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Expression of Recombinant Immunoglobulin Genes to Produce Novel Molecules with Specific Functions

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Introduction

Recombinant antibodies, which are the products of immunoglobulin (Ig) genes constructed in vitro and expressed in transfected cells, represent the 'next generation' of antibodies compared to those produced by hybridomas. The technology used to produce hybridomas was introduced in 1975 [1] and made possible the immortalization of cells that produce antibodies directed against specific antigens. More recently, by combining genetic engineering techniques with gene transfer methods it has been possible to construct Ig genes of predetermined specificity and express these genes in myeloma cells [2–18].

In this review I present some of the results obtained with recombinant antibodies and discuss their significance in our understanding of Ig regulation of expression and structure-function relationships. The methods used to produce recombinant antibodies will be presented briefly since detailed reviews of the techniques involved have appeared elsewhere [19–21]. Finally, some of the applications and potential uses of recombinant antibodies in diagnostics, immunotherapy, and protein purification will be discussed.

Antibodies are tetrameric molecules that consist of two heavy (H) chains joined to two light (L) chains by disulfide bonds, and held together by inter-heavy chain disulfide linkages. Both H and L chains consist of a variable (V) and a constant (C) region. Antibodies have two functional elements: the Fab fragment, which includes V_H and V_L and the carboxy-terminal portion of C_H1, and the Fc fragment, which consists of the remaining C_H domains. Fab is responsible for idiotype and antigen binding while Fc determines isotype and effector functions. These protein domains are determined by the genetic elements that make up the Ig genes (fig. 1). Three genes, V, D, and J (for variable, diversity and joining) combine to form the rearranged V_H gene while only a V and J gene combination produces the rearranged V_L gene. The juxtaposition of these genetic elements during B cell differentiation contributes to the diversity of the V regions in creating the antigen binding site [for reviews see refs. 22–24].

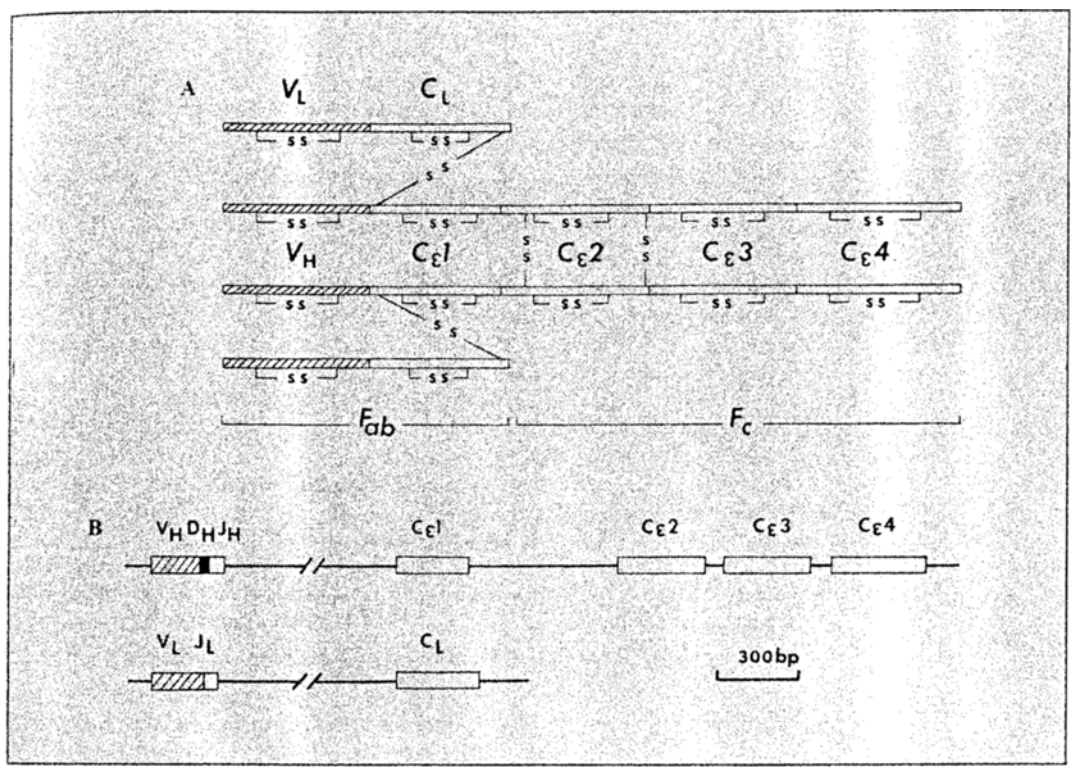

Fig. 1. A Schematic diagram of an IgE molecule with V regions cross-hatched and C regions open; intra- and interchain disulfide bonds are shown. The Fab and Fc portions are indicated by brackets below the antibody. **B** Organization of rearranged Ig genes. The upper diagram is for a switched ε gene and the lower for L chain genes. Exons are shown as boxes: V = cross-hatched; D_H = solid black; J and C = open. The major introns between the V and C regions are 2.1–2.3 kb for κ genes, 1.2–1.3 kb for λ genes, and 6.5 kb for C_H genes.

Experimental Design

Expression of recombinant antibodies involves construction of rearranged V genes proximal to C genes in a shuttle vector and transfection of these genes usually into myeloma cells to obtain measurable levels of secreted antibodies.

Types of Recombinant Ig Genes. Recombinant antibodies can be produced from V_H genes, from myelomas or hybridomas, combined with different isotype C_H genes to achieve intraspecies isotype switching [14, 15, 17, 18]. Interspecies V-C combinations result in chimeric antibodies [25–29]. Usually a murine V region is joined with a human C region to generate a 'nearly human' antibody of known specificity. Novel chimeric antibodies or hybrid molecules can be produced by joining V_H genes with C_L genes [30], by synthetically constructing a novel murine-human hybrid V gene [31], and by replacing C_H exons with different exons coding for nonantibody functions [32]. In addition to novel rearrangements, site-directed mutagenesis can be used to modify novel or existing Ig genes [33].

Vectors. Recombinant Ig genes are usually cloned into shuttle vectors that allow one to do the molecular genetic manipulations in vitro and then analyze and

amplify the genes in *Escherichia coli* prior to introducing the genes into the eukaryotic cells. The shuttle vector usually includes marker genes that can be selected for expression in *E. coli* and in mammalian cells. The most commonly used vectors are the pSV2 vectors [34, 35] and their derivatives [21]. A polyoma virus shuttle vector has also been used [11] with the advantages that (1) it replicates in most cells to generate more copies of the transferred genes and (2) transcription can initiate from polyoma regulatory sequences, obviating the need to clone the Ig control sequences along with the V and C genes. Retroviral vectors that transfer genes at high efficiency into a wide variety of mammalian cells [27, 36–38] may be useful in investigating Ig gene expression in different developmental stages of lymphoid cells and nonlymphoid cells.

Transfection. A variety of methods have been used successfully to introduce cloned Ig genes into mammalian cells. These include DNA transfer mediated by calcium phosphate precipitation and DEAE-dextran [for details see ref. 20], protoplast fusion [for details see ref. 21] and electroporation [8]. Transfection frequencies (10^{-3}–10^{-5}) depend on the vector, the recipient cell and the transfection method used.

Expression in Myeloma Cells. Myelomas are plasma cell tumors and therefore efficiently express Ig genes and secrete assembled antibodies. Expression in myelomas (or hybridomas) is efficient because (1) the Ig regulatory signals for transcription, mRNA splicing and translation are properly recognized, (2) the polypeptides are posttranslationally modified (glycosylation), which is important for effector functions [39], (3) the H and L chains are properly assembled into tetrameric molecules that are (4) efficiently secreted, thus simplifying antibody purification. In addition, IgM contains a covalently linked J chain which a hybridoma can provide [4]. Even novel Fab-like dimers [30, 32] have been assembled and secreted from transfected myeloma cells.

Although Ig molecules and Fc fragments can be produced in *E. coli* and yeast, posttranslational modification of the polypeptides, assembly and secretion are inefficient or absent in these systems [40–46]. Therefore, Ig molecules produced in this way require more difficult purification procedures and/or in vitro reconstitution and modification to produce functional antibody molecules.

Analysis of Transfectants for Ig Gene Expression. Cells can be assayed 48–72 h after transfection for the presence of mRNA specified by the transfected DNA (transient expression). Alternatively, the transformed cells can be grown in selective media (usually for 2 weeks) to obtain stable transformants that express the transfected marker gene(s). Cloned stable transformants can be grown in the absence of selective media. Transcription of transfected genes can be rapidly assayed using the cytoplasmic RNA dot blot method (fig. 2). Technically more difficult but qualitatively more sensitive approaches are Northern blot analysis and S1 nuclease mapping of transcription products to analyze the types of mRNAs produced by the transfected genes.

Analysis of the protein products from transfectants can be done using standard biochemical and immunochemical techniques including immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE), Western blotting (fig. 2), two-dimensional PAGE, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA). In addition, biological assays of effector functions can be used [17, 18, 26, 28]. The antibody produced can often be purified and concentrated using standard immunoprotein purification methods.

Regulation of Ig Expression

Our understanding of the regulation of Ig gene expression has increased substantially by experiments using recombinant Ig genes. Furthermore, by introducing Ig genes into a variety of cell lines, the role of these elements in the cell-specific and tissue-specific expression of Ig genes has been elucidated.

Many of the early investigations of recombinant Ig gene expression were done with κ light chain genes. Ochi et al. [3] showed that a transfected κ chain clone is expressed in a light-chain defective mutant hybridoma and functional antibody production is restored to the transfected hybridoma cells. They extended this work [4] by showing that a complete IgM could be produced in cells transfected with both cloned μ heavy chain and κ light chain genes. Rice and Bal-

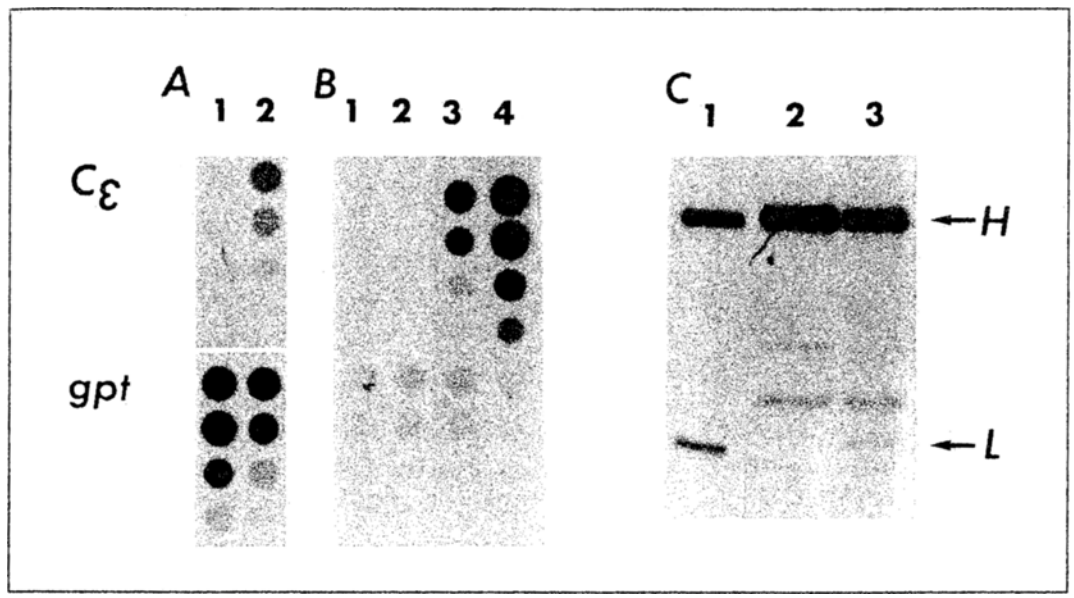

Fig. 2. **A** RNA dot blot for transient expression in: 1 = myeloma J558L cells transformed with the vector pSV2*gpt* and 2 = J558L cells transformed with a pSV2*gpt* vector containing a recombinant V_H-C_ε gene. Four dilutions (1:4, 1:16, 1:64 and 1:256 from top to bottom) were probed with DNA for the C_ε gene (upper half) and the vector's *gpt* gene (lower half). **B** RNA dot blot for: 1 = untransfected J558L cells; 2 = J558L stably transformed with pSV2*gpt*; 3 = J558L stably transformed with a pSV2*gpt*-V_H-C_ε clone, and 4 = an IgE-producing hybridoma. **C** Western blots of affinity-purified IgE from: 1 = an IgE-producing hybridoma; 2 = J558L cells stably transformed with a pSV2*gpt*-V_H-C_ε clone; 3 = J558L cells stably transformed with pSV2*gpt*-V_H-C_ε and pSV2*neo*V_κ-C_κ clones. Arrows mark heavy (H) and light (L) chains' positions. The other two bands seen in lanes 2 and 3 may be proteolytic cleavage products of the H chain. [For details see ref. 18.]

timore [2] showed that a transfected κ chain clone is expressed in Abelson murine leukemia virus-transformed lymphoid cells where it assembled with γ2b heavy chains to form a complete IgG2b. They further showed that mitogen stimulation of the transfected cells increased expression of both κ mRNA and protein, thus demonstrating regulated expression of the introduced gene. Oi et al. [5] also showed synthesis of a mouse myeloma κ chain from a transfected gene. This κ chain assembled with a γ1 heavy chain provided by the hybridoma recipient cells which was then secreted as IgG1. Light chain synthesis was not detected in a transformed thymoma cell line, indicating tissue-specific regulation of the introduced gene. Rat myeloma cells also did not synthesize the transfected mouse κ light chain. However, whether this is due to differences in interspecies regulation is unclear since others showed transcription of a human κ gene in mouse pre-B cells [8]. These early experiments led to the notion that the tissue specificity of Ig gene expression is regulated, at least in part, at the gene level.

The existence of regulatory DNA sequences for both light and heavy chain genes has been documented using recombinant Ig genes. By using different Ig gene constructs and deletion mapping, the promoters, enhancers and an intragenic control sequence have been located [2–14, 47, 48]. The contributions of each of these elements to expression in lymphoid cells of different stages of differentiation and in nonlymphoid cells has also been clarified [4–14, 47, 48]. Furthermore, sequences that regulate mRNA processing have been mapped [15, 16].

The promoters, DNA sequences responsible for initiation of transcription, for both IgH and IgL chains are located proximal to the rearranged V genes. Transcriptional enhancers, sequences first defined in viral systems, strongly stimulate transcription from promoters and are required for accurate transcription. The Ig enhancers are located in the major introns between the most 3′ J element and the switch recombination region, upstream of the C genes. While promoters exert their effects on the transcribed genes in a directional manner, enhancers can act in either direction and at distances several hundred to several thousand base pairs from the promoter.

To examine the DNA sequences involved in regulation of Ig gene expression various constructs of Ig genes are introduced into cells and the transfected cells are generally assayed for transient expression of mRNA using S1 nuclease mapping to determine the quality and quantity of Ig-specific mRNA present. Using this approach, both mouse and human κ light chain enhancers were mapped by analyzing transcripts produced from complete and major-intron deleted κ gene clones [7–9].

A similar set of constructs were used to demonstrate and locate the H chain enhancer. Gillies et al. [12] located the IgH enhancer by deletion mapping and by demonstrating enhancer activity when the enhancer-containing fragment was placed in novel positions relative to a complete IgM gene. They also showed the IgH enhancer (1) stimulates transcription activity from a heterologous SV40 promoter, (2) acts in a B-cell-specific manner, and (3) shares homology with nucleotide sequences found in viral enhancers. Banerji et al. [47] used a different approach to demonstrate the presence of the IgH enhancer. They linked the IgH enhancer to the SV40 T antigen gene and detected transient expression of the T antigen in transfected cells by indirect immunofluorescence. Using this technique, they demonstrated that the IgH enhancer behaves like the SV40 enhancer with the important exception that its activity was restricted to lymphoid cells. Using similar constructs, but with the enhancer linked to the β-globin gene, they showed by S1 mRNA mapping that transcripts produced using the IgH enhancer were identical to those from the SV40 enhancer. Their results show that the IgH enhancer activity is tissue-specific and demonstrate that the IgH enhancer acts independently of the structural gene that it affects.

To investigate the contributions of the light chain promoter and enhancer to tissue-specific expression of κ genes, Foster et al. [11] measured κ transcription from three independent constructs where the κ gene was linked to enhancers from the κ gene, a polyoma virus, and Moloney murine sarcoma virus (MSV). Although transcription initiating at the κ promoter was observed in myeloma cells for all three enhancers, little or no κ transcription occurred when the con-

structs were transfected into mouse liver or fibroblast cells where the polyoma and MSV enhancers are known to function. They conclude that the κ promoter is intrinsically inactive in nonlymphoid cells. Interestingly, only low level expression of transfected κ gene was seen in T cells, despite their lymphoid origin. Gopal et al. [48] also concluded that the κ promoter contributes to tissue-specific expression. They constructed a series of plasmids that linked the κ promoter to either the gene for chloramphenicol acetyl transferase or the *neo* gene that confers neomycin resistance to transformed cells. When these constructs, which included a 'neutral' enhancer from Harvey murine sarcoma virus, were transfected into myeloma, fibroblast and mouse L cells efficient expression of the chloramphenicol acetyl transferase or *neo* genes was only seen in myeloma cells.

Although a complete κ gene is efficiently expressed in B cells, it is not sufficient by itself to allow expression in nonlymphoid cells (e.g., fibroblasts, liver cells, monkey kidney cells) [5–8, 11]. Similar results have been obtained with complete Ig heavy chain genes expressed in transfected cells [4, 10, 12–14]. Thus, there must be factors present only in lymphoid cells that are required for transcription of Ig genes [49, 50]. Proteins required for Ig regulation can act on interspecies transfected genes (e.g., mouse proteins with human genes) and are present in pre-B cells, a stage of immunocyte development that precedes κ gene rearrangement [8].

Control signals important in Ig mRNA processing have also been identified using transfected recombinant Ig genes [15, 16]. The switch from membrane-bound to secreted antibody molecules is accomplished by producing alternate forms of mRNA from a single Ig gene. In myeloma cells, secretion-specific Ig mRNA is primarily produced. DNA sequences near the RNA cleavage/polyadenylation site of the secretion-specific mRNA for IgM [15] and IgG2b [16] have been implicated in the regulation of these two mRNA species. Regulatory sequences were located by deletion mapping: when the DNA in the region near the 3′ terminus of the secretion-specific mRNA was deleted the membrane-bound mRNA was primarily produced. DNA sequence analysis revealed a conserved sequence located 3′ of the polyadenylation site of the secreted mRNA. This sequence may form a stem-loop structure in the RNA in which the cleavage site is located in the exposed single-stranded loop. However, this is not the only mechanism for producing secreted Ig, since the sequence is absent in the α gene which produces a secreted IgA.

Chimeric Antibodies and Structure-Function Relationships

Most chimeric Ig genes constructed and expressed to date are of two types: (1) interspecies combinations of rearranged V and C genes to produce antibodies with predetermined functional Ig domains [25–29, 31], and (2) completely novel combinations of an antibody domain with a non-antibody domain [32]. In addition, an intraspecies chimeric molecule combining a heavy chain V domain with a κ light chain domain has been expressed in transfected myeloma cells [30]. This V_HC_κ chimeric could assemble in vivo with a $V_\kappa C_\kappa$ chain to form a secreted molecule with a functional hapten binding site; these dimers are analogous to a Fab fragment except that they have two C_L do-

mains. Secretion of another dimer Fab-like molecule, constructed with a V_H gene, the γ2b C_H1 domain and the 3′ secretion-specific polyadenylation signal sequence, has also been achieved [32].

Interspecies chimeric antibodies generally combine the V domain from a mouse myeloma or hybridoma with a human C domain to produce 'nearly human' antibody molecules of predetermined binding specificity [25–28]. Using chimeric Ig gene constructs, chimeric IgG1 heavy chains [27], chimeric antibodies for the isotypes IgG1 (γ1, κ), and IgG2, (γ2, κ) [25], IgM (μ, κ) [26], and IgE (ε, λ1) [28] were produced. Physiological effector functions (e.g., complement activation for IgM, and triggering histamine release from human basophils for IgE) have been demonstrated for some of the chimeric molecules [26, 28]. A novel chimeric IgE was recently constructed in which the mouse complementarity-determining regions (functional domains within the V region) were used to replace the human complementarity-determining regions in a human V_H gene [31]. This hybrid heavy chain, in combination with the corresponding mouse light chain, acquired the hapten affinity of the mouse antibody. This approach will be useful in defining functional domains of antibody molecules in a highly refined way. Indeed, site-directed mutagenesis to change a single invariant amino acid residue in a mouse V_H chain has been used to alter the hapten binding site while retaining idiotype [33].

A chimeric Ig gene combining a human V_H with a mouse C_κ has been constructed and expressed in mouse myeloma cells [29]. This latter chimera is primarily of interest in demonstrating that the human IgH promoter can function in mouse cells, opening up the possibility of efficient expression of human antibody genes in mouse cells.

Novel recombinant Ig genes have been constructed in which V_H-C_H1 genes (coding for an Fab) have been fused to a prokaryotic nuclease gene or the mouse c-*myc* gene [32]. In the first case, functional properties of both domains were retained on the secreted antibody-like molecule: the Fab portion bound to hapten and the nuclease domain digested DNA. In the second case, the Fab bound to hapten and the c-*myc* portion displayed antigenic determinants. One requirement of the gene replacing Fc exons in these novel chimeras is that the gene product be active as a monomer, contain no thiol residues that would interfere with assembly, and be able to fold properly as a protein domain.

Potential Uses and Prospects for Recombinant Antibodies

Monoclonal antibodies have been used in diagnostics and therapeutics almost since their production was first described [1, 51–53]. Although human monoclonals are preferred for therapeutic use, they are technically difficult to produce, to say nothing of the ethical considerations involved in acquiring human cells specific for a given antigen. Mouse monoclonal antibodies are often ineffective in immunotherapy because the patient develops antibodies against them after repeated injections [51, 53]. However, by combining mouse V region genes with human C region genes and producing chimeric 'nearly human' monoclonal antibodies these difficulties may be overcome. Because the C region is of human origin it is expected to be nonimmunogenic; the mouse V region should be no more immunogenic than its

human counterpart. However, the latter assumption may not prove to be true [53].

Cancer Immunotherapy. Monoclonal antibodies that recognize unique cell surface markers could be used to selectively destroy cells in vivo. An obvious candidate for this approach is cancer treatment [51, 52]. However, cancer immunotherapy may be limited to myelomas and leukemias due to the clonal nature of the diseases. Furthermore, antibody therapy may be effective only when the target cells are accessible; deep tissue tumors may not respond to treatment.

Organ Transplant Immunotherapy. Chimeric antibodies could be used to specifically neutralize alloreactive T cells and thus suppress transplanted organ rejections. This would be an improvement on conventional steroid treatment which causes overall immunosuppression. Chimeric antibody immunosuppression could presumably be used longer and safer since the patient would be able to respond immunologically to pathogens even while receiving treatment.

Treatment of Autoimmune Disorders with Recombinant Antibodies. For an autoimmune response that is limited to one or a few idiotypes (Id), anti-Id antibody (or Fab) could be used to bind to the autoimmune antibodies and thus depress the destructive immune response. Similarly, internal image anti-Id antibody (or Fab) which mimics the antigen could be used to compete with the self-destructive antibody. Whether such treatment will trigger a polyclonal expansion of other idiotype-producing cells is unknown. Finally, if specific regulatory idiotypes that suppress autoimmune responses could be identified, their genes could be cloned or synthesized and expressed to produce regulatory antibodies for immunotherapy of autoimmune disorders.

Vaccine Development. An anti-idiotype antibody that is an internal image of the pathogen's antigen could elicit an immune response to the pathogen without the dangers inherent in the use of pathogen-derived vaccines.

Novel Recombinant Antibody-Like Molecules. In diagnostics, linkage of a specific Fab to a domain with a readily assayed function could result in new ELISA reagents. For immunotherapeutics, the Fab recognizing specific cells could be linked to toxins to deliver chemotherapeutic agents exclusively to the target cells (the 'magic bullet' approach).

Treatment of Allergies with Recombinant Antibodies or Fab-Like Molecules. Fcε fragments can compete with allergen-specific IgE for receptors on human basophils in vivo and in vitro and block allergen-mediated histamine release [44–46]. Creation of genes, with secretion-specific signals in the constructs, coding for truncated Fcε-like antibodies, should make the secretion of such therapeutic dimers possible. Such molecules could be used in treatment without regard for an individual's specific allergen. For allergen-specific treatments, Fab-like molecules could be produced that would compete with the patients' IgE for allergen.

Hybrid Recombinant Antibody Molecules for Protein Purification. Linking Fab-coding genes with genes for other non-antibody products could be used to produce hybrid proteins which could be separated later into their two protein components by proteolytic cleavage. This approach could be especially useful for producing proteins that are normally available in small quantities (e.g., lymphokines). By linking the structural gene to the Ig regulatory sequences, protein expression would be under Ig control. Since up to

1% of myeloma protein may be Ig, this could produce significant quantities of a hybrid protein. Furthermore, by linking the protein to Fab the hybrid protein could be secreted from the myeloma and purified by affinity chromatography using an antigen column. In addition, linkage of Fab genes to genes coding for unknown proteins can be used to produce antisera against the gene products [32] which may help to identify the naturally occurring proteins, and define their function and cellular location.

Finally, recombinant Ig gene expression will continue to contribute to our understanding of Ig gene regulation, as well as elucidating the relationship between Ig structure and function. Use of this developing technology should generate exciting results and novel products in the next decade. Imagination may become the limiting factor in the use of these techniques to produce a new antibody and chimeric molecules.

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