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[54] **POLYNUCLEOTIDES ENCODING MODIFIED ANTIBODIES WITH HUMAN MILK FAT GLOBULE SPECIFICITY**

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[57] **ABSTRACT**

A polynucleotide encodes a modified antibody, or single chains thereof. The modified antibody has a non-antigen-binding peptide such as the constant regions of an antibody of a first species, peptide hormones, enzymes, and peptide transmitters; and a binding peptide such as the unsubstituted light and heavy chains of the variable region of an antibody of a second species which binds the human milk fat globule (HMFG) antigen. The non-antigen-binding peptide is linked to at least one chain of the binding peptide, the chains may be linked to one another at a site other than the antigenic binding site, and at least one chain of the binding peptide has 1 to 46 amino acids substituted with amino acids selected from specific ones assigned to each site. The polynucleotide and other products are also provided in the form of compositions, with a carrier. The polynucleotides may be RNAs and DNAs, and are also provided as hybrid vectors carrying them, and as transfected cells expressing the modified antibodies or their single chains.

63 Claims, No Drawings

POLYNUCLEOTIDES ENCODING MODIFIED ANTIBODIES WITH HUMAN MILK FAT GLOBULE SPECIFICITY

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the diagnosis, immunization, and therapy of neoplastic tumors, particularly carcinomas by means of specifically targeted analogue peptides comprising amino acid sequences encompassing, for instance, the complementarity determining regions (CDRs), and analogues of the variable (F_v) region of anti-carcinoma antibodies, among others. The carcinoma specific peptides are provided as a single amino acid chain having the specificity of the non-human antibody F_v regions of the light or heavy chains, or as paired chains, either by themselves or bound to other polymers such as synthetic polymers or oligopeptides resulting in chimeric antibodies, and more particularly analogues of human/non-human chimeric antibodies or other polymeric constructs. The analogue peptides comprise sequences derived from variable regions of heterogeneous antibodies specific for human carcinoma antigens that elicit a lesser immunological response in humans than whole heterologous non-human antibodies. The peptides of the invention are useful for in vivo and in vitro diagnosis and therapy of carcinoma. Anti-idiotype polypeptide and analogues thereof are suitable for immunizing humans or other animals against carcinoma. Polynucleotide sequences, hybrid vectors and host cells encoding the analogue peptide and anti-idiotype polypeptides, hybrid vectors and transfected hosts are useful for preparing the peptides disclosed herein.

2. Description of the Background

Carcinomas result from the carcinogenic transformation of cells of different epithelia. Two of the most damaging characteristics of carcinomas are their uncontrolled growth and their ability to create metastases in distant sites of the host, particularly a human host. It is usually these distant metastases that cause serious consequences to the host, since frequently the primary carcinoma may be, in most cases, removed by surgery. The treatment of metastatic carcinomas, that are seldom removable, depends on irradiation therapy and systemic therapies of different natures. The systemic therapies currently include, but not fully comprise, chemotherapy, different immunity-boosting medicines and procedures, hyperthermia and systemic monoclonal antibody treatment. The latter can be labeled with radioactive elements, immunotoxins and chemotherapeutic drugs.

Radioactively labeled monoclonal antibodies were initially used with success in lymphomas and leukemia, and recently in some carcinomas. The concept underlying the use of labeled antibodies is that the labeled antibody will specifically seek and bind to the carcinoma and, the radioactive element, through its decay, will irradiate the tumor in situ. Since radioactive rays travel some distance in tumors it is not necessary that every carcinoma cell bind the labeled antibody. The specificity of the monoclonal antibodies will permit a selective treatment of the tumor while avoiding the irradiation of innocent by-stander normal tissues, that could be dose limiting. Chemotherapy produces serious toxic effects on normal tissues, making the chemotherapy of carcinomas less than desirable, and the use of radiolabeled monoclonal antibodies a valid alternative.

Non-human antibodies raised against human epitopes have been used for the diagnosis and therapy of carcinomas as is known in the art. Also known are the methods for

preparing both polyclonal and monoclonal antibodies. Examples of the latter are BrE-2, BrE-3 and KC-4 (e.g., U.S. Pat. Nos. 5,077,220; 5,075,219 and 4,708,930.

The KC-4 murine monoclonal antibody is specific to a unique antigenic determinant, the "antigen", and selectively binds strongly to neoplastic carcinoma cells and not to normal human tissue (U.S. Pat. No. 4,708,930 to Coulter). The antigen appears in two forms in carcinoma cells, only the smaller of these forms being expressed in the cell membrane. The larger form appears only in the cytoplasm and has an approximate 490 Kdalton molecular weight (range of 480,000–510,000). The second form occurs at a higher density of expression, is found both in the cytoplasm and the membrane of carcinoma cells and has an approximate 438 Kdalton molecular weight (range of 390,000–450,000) as determined by gel electrophoresis with marker proteins of known molecular weights. Labeled KC-4 was applied to the diagnosis and medical treatment of various carcinomas, particularly adenocarcinoma and squamous cell carcinoma regardless of the human organ site of origin.

The BrE-3 antibody (Peterson et al., Hybridoma 9:221 (1990); U.S. Pat. No. 5,075,219) was shown to bind to the tandem repeat of the polypeptide core of human breast epithelial mucin. When the mucin is deglycosylated, the presence of more tandem repeat epitopes is exposed and the binding of the antibody increases. Thus, antibodies such as BrE-3 bind preferentially to neoplastic carcinoma tumors because these express an unglycosylated form of the breast epithelial mucin that is not expressed in normal epithelial tissue. This preferential binding combined with an observed low concentration of epitope for these antibodies in the circulation of carcinoma patients, such as breast cancer patients, makes antibodies having specificity for a mucin epitope highly effective for carcinoma radioimmunotherapy. A ^{90}Y-BrE-3 radioimmunoconjugate proved highly effective against human breast carcinomas transplanted into nude mice. Human clinical studies showed the ^{90}Y-BrE-3 radioimmunoconjugate to considerably reduce the size of breast tumor metastases without any immediate toxic side effects. Moreover, an ^{111}In-BrE-3 radioimmunoconjugate was successfully used for imaging 15 breast cancer patients, providing excellent tumor targeting in 13 out of 15 of the patients. Out of all the breast tumor metastases occurring in another study, 86% were detected by ^{111}In-BrE-3. Unfortunately, 2 to 3 weeks after treatment, the patients developed a strong human anti-mouse antibody (HAMA) response that prevented further administration of the radioimmunoconjugate. The HAMA response, which is observed for numerous murine monoclonal antibodies, precludes any long-term administration of murine antibodies to human patients. Similarly, other heterologous antibodies, when administered to humans, elicited similar antibody responses. The anti-heterologous human response is, thus, a substantial limiting factor hindering the successful use of heterologous monoclonal antibodies as therapeutic agents, which could, otherwise, specifically annihilate breast carcinomas, causing little or no damage to normal tissue and having no other toxic effects.

Chimeric antibodies are direct fusions between variable domains of one species and constant domains of another. Mouse/human chimeric antibodies prepared from other types of B cells binding to other types of antigenic determinants have been shown to be less immunogenic in humans than whole mouse antibodies. These proved to be less immunogenic but still in some cases there is a mounted immune response to the rodent variable region framework region (FR). A further reduction of the "foreign" nature of

the chimeric antibodies was achieved by grafting only the CDRs from a rodent monoclonal into a human supporting framework prior to its subsequent fusion with an appropriate constant domain. (European Patent Application, Publication No. 239,400 to Winter; Riechmann, et al., Nature 332:323–327 (1988). However, the procedures employed to accomplish CDR-grafting often result in imperfectly "humanized" antibodies. That is to say, the resultant antibody loses avidity (usually 2–3 fold, at best).

The ligand binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring residues also have been found to be involved in antigen binding (Davies, et al., Ann. Rev. Biochem. 59:439–473 (1990)).

The technologies of molecular biology have further expanded the utility of many antibodies by allowing for the creation of class switched molecules whose functionality has been improved by the acquisition or loss of complement fixation. The size of the bioactive molecule may also be reduced so as to increase the tissue target availability of the antibody by either changing the class from an IgM to an IgG, or by removing most of the heavy and light chain constant regions to form an F_v antibody. Common to all of these potentially therapeutic forms of antibody are the required complementary determining regions (CDRs), which guide the molecule to its ligand, and the framework residues (FRs) which support the CDRs and dictate their disposition relative to one another. The crystallographic analysis of numerous antibody structures revealed that the antigen combining site is composed almost entirely of the CDR residues arranged in a limited number of loop motifs. The necessity of the CDRs to form these structures, combined with the appreciated hypervariability of their primary sequence, leads to a great diversity in the antigen combining site, but one which has a finite number of possibilities. Thus, its hypermutability and the limited primary sequence repertoire for each CDR would suggest that the CDRs derived for a given antigen from one species of animal would be the same derived from another species. Hence, they should be poorly immunogenic, if at all, when presented to a recipient organism.

Accordingly, there is still need for a product of high affinity and/or specificity for carcinoma antigens suitable for the detection and therapy of carcinomas which elicits a lesser antibody response than whole non-human antibodies orchimeric antibodies containing, for instance the entire non-human variable region.

SUMMARY OF THE INVENTION

This invention relates to an analogue peptide or a glycosylated derivative which specifically binds to an antigen found on the surface or in the cytoplasm of carcinoma cells or released by the cells, the analogue peptide consisting essentially of at least one CDR or variable region of the light or heavy chains of an antibody of a first species having affinity and specificity for an antigen found on the surface or the cytoplasm of a carcinoma cell or released by the cells, wherein preferably about 1 to at least 46 amino acids in the FR are substituted per chain with amino acids selected from the group consisting of amino acids present in equivalent positions in antibodies of a second species, or fragments thereof comprising 1 to 3 CDRs per chain and flanking regions thereof, each of about 1 to at least 10 amino acids, alone or with an N-terminal fragment of to about 1 to at least 10 amino acids, combinations thereof, combinations thereof with variable regions or analogues thereof, wherein each analogue peptide is operatively linked to at least one other peptide or analogue thereof, or mixtures thereof. The analogue is also provided as a fusion protein, their corresponding DNAs hybrid vectors, transfected hosts and RNAS. This invention also encompasses a method of producing an analogue peptide or hybrid analogue peptide by recombinant technology. Also provided herein are in vivo an in vitro methods of diagnosing and for the therapy of a carcinoma.

Also disclosed herein is an anti-idiotype polypeptide, comprising polyclonal antibodies raised against the analogue peptide of this invention, monoclonal antibodies thereof, fragments thereof selected from the group consisting of Fab, Fab', $(Fab')_2$, CDRs, variable regions and analogues thereof described above, an anti-carcinoma vaccine, a vaccination kit, a method of vaccinating against carcinoma, and a method of lowering the serum concentration of a circulating antibody with the anti-idiotype polypeptide of this invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire by the inventors to improve on antibody technology suitable for use in diagnostic, vaccine and therapeutic applications. The monoclonal antibodies obtained up to the present time have been prepared by fusing immortalized cell lines with B-cells of non-human origin such as murine, rat, rabbit, goat, and the like. Many of these hybridomas can produce monoclonal antibodies that have desirable binding properties such as high affinity and/or specificity for human carcinoma antigens, and are also produced in large amounts. However, in general, non-human antibodies may only be administered once to humans due to the detrimental effects they produce. This is true for most xenogeneic antibodies being administered to a mammalian animal. For example, the repeated administration of mouse antibodies to a human subject elicits a strong human anti-mouse antibody (HAMA) response, which precludes their further utilization as therapeutic agents in humans. These non-human antibodies initiate an immediate adverse reaction in many patients and are, thus, rendered ineffective as therapeutic agents. Non-human-human chimeric antibodies and non-human CDR "grafted"-human antibodies may have low affinity and/or specificity for their antigens. On the other hand, human monoclonal hybridoma cell lines have not been very stable and have, therefore, not been suitable for the large scale, repeated production of monoclonal antibodies.

The present inventors, thus, have undertaken the preparation of anti-carcinoma human and non-human CDRs and non-human variable regions of antibodies, having affinity and specificity for an antigen found on the surface or the cytoplasm of a human carcinoma cell or released by the cells, wherein about 1 to 46 amino acids in the FR are substituted per chain with amino acids selected from the group consisting of amino acids present in equivalent positions in human antibodies, or fragments thereof comprising 1 to 3 CDRs per chain and flanking regions thereof, each of about 1 to at least up to 10 amino acids, alone or with an N-terminal fragment of about 1 to at least up to 10 amino acids, to lower or even circumvent the anti-xenogeneic response. To preserve substantial binding specificity the present invention utilizes CDRs and/or analogues of varying lengths of the variable regions of light and/or heavy chains of mouse, rat, rabbit, goat, horse, primate such as human and simian, bovine, and guinea pig antibodies, among others.

The present inventors have found, surprisingly, that these analogue antibody fragments substantially preserve the

binding and specificity characteristics of the whole antibody while eliciting a lesser detrimental immunological. However, the simple preservation of the binding region of an antibody does not ensure that the binding characteristics of the antibody will be maintained. Antibodies are glycopolypeptides that are folded into specific conformations. When the glycoside portion of the molecule or portions of the amino acid sequence are perturbed or excised, the folding pattern of the molecule is generally perturbed. Thus, any deletion or modification of sequences of an antibody must be made taking into consideration that its folding-dependent properties may be diminished or even obliterated if the folding is affected, even though the amino acid sequences involved in the binding of the antigen are preserved.

The present inventors have selected the following strategy for the preparation and manufacture of the analogue peptides and hybrid peptides of this invention. The cDNAs that encode the variable chains of an antibody may be obtained by isolation of mRNA from a hybridoma cell and reverse transcription of the mRNA, amplification of the cDNA by PCR and insertion of the DNA into a vector for sequencing and restriction enzyme cutting. The cDNAs encoding the CDR or F_v region fragments of the light (V_L) and heavy (V_H) chains of an antibody having affinity and specificity for a carcinoma cell antigen may be reverse transcribed from isolated mRNA. The variable region cDNAs may then be modified with predesigned primers used to PCR amplify them, cloned, into a vector optionally carrying DNA sequences encoding, e.g., a constant region(s), optionally sequenced, and then transfected into a host cell for expression of the analogue gene products. The binding specificity characteristics of the analogue peptides may be then determined and compared to those of the whole antibodies.

X-ray crystalographic studies have repeatedly demonstrated that the framework structures of the F_Vs of different antibodies assume a canonical structure regardless of the species of origin, amino acid sequence, or ligand specificity. This is generally taken as evidence that the ligand-binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring framework residues also have been found to be involved in antigen-binding. Thus, if the fine specificity of an antibody is to be preserved, its CDR structures, and probably some of the neighboring residues, their interaction with each other and with the rest of the variable domains, must also be maintained. These crystallographic studies point to the possible need for retaining most, if not all, of the many interior and inter-domain contact residues since the structural effects of replacing only a few of them cannot be predicted.

While at first the necessity of keeping these amino acids might seem to defeat the humanization goal of decreasing immunogenicity, the actual number of amino acids that must be retained is usually small because of the striking similarity between human and murine variable regions. Moreover, many, if not most, of the retained amino acids possess side chains that are not exposed on the surface of the molecule and, therefore, may not contribute to the antigenicity.

The challenge in humanizing the variable regions of a non-human antibody, e.g., a murine antibody, thus begins with the identification of the "important" xenogenenic amino acids. "Important" amino acids are those that are involved in antigen binding, contact the CDRs and the opposite chains, and have buried side-chains. Ideally, these residues would be readily identified from a well characterized three-dimensional structure. When direct structural data are not available, it is, fortunately, still possible to predict the location of these important amino acids from the knowledge of other antibody structures, especially those whose variable light and heavy regions belong to the same class. The classes of variable regions can be determined from their amino acid sequence.

A method by which these important amino acids are identified has been described for the case of the amino acids with buried side chains by Padlan, E. A. (Padlan, E. A., "A Possible Procedure for Reducing the Immunogenicity of Antibody Variable Domains While Preserving Their Ligand-Binding Binding Properties", Molecular Immmunology, 28:489–4948 (1991)). Various antibody variable region structures were compared using a computer program that determines the solvent accessibility of the framework residues as well as their contacts with the opposite domain (Padlan, E. A. (1991), supra). Surprisingly, a close examination of the fractional solvent accessibility reveals a very close similarity in the exposure patterns of the V_H and the V_L domains. Put in simple terms, this means that regardless of the particular antibody in question, and of its amino acid sequence, the buried residues occupy the same relative positions in most antibodies.

A similar analysis can be done by computer modeling, to determine which amino acids contact the CDRs and which contact the opposite domain. At this point, the Fab structures that are currently in the Protein Data bank (Bernstein, F. C., et al., J. Mo. Biol. 112:535–542 (1977)) may be examined to determine which FRs are probably important in maintaining the structure of the combining site. Thus, after a close inspection of many high resolution three-dimensional structures of variable regions, the positions of all important framework amino acids, that is, those that contact the CDRS, the opposite domain, and those whose side chains are inwardly pointed, may be tabulated. Keeping these amino acids, as well as those from the CDRs, and finally those FR amino acids that may be involved in ligand binding, should insure to a great extent the preservation of affinity. The precise identification of FR amino acids that are involved in ligand-binding cannot be generalized since it varies for different antibodies. Nevertheless, conservative decisions can be made to preserve the amino acids located in FR that have a high probability of contacting the antigen. These regions are located immediately adjacent to the CDRs and at the N-terminus of both chains, because the surfaces of these regions are contiguous with the CDR surfaces.

Surprisingly, it is possible to keep all of these important amino acids in a heterologous humanized antibody and still increase dramatically the similarity with a human consensus sequence. That is, the final number of amino acids with mouse identities differing from human identities that are kept is typically small. This is usually possible because human frameworks that are similar to the mouse frameworks, especially at the positions of the important amino acids, can be found. This is because many of the important amino acids have the same identities in both mouse and human antibodies.

All the amino acids that are determined to be not important by the method described above may be completely replaced by their corresponding human counterparts. The surface of the finally humanized antibody should look very much like that of a human antibody except for the antigen binding surfaces. The original shape of those binding surfaces, however, is maintained by leaving the internal composition of the antibody intact, preserving inter-domain contacts and by keeping very few key amino acids that contact the CDRs.

a) Choosing the Best Human Framework to Use in the "Humanization" of an Antibody When Its Structure Is Known

At the present time, there are 11 Fab structures for which the atomic coordinates are known and have been placed in the Protein Data Bank as shown in Table 1 below, 2 from human and 9 from murine antibodies.

TABLE 1

Fab Structures for Which Coordinates are in the Protein Data Bank

	ANTIBODY	RESOLUTION (A)	R-VALUE	PDB CODE
HUMAN:	NEWM	2.0	0.46	3FAB
	KOL	1.9	0.189	2FB4
MURINE:	McPC603	2.7	0.225	1MCP
	J539	1.95	0.194	2FBJ
	HyHEL-5	2.54	0.245	2HFL
	HyHEL-10	3.0	0.24	3HFM

TABLE 1-continued

Fab Structures for Which Coordinates are in the Protein Data Bank

ANTIBODY	RESOLUTION (A)	R-VALUE	PDB CODE
R19.9	2.8	0.30	1F19
4-4-20	2.7	0.215	4FAB
36-71	1.85	0.248	6FAB
B13I2	2.8	0.197	1IGF
D1.3	2.5	0.184	1FDL

The contacts between side chains in the variable domains of the 11 Fabs have been collected and are presented in Tables 2 to 4 below. The FR in the V_L domains that contact CDRs are listed in Table 2 below.

TABLE 2

V_L Framework Residues That Contact CDR Residues in Fabs of Known Three-Dimensional Structure

	ANTIBODY										
POSITION	J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	36-71	B13I2	D1.3	NEWM	KOL
1	GLU(2)	ASP(5)	ASP(10)	ASP(3)		ASP(8)	ASP(4)		ASP(11)		
2	ILE(11)	ILE(15)	ILE(17)	ILE(13)	ILE(5)	VAL(9)	ILE(20)	VAL(9)	ILE(10)	SER(3)	
3		VAL(3)	VAL(2)	VAL(3)	GLN(2)	VAL(2)	GLN(2)	LEU(6)		VAL(2)	
4	LEU(7)	MET(6)	LEU(6)	LEU(10)	MET(9)	MET(13)	MET(7)	MET(6)	MET(7)	LEU(4)	LEU(6)
5		THR(1)			THR(1)	THR(2)				THR(1)	
7								THR(4)			
22								SER(6)			
23	CYS(1)	CYS(1)	CYS(2)	CYS(2)	CYS(1)	CYS(1)	CYS(1)				CYS(1)
35	TRP(3)	TRP(2)	TRP(4)		TRP(2)		TRP(6)	TRP(4)	TRP(4)	TRP(1)	TRP(2)
36	TYR(12)	TYR(16)	TYR(8)	TYR(10)	TYR(22)	TYR(13)	TYR(15)	TYR(8)	TYR(14)	TYR(13)	TYR(11)
45					LYS(12)	LYS(5)					
46	PRO(3)	LEU(6)	LEU(4)	ARG(15)	LEU(5)	VAL(14)	LEU(5)	LEU(10)	LEU(6)	LEU(2)	LEU(6)
48	ILE(1)	ILE(1)	ILE(1)				ILE(3)	ILE(2)	VAL(1)		ILE(1)
49	TYR(28)	TYR(29)	LYS(13)	TYR(12)	TYR(40)	TYR(22)	TYR(22)	TYR(16)	TYR(25)		TYR(25)
58	VAL(3)	VAL(3)	ILE(1)	VAL(6)	VAL(6)	VAL(5)	VAL(4)	VAL(5)	VAL(1)		VAL(6)
60		ASP(1)				ASP(2)		ASP(4)			ASP(2)
62				PHE(1)		PHE(1)	PHE(1)				
66										LYS(2)	LYS(11)
67		SER(3)							SER(1)		
69		THR(3)	THR(3)			THR(5)	THR(1)	THR(4)	THR(1)	SER(1)	
70		ASP(2)			ASP(1)		ASP(6)			SER(2)	
71	TYR(14)	PHE(23)	PHE(17)	TYR(17)	TYR(24)	PHE(17)	TYR(17)	PHE(19)	TYR(16)	ALA(3)	ALA(4)
88	CYS(1)		CYS(2)		CYS(1)	CYS(1)	CYS(1)	CYS(1)	CYS(2)		CYS(1)
98	PHE(8)	PHE(8)	PHE(10)	PHE(5)	PHE(8)	PHE(4)	PHE(8)	PHE(14)	PHE(14)	PHE(3)	PHE(7)

Those FR in the V_H domains that contact CDRs are listed in Table 3 below.

TABLE 3

V_H Framework Residues That Contact CDR Residues in Fabs of Known Three-Dimensional Structure

	ANTIBODY										
POSITION	J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	36-71	B13I2	D1.3	NEWM	KOL
1							GLU(3)				
2	VAL(11)	VAL(3)	VAL(8)		VAL(1)		VAL(7)	VAL(3)	VAL(12)		VAL(9)
4	LEU(2)	LEU(5)	LEU(5)		LEU(2)	LEU(1)	LEU(1)	LEU(1)	LEU(1)		LEU(1)
24		THR(2)	VAL(6)			ALA(1)					
27	PHE(3)	PHE(2)		TYR(14)	TYR(11)	PHE(26)	TYR(4)	PHE(4)	PHE(4)	THR(1)	PHE(3)

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