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[54] **HYBRID DNA PREPARED BINDING COMPOSITION**

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[57] **ABSTRACT**

Proteinaceous binding compositions are prepared employing hybrid DNA technology, where the variable region polypeptides of immunoglobulins are substantially reproduced to provide relatively small protein molecules having binding specificity and lacking the undesirable aspects of the heavy regions of immunoglobulins. The compositions find a wide range of use, particularly for physiological purposes for diagnosis and therapy. The binding compositions may be modified by labeling with radioisotopes, fluorescers, and toxins for specific applications in diagnosis or therapy.

2 Claims, No Drawings

HYBRID DNA PREPARED BINDING COMPOSITION

This application is a division of application Ser. No. 08/394,923, filed Feb. 23, 1995, now abandoned, which is a continuation of application Ser. No. 08/210,540, filed Mar. 17, 1994, now abandoned, which is a continuation of application Ser. No. 08/061,760, filed May 13, 1993, now abandoned, which is a continuation of application Ser. No. 07/928,526, filed Aug. 11, 1992, now abandoned, which is a continuation of application Ser. No. 07/740,862, filed Jul. 31, 1991, now abandoned, which is a continuation of application Ser. No. 07/235,835, filed Aug. 18, 1988, now abandoned, which is a continuation of application Ser. No. 06/558,551, filed Dec. 5, 1983, now U.S. Pat. No. 4,642,334, which is a continuation of application Ser. No. 06/358,414, filed Mar. 15, 1982, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The mammalian immunological system is unique in its broad ability to produce protein compounds having extremely high specificity for a particular molecular structure. That is, the proteins or immunoglobulins which are produced have a conformation which is specifically able to complement a particular structure, so that binding occurs with high affinity. In this manner, the mammalian immune system is able to respond to invasions of foreign molecules, particularly proteins in surface membranes of microorganisms, and toxins, resulting in detoxification or destruction of the invader, without adverse effects on the host.

The primary immunoglobulin involved in the defensive mechanism is gamma-globulin (IgG). This immunoglobulin, which is a glycoprotein of about 150,000 daltons, has four chains, two heavy chains and two light chains. Each of the chains has a variable region and a constant region. The variable regions are concerned with the binding specificity of the immunoglobulin, while the constant regions have a number of other functions which do not directly relate to the antibody affinity.

In many situations it would be desirable to have molecules which are substantially smaller than the immunoglobulins, while still providing the specificity and affinity which the immunoglobulins afford. Smaller molecules can provide for shorter residence times in a mammalian host. In addition, where the immunoglobulin has to be bound to another molecule, it will be frequently desirable to minimize the size of the final product. Also there are many economies in being able to produce a smaller molecule which fulfills the function of a larger molecule.

There are situations where it will be desirable to be able to have a large number of molecules compactly held together. By having smaller molecules, a greater number can be brought together into a smaller space. Furthermore, where the binding molecule can be prepared by hybrid DNA technology, one has the opportunity to bind the binding portion of the molecule to a wide variety of other polypeptides, so that one can have the binding molecule covalently bonded at one or both ends to a polypeptide chain.

Where immunoglobulins are used in in vivo diagnosis or therapy, antisera from an allogenic host or from a monoclonal antibody may be immunogenic. Furthermore, when conjugates of other molecules to the antibody are employed, the resulting conjugate may become immunogenic and elicit

host antibodies against the constant region of the immunoglobulin or against any other part of the molecule.

It is therefore important that methods be developed which permit the preparation of homogeneous compositions having high specificity for a particular ligand, while avoiding the shortcomings of complete immunoglobulins, and providing the many advantages of lower molecular weight.

2. Description of the Prior Art

Discussions concerning variable regions of heavy and light chains of immunoglobulins may be found in Sharon and Givol, Biochem. (1976) 15:1591–1594; Rosemblatt and Haber, Biochem. (1978) 17:3877–3882; and Early and Hood, Genetic Engineering (1981) 3:157–188. Synthesis of part of a mouse immunoglobulin light chain in a bacterial clone is described by Amster et al., Nucleic Acids Res. (1980) 8:2055–2065. See also the references cited throughout the specification concerning particular methodologies and compositions.

SUMMARY OF THE INVENTION

Novel protein complexes are provided by producing homogeneous compositions defining the variable regions of the light and heavy chains of an immunoglobulin, which individually or together form a specific binding complex to a predetermined haptenic or determinant site. Employing hybrid DNA technology, cDNA is tailored to remove nucleotides extraneous to the variable regions of the light and heavy chains. The resulting tailored ds cDNA is inserted into an appropriate expression vector which is then introduced into a host for transcription and translation. The resulting truncated light and heavy chains define at least a major portion of the variable regions and are combined to form a complex capable of specifically binding to a predetermined haptenic site with high affinity.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The subject invention concerns a hybrid DNA strategy for the preparation of specific binding polypeptides, normally comprised of two different polypeptide chains, which together assume a conformation having high binding affinity to a predetermined ligand or haptenic site thereof. The polypeptide chains form binding sites which specifically bind to a predetermined ligand to form a complex having strong binding between the ligand and the binding site. The binding constant or avidity will generally be greater than 10^5, more usually greater than 10^6, and preferably greater than 10^8. The haptenic binding site or determinant binding site of the polypeptide chain may be associated with a hapten or antigen.

One or both of the different polypeptide chains derived from the variable region of the light and heavy chains of an immunoglobulin may be used to provide specific binding to a ligand. For the most part each of the polypeptide chains of the light and heavy variable regions would be employed together for binding to the ligand. In describing this invention, it will be understood that while the two different chains are indicated as forming a complex, either of the chains could be used individually, where feasible due to sufficient binding affinity of the particular chain to the reciprocal ligand.

The two polypeptide chains which, individually or together, provide the compositions of this invention will form a receptor site, analogous to the binding site of an immunoglobulin. The composition will be referred to as an

rFv with the individual chains referred to as L-rFv or H-rFv. The L- and H- designations will normally mean light and heavy respectively, but in some instances the two chains may be the same and derived from either the light or heavy chain sequences. The polypeptide chains of the rFv will generally have fewer than 125 amino acids, more usually fewer than about 120 amino acids, while normally having greater than 60 amino acids, usually greater than about 95 amino acids, more usually greater than about 100 amino acids. Desirably, the H-rFv will be from about 110 to 125 amino acids while the L-rFv will be from about 95 to 115 amino acids.

The amino acid compositions will vary widely, depending upon the particular idiotype involved. Usually there will be at least two cysteines separated by from about 60 to 75 amino acids and joined by a disulfide bond to form cystine. The two chains will normally be substantial copies of idiotypes of the variable regions of the light and heavy chains of immunoglobulins, but in some situations it may be sufficient to have combinations of either the light or the heavy variable region chains.

In many instances, it will be desirable to have one or both of the rFv chains labeled or bound to a support. Various labels may be employed, such as radioisotopes, fluorescers, or toxins. In other situations, one or both of the chains may be bound to an inert physiologically acceptable support, such as synthetic organic polymers, polysaccharides, naturally occurring proteins, or other non-immunogenic substances.

In some situations, it may be desirable to provide for covalent crosslinking of the two chains, which could involve providing for cysteine residues at the carboxyl termini. The chains will normally be prepared free of the constant regions, including or being free of all or a portion of the J region. The D region will normally be included in the transcript of the H-rFv.

For the most part only a relatively small percent of the total amino acids will vary from idiotype to idiotype in the rFv. Therefore, there will be areas providing a relatively constant framework and areas that will vary, namely, the hypervariable regions.

The C-terminus region of the rFv will have a greater variety of sequences than the N-terminus and, based on the present strategy, can be further modified to permit variation from the naturally occurring heavy and light chains. A synthetic oligonucleotide can be employed to vary one or more amino acids in a hypervariable region.

The preparation of the rFv employing hybrid DNA technology will now be described in greater detail.

The preparation of the rFv will be divided into three parts: (1) isolation of appropriate DNA sequences; (2) introduction of the DNA sequences coding for the members of the rFv into an appropriate expression vector; and (3) expression and isolation of the mimetic variable regions of the light (L-rFv) and heavy (H-rFv) chains to provide the rFv.

I. Isolation of Appropriate DNA Sequences.

In preparing the DNA sequences, a source of the genes encoding the variable region will be required. The variable regions may be derived from IgA, IgD, IgE, IgG or IgM, most commonly, from IgM and IgG. This can be achieved by immunizing an appropriate vertebrate, normally a domestic animal, and most conveniently a mouse. The immunization may be carried out conventionally with one or more repeated injections of the immunogen into the host mammal, normally at two to three week intervals. Usually three days after the last challenge, the spleen is removed and dissociated into single cells to be used for cell fusion to provide hybridomas.

The immunogen will be the antigen of interest, or where a hapten, an antigenic conjugate of the hapten to an antigen.

In order to prepare the hybridomas, the spleen cells are fused under conventional conditions employing a fusing agent, e.g. PEG6000, to a variety of inter- or intra- species myeloma cells, particularly mouse cells such as SP-2/0, NS-1, etc. and then suspended in HAT selective media. The surviving cells are then grown in microtiter wells and immunologically assayed for production of antibodies to the determinant site(s) of interest.

Assays for antibodies are well known in the art and may employ a variety of labeled antigens or haptens, where the labels are conveniently radioisotopes, fluorescers, enzymes, or the like. Other techniques may also be employed, such as sandwich techniques involving two antibodies, one bound to a support and the other being labeled. The cells from microtiter wells scored as positive are cloned either by limiting dilution or cloning in soft agar. The resulting cloned cell lines are then propagated in an appropriate nutrient medium and, if necessary, may be stored frozen in liquid nitrogen.

After selection of a particular cell line providing a monoclonal antibody of interest, the cells are expanded. Conveniently, the cells may be grown to a density of about 1×10^6 cells/ml in a 1 L culture. The cells are then harvested by centrifugation and lysed.

In order to obtain the desired DNA sequence, one can look to either the gene expressing the variable region or the messenger RNA, which expresses the variable region. The difficulty with employing genomic DNA is in juxtaposing the sequences coding for the variable region, where the sequences are separated by introns. One must isolate the DNA fragment(s) containing the proper exons, excise the introns and then splice the exons in the proper order and orientation. For the most part, this will be difficult, so that the alternative technique employing the messenger RNA will be the method of choice.

Where the messenger RNA is to be employed, the cells will be lysed under RNase inhibiting conditions. The messenger RNA has the advantage that the mature messenger is free of introns, so that the sequence is continuous for the entire variable region. Difficulties with messenger RNA have been encountered, due to incomplete reverse transcription but these difficulties can be minimized. The first step is to isolate the messenger RNA. Conveniently, messenger RNA can be separated from other RNA because of its polyadenylation, employing an oligo-(dT) cellulose column. The mixture of messenger RNAs will be obtained free of other RNA. The presence of messenger RNAs coding for the heavy and light chain polypeptides of the immunoglobulins may then be assayed by hybridization with DNA single strands of the appropriate genes. Conveniently, the sequences coding for the constant portion of the light and heavy chains may be used as probes, which sequences may be obtained from available sources (see, for example, Early and Hood, Genetic Engineering, Setlow and Hollaender eds. Vol. 3, Plenum Publishing Corp., New York (1981), pages 157–188.)

Whether the messenger RNA codes for the correct immunoglobulin may be determined by in vitro translation employing a rabbit reticulocyte cell-free extract (Pelham and Jackson, Eurp. J. Biochem. (1976) 66:247–256). The resulting translation product may then be isolated by employing antibodies specific for one or more of the regions of the

chain of interest, for example, using rabbit anti(mouse IgG) where the chains are derived from mouse immunoglobulin.

The immunoprecipitate may be further analyzed by polyacrylamide gel electrophoresis, and the presence of complexes determined by using radiotagged receptors for antigen-antibody complexes, such as *S. aureus* protein A, Rf factor, or the like. In addition, RNA blot hybridization can be employed to further insure that the correct messenger RNA is present.

The crude mixture of mRNA sequences containing the desired mRNA sequences will be treated as follows. In order to enhance the probability that full length cDNA is obtained, the method of Okayama and Berg, Mol. Cell. Biol. (1982) may be employed. Alternatively, the methods described by Efstradiadis and Villa-Komaroff (1979) in Genetic Engineering: Principles and Methods 1, Setlow and Hollaender, eds., New York, Plenum Press, pages 15–36, or Steinmetz et al. (1981) Cell 24:125–134, may be employed. The first strand of cDNA is prepared employing a virus reverse transcriptase in the presence of primer. A second strand may then be prepared employing reverse transcriptase, the Klenow fragment of DNA polymerase I or T4 polymerase. If necessary, the resulting ds cDNA may then be treated with a single-strand-specific nuclease, such as S1 nuclease for removal of single stranded portions to result in ds cDNA, which may then be cloned.

II. Preparation of Genes Coding For L-rFv and H-rFv and Introduction into an Expression Vector For Amplification

A wide variety of vectors may be employed for amplification or expression of the ds cDNA to produce the light and heavy chains of the immunoglobulin. A vector having an appropriate restriction site is digested with the appropriate endonuclease. The ds cDNA obtained from the reverse transcription of the mRNA may be modified by ligating linkers, treatment with terminal transferase or other techniques to provide staggered (complementary) or blunt ended termini. The vectors may have one, two or more markers for selection of transformants. Desirably, the vector will have a unique restriction site in one of multiple markers, so that the transformants may be selected by the expression of one marker and the absence of expression of the other marker. Various markers may be employed, such as biocide resistance, complementation of an auxotroph, viral immunity, or the like.

After transforming an appropriate host with the ds cDNA prepared from the mRNA, e.g. *E. coli, B. subtilis, S. cerevisiae*, etc., in accordance with conventional ways, the transformants are plated and selected in accordance with the particular markers. The resulting colonies are screened, by restriction electrophoretic pattern, hybridization to a labeled probe or by any other conventional means. See, for example, Hanahan and Meselson (1980), Gene 10:63–67. One procedure employs colony hybridization, where the transformants are grown on a solid medium to produce colonies. Cells from the colonies are transferred to a nitrocellulose replica filter, the transferred cells incubated for further growth, lysed, dried and baked. The replica filter is then hybridized with appropriate radio-isotope labeled probes. conveniently, there are readily available probes for the determinant sites present in the constant regions of a variety of mammalian immunoglobulins. The colonies may be probed based on the nature of the particular immunoglobulin, as well as the different determinant sites, which may be present with the particular immunoglobulin.

The host colonies, usually bacterial, which have DNA which hybridizes to either the light or heavy chain probes are picked and then grown in culture under selective pressure. In order to maintain selective pressure, it is desirable that the vector which is employed have biocidal, particularly antibiotic, resistance. After sufficient time for expansion of the host, the host cells are harvested, conveniently by centrifugation. The hybrid plasmid DNA may then be isolated by known procedures. (Gunsalus et al., J. Bacteriol. (1979) 140:106–133).

The isolated plasmid DNA is then characterized by restriction enzyme digestion and DNA sequence analysis. These analyses insure that the isolated cDNA clones completely encode the variable region and, optionally, the leader sequences for the light or heavy chain of the desired immunoglobulin. Furthermore, by having a restriction map of the variable regions and leader sequences, as well as the flanking sequences, one can determine the appropriate restriction sites for excising a DNA fragment which will allow for appropriate modification of the DNA sequence for insertion into a vector and expression of the polypeptide of interest. Where no unique restriction site is available at an appropriate position in the flanking regions, partial digestion may be employed, with selection of fragments having the variable region and, optionally, the leader sequence intact. Where the 5' and 3' flanking regions are too extended, these can be chewed back using Bal 31 to varying degrees by varying the period of digestion.

Furthermore, by knowing the DNA sequence of the coding strand in the region of the C-terminus of the heavy and light chain variable regions, a stop codon may be introduced at the C-terminus by the following procedure of in vitro mutagenesis. The cDNA is restricted with the appropriate enzyme(s) to provide a variable region coding segment with additional 5' and 3' flanking sequences. This segment is purified, for example, by gel electrophoresis, gradient density centrifugation, etc. After isolating the desired segment, the two strands of the segment are dissociated, conveniently by boiling. Alternatively, the undesired strand of the intact cDNA-plasmid clone may be nicked and digested.

A synthetic, single-stranded DNA oligomer is prepared, conveniently by synthesis, which will have at least about 12 nucleotides, more usually about 15 nucleotides, and will generally have fewer than about 50 nucleotides, usually fewer than 30 nucleotides, since a more extended oligomer is not required.

Where heteroduplexing is involved, the non-complementary nucleotides will usually be flanked by at least about three, more usually at least about six nucleotides complementary to the hybridized strand. The heteroduplexing oligonudleotide will be complementary to the sequence at or about a significant juncture i.e. between the leader sequence and the variable region or the variable region and the constant region. The synthetic DNA oligomer will be complementary to the coding ("sense") strand of the variable-region sequence, but altered to encode a termination codon at the C-terminus of the variable region. That is, the oligomer will be complementary to the coding strand except at or about the amino acid which is involved at the juncture of the variable region and the D-, J- or C-regions of the light and heavy chains, particularly at or intermediate the D- or J-regions or intermediate the J-region, or at the J-region and C-region juncture. It is intended that there will be some variation in the polypeptides which are prepared, so far as extending beyond the variable domains or not including all of the amino acids at the C-terminus of the variable region.

An excess amount of the oligomer is combined with the denatured strands of the restriction fragment under sufficiently stringent hybridization conditions. Thus, the oligomer specifically heteroduplexes to the complementary portions of the coding strand, while providing one or more stop and/or nonsense codons to insure the termination of expression at the desired amino acid at the C-terminus.

After sufficient time for hybridization at the desired level of stringency, sufficient amounts of the four deoxynucleotides are added in conjunction with the Klenow fragment of DNA polymerase I. A strand complementary to the coding sequence of the variable-region and any 5'-flanking sequence is synthesized by enzymatic elongation of the primer resulting in a sequence complementary to the strand to which the oligonucleotide is bound. The single-stranded DNA sequence on the coding strand located 3' to the region hybridized to the synthetic oligonucleotide is degraded by the 3'–5' exonuclease activity of the DNA polymerase. In this manner, ds cDNA is obtained which specifically codes for the variable-region and upstream flanking regions associated with the light and heavy chains. Each of the heavy and light chains is encoded to terminate expression at a predetermined codon in the V, D or J region.

The resulting heteroduplexed blunt-ended ds CDNA fragments are then employed for preparation of homoduplexed ds CDNA coding for the light and heavy variable regions with the stop codons at the desired sites. Conveniently, the blunt ended fragments are modified as described previously, e.g. joined to linkers which code for restriction sites which are absent in the variable region sequences, or may be tailed e.g. polyG or polyC, or used directly for insertion. With restriction site linkers, after insertion of the fragment into an appropriate vector having complementary termini, the fragment can be recovered by restriction at the linker sites. The linkers are joined to the coding sequences with an appropriate ligase, e.g. T4 ligase, the resulting fragment restricted to provide cohesive ends, and the product annealed to the complementary ends of a vector.

At this stage, the vector which is employed provides for amplification and convenient isolation of transformants having the variable region coding sequence insert. Numerous vectors for amplification in bacteria or other hosts exist such as pBR322, pSC101, pRK290, 2 μ-plasmid, etc. The hybrid plasmid containing the mismatched sequences will replicate in the host to generate two different plasmid molecules, one with the original sequence and one with the "tailored" or "site mutated" sequence derived from the synthetic oligonucleotide. Therefore, each transformant colony is grown in small (approximately 2 ml) culture for plasmid isolation.

The transformants are grown, the plasmid DNA isolated in accordance with known procedures, and used for a second cycle of transformation to provide individual clones replicating the tailored sequence. The clones may be screened by filter blot hybridization, probing with a labeled synthetic oligonucleotide which will include the synthetic oligonucleotide employed in tailoring the variable region sequence, or other convenient technique. Thus, plasmids are obtained having ds cDNA flanked by appropriate restriction sites and having a stop codon at a predetermined site.

Having now defined the 3'-terminus of the coding strand or, alternatively, the C-terminus amino acid, the 5'-region or N-terminus of the polypeptide is now defined. Of course, the particular order in which the two termini are modified is primarily one of convenience, and can even be done simultaneously, where primer repair is used at the 5'-end of the coding strand in conjunction with site mutation at the 3'-end.

Different strategies may be evolved, depending upon the nature of the host in which expression is to be obtained, and whether such host recognizes the leader sequence as a secretory signal for secretion of the polypeptide with concomitant removal of the leader sequence polypeptide. Where this opportunity is not available, the strategy will involve removal of the leader sequence to provide a start codon at the 5'-terminus of the sequence of the coding strand coding for the variable region, which sequence can be inserted into an expression vector, so as to be under the control of a predetermined promoter and ribosomal start site.

Based on the sequence of the leader region or the sequence coding for the N-terminus of the variable region, different oligonucleotides for homo- or heteroduplexing can be prepared.

Where the leader sequence is retained, primer repair is employed to remove the 5'-flanking sequence of the coding strand. When the primer repair of the N-terminus is performed simultaneously with the C-terminus mutagenesis, after treatment with the DNA polymerase, the resulting partial double stranded DNA will be treated with a 5'-3'-single strand exonuclease to remove the 5'-flanking region as well as a ligase to provide for covalent linking of the replicated strand to the N-terminus oligonucleotide.

Where the leader sequence is to be removed, in vitro mutagenesis is employed to introduce an f-met codon at the N-terminus of the DNA sequence coding for the variable region.

Alternative strategies may be employed for recovering the desired ds cDNA and performing the in vitro mutagenesis. If useful restriction sites are distant from the coding regions, the plasmid may be digested with the appropriate restriction endonuclease, followed by digestion with a double-strand exonuclease e.g. Bal 31. The resulting ds cDNA may be cloned and the proper sequence selected and modified, as appropriate, as described above. If the non-coding flanking region at the 5'-terminus of the coding strand is too long, it may be digested with an endonuclease, where a convenient restriction site is available or by digestion with an exonuclease e.g. Bal 31.

By repeating the above described procedure for modifying the 3'-terminus, except that the oligonucleotide is now complementary to the non-coding (nonsense) strand, and includes an initiation codon at the 5'-end (primer repair) or within the oligonucleotide (in vitro muta-genesis), the 5'-terminus of DNA sequence encoding the variable regions may be tailored. Normally, the oligonucleotide homoduplexes for primer repair and heteroduplexes for in vitro mutagenesis. In this way, "tailored" ds-cDNA is obtained which has start and stop codons properly positioned to define the variable regions of both the light and heavy chains of immunoglobulins. The resulting blunt ended ds cDNA may be modified, e.g. by addition of linkers, to provide complementary termini for insertion into an expression vector in proper spacing to the regulatory signals which are ligated to the ds cDNA or are present in the vector.

The ds cDNA is now ready to be used for insertion into a vector for expression. As distinguished from the earlier vectors, which were solely concerned with replication of the ds cDNA, the vector which is employed at this stage requires the presence of the regulatory signals for transcription and translation.

A vector is chosen having an appropriate promoter, as well as other transcriptional regulatory signal sequences, such as an operator, attenuator, or activator. Also, the vector will have been at least partially sequenced, so as to deter-

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