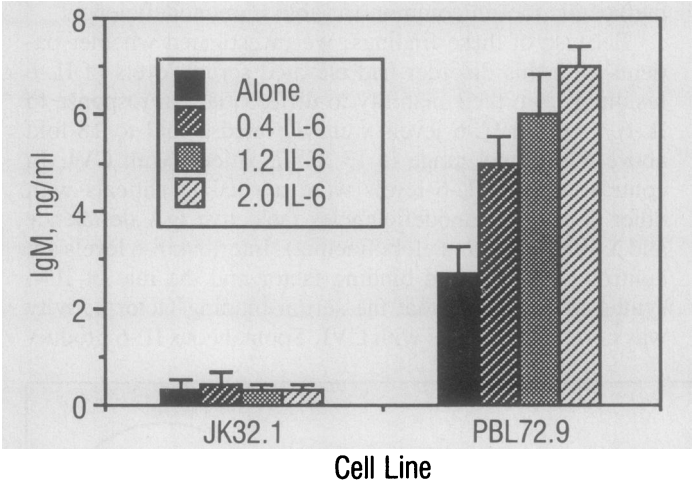

Figure 3.—Immunoglobulin (Ig) M production by common variable immunodeficiency (JK32.1) or normal (PBL72.9) hybridoma B cells is shown. Cells (10,000) were cultured for 3 days in 200 ml of complete medium alone or an increasing amount of recombinant interleukin 6 (IL-6). The amount of IgM in the supernatant at day 7 was measured by isotype-specific solid-phase enzyme-linked immunosorbent assay. The normal hybridoma cells show a clear dose response to IL-6, whereas this was not found with the common variable immunodeficiency cells. The error bars represent 1 standard deviation.

A Maturational Block at the Germinal Center Stage

Diverse functional abnormalities, such as those seen in CVI, raise the possibility that the disease actually involves a differentiative arrest at a specific stage in B-cell development. This idea was addressed by analyzing the expression of surface molecules known to be related to B-cell activation and maturation.[32] The striking difference between normal subjects and patients was a pronounced increase in the abundance of Leu-8^{dim} B cells, which also typically had elevated CD20 expression (Figure 4). A less striking but significant reduction in CD21 expression was also found. There was almost no overlap between 10 control subjects and the 15 patients with the disorder analyzed in this manner, and the abnormality was restricted to the B-cell population.

Remarkably, this unusual differentiation antigen phenotype of blood B cells in patients with CVI is in key respects the *normal* phenotype of B cells found in the germinal center, a critical site of memory B-cell development.[52] In addition, the unusual pattern of developmentally regulated B-cell clones (for example, V_H3_L) common to blood B cells in patients with CVI is that found with normal germinal center B cells (see preceding discussion by Braun). Moreover, a subpopulation of antigen-responsive B cells apparently representing recent germinal center emigrants[53] is profoundly deficient in patients with this immune deficit.[54] Taken together, these findings strongly suggest that B cells in most patients with CVI are arrested at a germinal center stage of differentiation.

The inability of these patients' B cells to proceed beyond this stage is consistent with the B cells' functional abnormalities in terminal differentiation and the corresponding deficits in qualitative and quantitative antibody production. The Leu-8 antigen is now known to represent a lymph node homing molecule,[55,56] and deficient expression may hinder effective lymphoid localization of B cells needed for antigen-specific responses. The CD20 molecule (a probable B cell-specific calcium channel) transmits a co-competence proliferative signal and may normally prevent cells from progressing through their differentiation pathway.[57] Its constitutive overexpression may thus directly contribute to the maturational arrest in CVI and perhaps to the associated B-cell hyperplasia and lymphoproliferative disease.

Thus, CVI may involve a relatively uniform pathophysiology related to a specific maturational arrest in B-cell development rather than a wide variety of disparate disease processes as previously thought. This primary defect remains to be defined and may include either an intrinsic unresponsiveness of B cells to the cytokines and regulatory cells found in the germinal center or to the dysfunction or destruction of these regulatory cell types. Secondary processes, such as the overexpression of IL-6 and possibly other potent

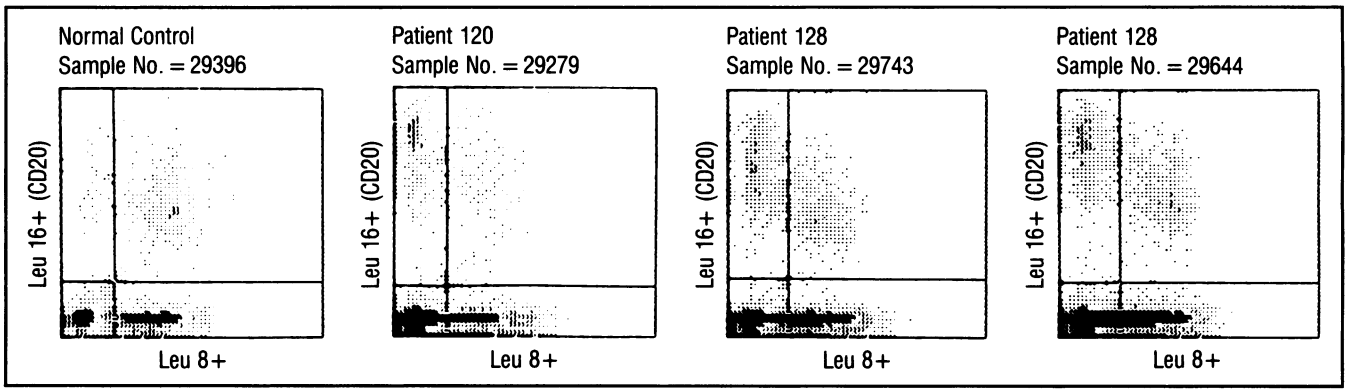

Figure 4.—Dual color flow cytometric analysis is shown of the expression of CD20 and Leu-8 on normal and common variable immunodeficiency (CVI) B cells. In a normal donor, only 10% of B cells ($CD20^+$) are Leu-8^{dim}. In contrast, in the subjects with CVI (120 and 128), most of the $CD20^+$ cells are Leu-8^{dim} (77% and 62%, respectively). Leu-8 expression in $CD20^-$ cells (mainly T cells) is unaffected. Compared with normal subjects, CD20 expression is also elevated in many CVI B cells. The right 2 panels show data from the same subject 4 months apart, showing the reproducibility of the staining pattern (from Saxon et al[36]; adapted with permission from *Journal of Allergy and Clinical Immunology*).

cytokines, may account in a large measure for the widely variable clinical phenotype of the disease.

Molecular Pathways in Immunoglobulin κ Light-Chain Gene Activation

RANDOLPH WALL, PhD*: Immunoglobulin heavy- and light-chain genes are rearranged and expressed at different stages in B-cell development.[58] Pre-B cells contain rearranged μ genes and synthesize Ig μ heavy chains.[59] When pre-B cells develop into B cells, light-chain genes are rearranged and expressed, and the light chains produced are assembled into membrane IgM.[60] This process involves the juxtaposition and activation of several transcriptional regulatory DNA regions found in the regions flanking the light-chain coding segments (Figure 5). The murine pre-B leukemic cell line,

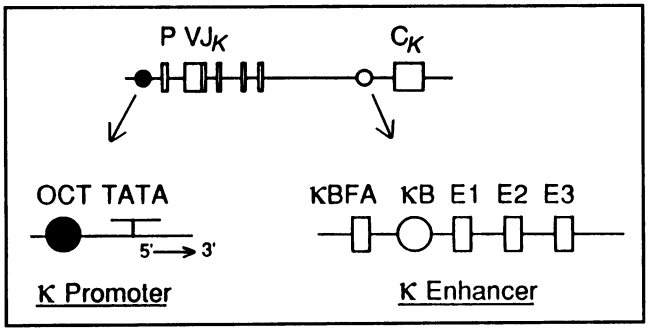

Figure 5.—The schematic shows the transcriptional regulatory elements found in promoter and enhancer regions of the immunoglobulin light-chain κ gene. See text for details.

70Z/3, has been extremely useful in defining the molecular events in Igκ gene activation.[61] The 70Z/3 cells contain an unrearranged κ-chain gene and a productively rearranged light-chain gene with a variable region (V_κ) joined to a joining region (J_κ) upstream of the constant region (C_κ).[62] These κ genes in 70Z/3 cells are normally not expressed. Both κ genes in 70Z/3 pre-B cells, however, are activated and expressed in response to bacterial lipopolysaccharide, IL-1, interferon gamma (IFN-γ), and several physiologic inducers, which mimic different intracellular second messengers.[61,63,64] I will focus on the lymphokine- or mitogen-inducible *trans*-acting factors that bind to κ enhancer DNA sequence motifs to control the developmental stage-specific activation of κ-gene transcription.

Lipopolysaccharide (LPS) induction of 70Z/3 cells results in the appearance of deoxyribonuclease hypersensitivity at a region in both κ genes[62] later shown to be the κ-gene enhancer.[59] The κ enhancer is now one of the best-studied transcription control segments of any mammalian gene. The κ enhancer contains multiple DNA sites that regulate light-chain gene transcription through their interaction with specific *trans*-acting DNA-binding proteins.[65] Some of these are constitutive regulators (for example, E1, E2, E3) that are not regulated by activation.[59,65] Others, such as the κB DNA and κBF-A, are key sites in the induction of κ gene transcription by endotoxin (LPS) and IL-1.[66,67]

The first clue to the molecular processes in κ induction by LPS came from studying the requirements for new macromolecular synthesis in LPS-induced κ-gene transcription in 70Z/3 pre-B cells. It was found that the protein synthesis inhibitor, cycloheximide, failed to block κ induction by LPS.[68] In fact, treatment of 70Z/3 cells with cycloheximide alone activated the transcription of κ genes.[68] This unexpected finding showed that LPS induction of κ-gene transcription occurred through a novel post-translational mechanism involving the activation or modification of existing cell proteins. We predicted that κ-gene expression in untreated 70Z/3 pre-B cells was blocked by labile inhibitory protein(s) and that induction by LPS (or cycloheximide) activated κ transcription by causing turnover of the labile inhibitor(s).

Sen and Baltimore subsequently determined that the induction of κ transcription by LPS and cycloheximide occurred through the post-translational activation of the κ enhancer binding factor, NF-κB.[69] Interleukin 1, PMA (12-*O*-tetradecanoylphorbol 13-acetate), and cyclic adenosine monophosphate (cAMP) analogues also activate NF-κB transcription factor binding to the κB DNA motif.[66] This factor exists in the cytoplasm of uninduced cells in an inactive state complexed to an inhibitor called IκB.[70] Strong evidence indicates that NF-κB activation occurs through protein kinase-mediated phosphorylation of the labile IκB inhibitor.[66] This phosphorylation is activated through distinct intracellular signaling pathways involving either protein kinase C (activated by LPS or PMA) or cAMP-dependent protein kinase (activated by IL-1).[66] NF-κB also can be activated by protein synthesis inhibitors like cycloheximide, presumably through a turnover of the IκB inhibitor.[66,70] Thus, the IκB inhibitor[70] defined by Sen and Baltimore[69] is likely to be the labile inhibitor of κ-gene expression proposed in our earlier study.[68]

Two lines of evidence from our laboratory indicate that NF-κB activation is only one of several collaborative molecular events needed for κ induction after LPS (or IL-1) induction. With Paul Kincade, PhD (Oklahoma Medical Research Foundation), we found that transforming growth factor β (TGF-β) efficiently blocked κ induction.[71] We then determined that NF-κB activation and binding occurred normally in the presence of TGF-β,[72] indicating that the TGF-β sensitive step in κ induction parallels or follows NF-κB activation. The recently discovered LPS-induced factor, κBF-A, provides a compelling candidate target for the inhibitory effect of TGF-β because κBF-A activation follows the early induction of NF-κB binding directly after LPS stimulation.[67]

The second line of evidence comes from our studies analyzing the molecular defects in 70Z/3 mutant cell lines, which are unresponsive to LPS and other inducers used to activate κ-gene transcription. We found two classes of LPS-unresponsive mutants with regard to NF-κB function and activation of κ transduction. One class (composed of three 70Z/3 variant lines) contains functional NF-κB factor (as shown by in vitro activation with detergent), which cannot be induced in vivo by any agent.[73] These variants seem to be defective in the intracellular signaling events leading to IκB phosphorylation. This has been confirmed by showing that purified cAMP-dependent protein kinase added to variant cell extracts activates NF-κB in vitro. In the second class of unresponsive mutant 70Z/3 cells (represented by one 70Z/3 variant cell line), NF-κB is activated normally by LPS, but κ expression is not induced. The behavior of this latter mutant line clearly mimics the inhibitory effect of TGF-β. The defect in this class of LPS-unresponsive 70Z/3 mutant cells may affect κBF-A activation. Experiments in progress are directed at determining if LPS-induced κBF-A binding to the

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κ enhancer is inhibited by TGF-β or deficient in this second class of variant 70Z/3 cells that activate NF-κB normally.

Interferon gamma is another potent inducer of κ-gene transcription in 70Z/3 cells.[61] Unlike LPS and other inducers, however, κ induction by IFN-γ is not affected by TGF-β.[71] Because of this, it seemed likely that NF-κB might not be induced by this lymphokine. Subsequent experiments confirmed that NF-κB is not activated in 70Z/3 cells expressing κ after IFN-γ induction.[72] These results indicate that the induction of κ transcription by LPS (or IL-1) proceeds by a different molecular pathway than that used by IFN-γ. The finding that mutant cell lines of 70Z/3, selected for a loss of responsiveness to LPS, retain inducibility of κ expression by IFN-γ[73] is consistent with this proposal.

What DNA sequences control κ-gene induction by IFN-γ? One candidate is the octamer motif in the κ promoter, which binds a transcription factor found in all cells (called oct-1) and an LPS-induced factor restricted to B cells (called oct-2).[74] The genes encoding the oct-1 and oct-2 factors have been cloned,[74] but it is unlikely that the oct-2 factor is involved in IFN-γ induction because it is not increased by IFN-γ treatment.[72] A promising candidate DNA sequence for mediating IFN-γ induction of κ transcription is the κBF-A site in the κ enhancer, which is notably similar to a DNA sequence motif known to be involved in IFN-γ induction of major histocompatibility complex class I genes and shown to bind IFN-γ–induced *trans*-acting factors. We have recently determined that this sequence in the κ enhancer binds an IFN-γ–induced factor, which is different from the LPS-induced κBF-A factor binding to this same sequence (R. Wall, PhD, unpublished data, June 1991). This new finding suggests that LPS and IFN-γ activate different *trans*-acting DNA-binding factors that bind to the same DNA sequence. This mimics the situation with the octamer DNA motif whose activity is determined by different DNA-binding factors reacting with one DNA sequence.[74]

Even with the present state of knowledge of the molecular events in κ light-chain gene activation, several issues remain to be resolved before the different molecular pathways to κ-gene expression are fully delineated. B cells and plasma cells, which constitutively express κ genes,[66] contain an active DNA-binding form of NF-κB. It is not known how this constitutively activated form of NF-κB is generated. The recent finding of a second κ enhancer segment located far downstream of the C_κ region adds further complexity to be resolved.[75] Clearly studies on the κ enhancers and their roles in κ induction and expression will continue to yield interesting surprises.

Genetically Engineered Antibody Molecules

SHERIE L. MORRISON, PhD*: Antibodies have long been appreciated for their specificity in binding their ligand, an antigenic determinant. Because of this specificity, antibodies seem ideal for discriminating between the minor differences that occur between cell-surface antigens of tumor cells versus normal tissues. A second, perhaps equally important, characteristic of antibody molecules is their ability to carry out biologic effector functions, such as complement activation and antibody-dependent cellular cytotoxicity. The combining specificity of the antibody molecule can therefore be used to target these naturally occurring effector functions to particular cellular or subcellular locations. Alternatively, antibodies can be used to deliver cytotoxic agents.

Antitumor antibodies were initially obtained from immunized animals, but because different antisera represented pools of heterogeneous antibodies with differing specificities and effector functions, it was difficult to carry out reproducible studies using serum as the source of antibodies. A significant breakthrough was the development of the hybridoma technology, whereby it became possible to generate a potentially endless supply of monoclonal antibodies of defined binding specificity.[76]

Even the monoclonal antibodies produced by hybridoma cell lines, however, have some properties that make them less than ideal: It has been difficult to produce monoclonal human antibodies,[76] and the isotype of the resulting antibodies often is inappropriate for the desired biologic properties.[77] Genetically engineered antibodies produced by expressing cloned genes in the appropriate cell type provide an approach for producing antibodies with superior properties. It is now possible to produce chimeric antibodies with gene segments derived from diverse sources. Because genes can be modified before they are expressed, constant regions with improved biologic properties can be produced. In addition, molecules that bind antigen, but that are never found in nature, such as fusions of antibodies with nonantibody proteins, can be manufactured.

All antibodies have a basic structure of two pairs of identical heavy (H) and light (L) chains joined together by disulfide bonds to form a bilaterally symmetric structure (Figure 6). The polypeptide chains fold into globular domains separated by short peptide segments. The *N*-terminal domain of each chain constitutes the variable region that carries the antigen combining site, which determines the specificity of the antibody. The remainder of the antibody constitutes the constant (C) region, which is responsible for the effector functions, such as Fc-receptor binding, complement fixation, catabolism, and placental transport. Immunoglobulins with different constant regions and therefore of differing isotypes—in humans they are IgM, IgD, IgG1-4, IgA1, IgA2, and IgE—show different biologic properties. In some isotypes a hinge region separates C_H1 and C_H2 and provides the molecule with segmental flexibility. Antibodies are glycoproteins with the carbohydrate content varying among different isotypes. One uniform feature of all IgG isotypes is that each H chain contains a single carbohydrate moiety at Asn 298 in C_H2, which is essential for some effector functions.

Antibodies are unusual proteins in that the genes that encode them must be somatically assembled to produce a functional protein. For a variable region to be expressed, a rearrangement of DNA must take place and the expressed variable region positioned next to a J segment for the L chain or a DJ segment for the H chain. The domain structure seen in the antibody molecule is reflected in the structure of the genes that encode it, with each antibody domain being encoded by a discrete exon (Figure 6).

One approach to producing improved antibody molecules is to use recombinant DNA techniques and gene expression to produce antibodies with improved antigen binding specificities and effector functions. An advantage of this approach is not being limited to producing antibodies as they exist in nature. Instead, antibody molecules can be designed to have optimized properties, such as binding specificity pharmacokinetics, and effector functions, such as complement activa-

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tion and Fc-receptor binding. In addition, novel functions can be introduced into the antibody molecule that are not normally found there. Mouse-human chimeric antibodies, in which the variable region is derived from a murine hybridoma cell line and the constant regions are human, provide reagents with reduced immunogenicity when used in vivo in humans.

To produce genetically engineered antibody molecules, the following must be available:

- A recipient cell that will produce a functional antibody molecule;
- A means to efficiently introduce DNA into the recipient cells; and
- Expression vectors that permit the expression of the antibody genes and the isolation of the rare recipient cells expressing the introduced genetic information.[78]

Gene transfection into eukaryotic recipients is one way to produce genetically engineered antibodies.[77] Appropriate mammalian cells for immunoglobulin gene expression are myeloma and hybridoma cell lines, which are capable of high-level expression of endogenous heavy- and light-chain genes; these cells lines can also glycosylate, assemble, and secrete functional antibody molecules. Transfected genes are not always expressed at the level found in hybridomas and myelomas. When all of the important features controlling the expression of transfected genes are understood, however, there is no reason that high levels of antibody expression by gene transfection cannot be achieved routinely. Several myeloma cell lines are available for gene transfection and expression. The cell lines routinely used to produce transfectoma cell lines are the non-Ig-producing hybridoma cell-line parents, SP2/0 and P3X63.Ag8.6.5.3.

Several methods are available for introducing foreign DNA into eukaryotic cells. Although lymphoid cells are inefficient in taking up and expressing calcium phosphate-precipitated DNA, both electroporation[78] and protoplast fusion[79,80] are effective methods of introducing genes into lymphoid cells. There is variability in transfection frequency between different lymphoid cell lines and even between clones of the same cell line. With both electroporation and protoplast fusion, transfection frequencies between 10^{-3} and 10^{-6} can be achieved routinely. Thus, it is feasible to isolate the desired transfectant cell line or transfectomas.

Even under optimal conditions, gene transfection into eukaryotic cells is an inefficient procedure, with only rare recipient cells becoming stably transfected. Thus, vectors must be used that contain biochemically selectable markers, which permits the selection of the rare, stably transfected cell lines. The most commonly used markers are genes derived from bacteria that permit cells expressing them to survive in the presence of particular drugs. These markers are dominant drug-resistance markers; that is, the expression of their phenotype is a dominant trait, and they can be used in cells that have not been drug-marked previously. The bacterial genes used in these vectors are provided with a viral (SV40) promoter, splice junction, and polyadenylate addition signal so that they can be expressed in eukaryotic cells. The bacte-

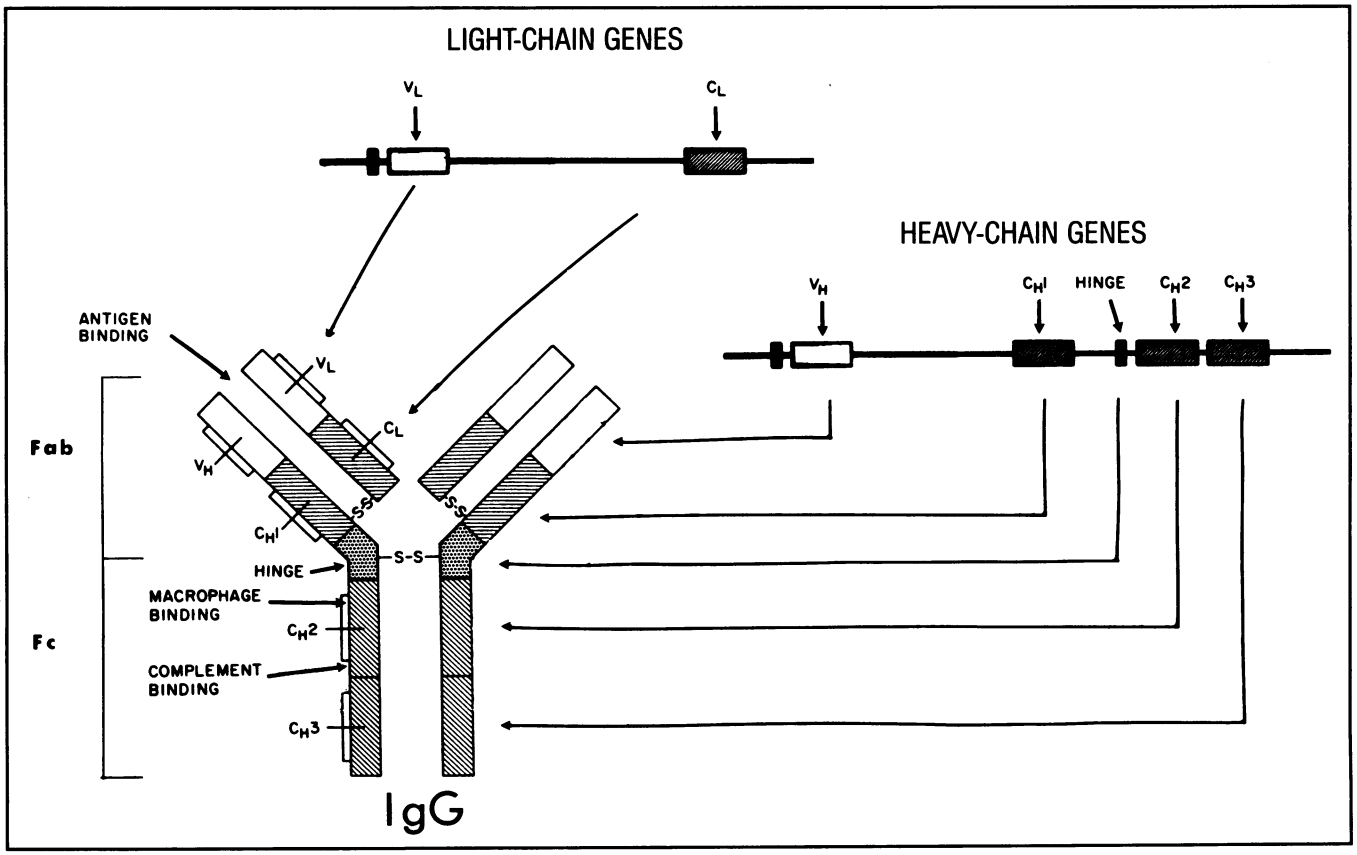

Figure 6.—The structure of an immunoglobulin molecule and the genes that encode it is shown. The regions of the molecule that participate in antigen binding (Fab) or different effector functions (Fc) are indicated. The arrows show the correspondence between the DNA segments and the different domains of the immunoglobulin polypeptide chain that they encode. The hydrophobic leader sequence of both heavy and light chains is removed immediately after synthesis and is not present in the mature immunoglobulin molecule (from Morrison[77]; reproduced with permission from *Science*).

rial genes usually used include the phosphotransferase gene from the Tn5 transposon (designated *neo*),[81] the xanthine-guanine phosphoribosyltransferase gene (*xgprt* or *gpt*),[82] and genes encoding key steps in the synthesis of essential amino acids, *his* and *trp*.[83]

To obtain DNA in sufficient quantities for genetic manipulation, it is essential that the vectors can be propagated as plasmids in bacteria. To achieve this objective, the original plasmids contain the origin of replication and β-lactamase gene from the plasmid pBR322. Bacteria propagating these plasmids can be selected for their resistance to ampicillin. A second family of vectors contains the origin of replication from the plasmid pACYC184 and the chloramphenicol acetyltransferase gene[84]; these plasmids are propagated in bacteria selected for resistance to chloramphenicol. With both vectors, it is easy to obtain large quantities of DNA for in vitro manipulation. Because pBR322 and pACYC184 have compatible replication origins, both plasmids can be maintained together at high copy number within the same bacterium. When protoplast fusion is used, both vectors can be introduced simultaneously into a recipient cell.

To create functional antibody molecules, the genes encoding heavy and light chains must be transfected into the same cell, and both polypeptides must be synthesized and assembled. Several methods have been used to achieve this objective. Both the heavy- and light-chain genes have been inserted into a single vector and then transfected[85]; this approach generates large, cumbersome expression vectors, and further gene manipulation of the vector is difficult. A second approach is to transfect sequentially the heavy- and light-chain genes, using different drug resistance markers to select for the expression of the different vectors.[86] Sequential selection of transfectants is time-consuming. Alternatively, both genes can be introduced simultaneously on different DNA fragments using either electroporation or protoplast fusion and using the compatible plasmid vectors described earlier; this approach is usually the most efficient in creating complete antibody molecules.

To produce antibodies of interest, it is necessary to obtain the gene sequences for the variable regions of interest and then to join them to the appropriate constant region in an expression vector. The variable regions desired for expression can be obtained from the appropriate antibody-producing cell line as either complementary DNA or genomic clones. By obtaining variable regions from human antibody-producing cell lines, it is possible to produce completely human antibodies. Thus, this approach is also appropriate for rescuing low-production human hybridomas and lymphoblastoid lines and for obtaining isotype switch variants of human immunoglobulin genes.

A major reason for developing Ig gene transfections and expression systems is to be able to engineer and express novel hybrid, chimeric, and mosaic genes using recombinant DNA techniques. This technology should have an important effect on our understanding of the structures and mechanisms involved in the biologic functions of antibody and should provide invaluable reagents.

Genomic clones of Ig variable- and constant-region genes are easy to manipulate using genetic engineering techniques because of their division into exons. The distinct Ig polypeptide domains are each encoded by a discrete exon; for example, the variable *and* constant domains of the light chain, the variable region, the hinge, and the three constant-region domains of the IgG heavy chain are each encoded by distinct exons. The intervening DNA sequences (introns) separating each domain provide ideal sites for manipulating the antibody gene shown in Figure 6. Because the intervening sequences are removed by splicing from the mature mRNA, alterations within them will not affect the structure of the protein. In addition, the RNA splice junction between variable- and constant-region exons in heavy- and light-chain genes is conserved, making it easy to manipulate the Ig structure by deleting, exchanging, or altering the order of exons.

The exon structure of the Ig gene can be exploited to construct antibody-cassette expression vectors. Oligonucleotide linkers can be used to flank the constant-region exons with unique restriction sites.[84] In addition, linkers can be placed between the domains of the human IgG heavy-chain genes, making exon and domain shuffling of these straightforward.[86] Oligonucleotide-directed mutagenesis can also be used to produce mutations in the Ig genes.

Biologic effector functions, such as complement fixation, antibody-dependent cellular cytotoxicity, and Fc-receptor binding, are mediated by the heavy-chain constant region, and different isotypes have different biologic properties. Although some structures important for these activities have been defined, the exact molecular correlates of many antibody effector functions remain unknown. With the availability of recombinant DNA technology and gene transfection, the study of structure-function relations within the antibody molecule can now be approached systematically. Chimeric proteins in which the murine variable region is expressed joined to different human constant regions show the biologic properties previously defined for naturally occurring nonchimeric antibodies. Therefore, chimeric antibodies can be used to determine the amino acid sequences responsible for the effector functions of antibody molecules. The ultimate objective of these experiments is to design and produce human immunoglobulin molecules with improved effector functions.

All IgG molecules contain a consensus glycosylation sequence (Asn-X-Thr-, where X represents any amino acid) in the C_H2 domain. The presence of this carbohydrate is important for some of the effector functions of Ig, including Fc-receptor binding and complement activation[87]; however, aglycosylated antibodies are properly assembled, secreted, and bind antigen and *Staphylococcus* protein A. The serum half-life in mice of an aglycosylated chimeric human IgG1 was the same as the wild type (6.5 days), but aglycosylated IgG3 has a shorter half-life (3.5 days) than does wild-type IgG3 (5.1 days). Thus, the absence of carbohydrate profoundly changes some properties of the antibody molecule while leaving others intact.

A property of antibody molecules critical for their in vivo function is the ability to bind to cellular Fc receptors. Chimeric IgG1 and IgG3 bind the high-affinity Fc receptor (IgFcg1) with a binding constant of 1×10^{-9} mol per liter^{-1}; chimeric IgG4 binds with approximately tenfold reduced affinity, and binding by chimeric IgG2 is not detectable. Using site-directed mutagenesis, residue 235 of mouse IgG2b has been pinpointed as interacting with this Fc receptor.[86] Equivalent experiments are in progress with chimeric human antibodies.

Novel proteins that possess the binding specificity of antibody molecules can be created by replacing the constant-region DNA sequence of an Ig molecule with a sequence

derived from another molecule, such as an enzyme, toxin, growth factor, or other biologic response modifier.[89-92] Such molecules have potential use in immunoassays, diagnostic imaging, and immunotherapy.

The possible uses of genetically engineered monoclonal antibodies are many. Perhaps the most immediate advantage of chimeric monoclonal antibodies will be the reduction of the human antimurine antibody response. The human antimurine response creates two problems: the potential of an allergic response with anaphylaxis and the rapid clearance of the administered antibody, preventing most of the antibody from reaching the tumor site. Another advantage of chimeric antitumor antibodies is the potential for enhancing effector cell killing and complement-mediated tumor cell killing. By using genetically engineered monoclonal antibodies, it is also feasible to modify the Ig molecule to alter pharmacokinetics. The plasma clearance of an unconjugated antibody can be slowed down so that it will have the opportunity to mediate antibody-dependent cellular cytotoxicity or complement-mediated cytolysis, or speed up the plasma clearance of a monoclonal antibody conjugated to a radioisotope, in which the circulating conjugate may cause damage to normal cells.

Finally, antitumor monoclonal antibodies may be modified so that they can act as more efficient vehicles for the delivery of antitumor drugs, toxins, radionuclides, or biologic response modifiers. This can be achieved either by directly ligating the antitumor agent into the Ig molecule or by ligating efficient linkers for drugs, radioisotopes, or bioresponse modifiers into the Ig molecule.

Many possible applications of chimeric or genetically engineered antibodies exist in addition to their use as anticancer agents. Monoclonal and chimeric antibodies to cell surface antigens have been produced that are important for immune interactions. These antibodies have great potential as immune response modifiers to facilitate organ transplantation and address problems of autoimmunity. It is also possible that antibodies may be developed to approach the problem of immune nonreactivity, such as is seen in the acquired immunodeficiency syndrome. Chimeric antibodies also may have applications in the treatment of infectious diseases where they should be far superior to antiserum derived from heterologous sources and should address the heterogeneity and limited availability of antiserum derived from human sources. Chimeric antibodies also have great potential for use as vaccines devised to exploit the anti-idiotypic network. The possible uses of chimeric antibodies and antibodies modified by recombinant DNA techniques are limited only by the imagination of the investigators exploiting this family of reagents.

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