

# Development of More Efficacious Antibodies for Medical Therapy and Diagnosis

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**Two procedures for improving the efficacy of medically important antibodies are described. The first procedure is designed to reduce the immunogenicity of nonhuman antibodies to the barest minimum—the "humanization" is accomplished by transplanting only the specificity-determining residues of the nonhuman antibody onto a human antibody template. The second procedure is designed to permit the easy production of multispecific/multivalent antibodies via heterodimer formation of electrostatically complementary Fc regions. © 1998 Academic Press**

## I. Introduction

Antibodies represent a major factor in our defense against invading pathogens and noxious substances. Antibodies are generated to bind specifically to the foreign substance (antigen) and to neutralize it and facilitate its

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elimination by normal biological processes. Antibodies are multivalent (at least bivalent) molecules, thus they can cross-link antigens, thereby immobilizing them. The binding of antibodies to antigens also may cause the recruitment of other molecules or of certain cells—additional components of the immune system—which would then act to dispose of the invading substance or organism.

All antibodies share the same basic structural unit that consists of two identical heavy chains (MW of each,  $\sim 50,000$  to  $\sim 77,000$ ) and two identical light chains (MW of each,  $\sim 25,000$ ). Each light chain is usually linked to a heavy chain by a disulfide bond and the heavy chains are usually linked together by one or more disulfide bonds. Each chain has variable and constant domains. The N-terminal domain of both light and heavy chains is variable and is followed by one constant domain in the light chain ( $C_L$ ) and by three or four constant domains in the heavy chain ( $C_{H1}$ ,  $C_{H2}$ ,  $C_{H3}$ , and  $C_{H4}$ ) depending on antibody class.

The antibody class, or isotype, is determined by the constant domains. The light chain exists in two distinct isotypes called kappa ( $\kappa$ ) and lambda ( $\lambda$ ). The heavy chain may be  $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , or  $\mu$  type, which defines the antibody class as IgA, IgG, IgD, IgE, or IgM, respectively. IgG is the major antibody class in human serum; IgE is the antibody responsible for allergic reactions. The constant domain of the heavy chain determines the effector function(s) of an antibody, e.g., complement activation, and Fc receptor binding. Different classes have different biological properties.

The variable region contains the antigen-binding site. Each variable domain consists of three hypervariable segments, called the complementarity-determining regions (CDRs) (1), flanked by four relatively less variable framework regions. The antigen-binding site is built mainly with CDR residues, with occasional contribution from neighboring framework residues. The lengths and sequences of the CDRs vary from antibody to antibody, resulting in different antigen-binding specificities. The  $V_L:V_H$  module is often referred to as the Fv fragment and the  $V_L C_L:V_H C_H1$  as the Fab fragment. The  $C_{H2}$  and  $C_{H3}$  domains of the two heavy chains, plus the  $C_{H4}$  in the case of IgE and IgM, constitute the Fc fragment.

The exquisite specificity of the binding of an antibody to its antigen and the ability of the immune system to respond to challenge by all sorts of antigens have found many uses in medical therapy and diagnosis. The use of antivenom against snake bites, antitoxins against bacterial infections, immune serum globulin against certain diseases, and so forth, are some of the well-known uses of specific antisera (see Ref. 2 for a review). Among the more recent uses of antibodies in medicine is the specific targeting of cells or tissues, e.g., tumor cells, either for location (*in vivo* imaging) or for destruction (3).

With the advent of hybridoma technology (4), monoclonal antibodies of virtually any desired specificity can be produced. Further, the development of novel expression systems has permitted the generation of pure antibodies in large amounts. For various reasons, including ethical considerations, monoclonal antibodies are usually obtained from nonhuman sources. Unfortunately, the human immune system will react to, and attempt to eliminate, any nonhuman (or nonself) entity. This necessitates the "humanization," i.e., the reduction of the immunogenicity, of the nonhuman antibodies prior to their use in human patients, especially if the treatment protocol requires protracted use of such molecules. Various procedures have been devised to humanize nonhuman antibodies (5–11). Here, we present a new procedure, currently being developed in our laboratory, that seeks to reduce immunogenicity to the barest minimum while at the same time preserving the antigen-binding properties of the original antibody.

Another topic of interest is the generation of multispecific/multivalent antibodies. A molecule that can bind different ligands has many potential uses. For example, a molecule of a desired reactivity can be brought to close proximity to a target cell by using a bispecific antibody that can bind the molecule via one site and an antigen on the surface of the cell via the other. Likewise, two different cells can be brought together with the use of a bispecific antibody. Further, there are instances when an antigen has only one site (epitope) to which a given antibody type can bind. Such an antigen cannot be cross-linked by antibodies, the binding sites of which have the same binding properties; at least one other antibody type, with a different and nonoverlapping specificity, will be required to cross-link the antigen. In nature, several different antibody types are elicited by a single antigen (in a normal polyclonal response). If an antibody could be engineered so that its binding sites have different specificities, such an antibody could by itself produce the same effect as two, or more, different antibodies. Various techniques are currently in use for the generation of bispecific antibodies (12–26).

Multivalency amplifies the affinity of antibodies for their specific antigens. Usually, antibody–antigen reactions are characterized by nanomolar (or better) affinity constants, when the antigen is a protein. With carbohydrate antigens, the binding constants are often much lower. The binding of an antibody to its antigen can be improved by increasing the number of combining sites on the antibody (the antigen-binding sites) so that, even with a low intrinsic binding affinity per site, the avidity due to the presence of multiple sites can be substantial. This is observed in nature in antibodies of the IgM class, which can have as many as 12 identical antigen-binding sites.

Judicious engineering can generate multispecific and multivalent antibodies. Here, we present a procedure that we are developing for the easy generation of multispecific/multivalent molecules.

## II. Procedure for Reducing the Immunogenicity of a Nonhuman Antibody to a Minimum

The goal of humanization is to produce a molecule that has the same antigen-binding properties as the original antibody, but that is nonimmunogenic in humans. This can be accomplished by transplanting the structures that determine binding properties from the nonhuman antibody to a human scaffold. The feasibility of transplanting a combining site from one antibody to another was recognized (27) as soon as the first antibody structures had been determined.

Various physical and chemical techniques have shown that antigen binding occurs via the variable domains of the light and heavy chains of the antibody. Thus, the first attempt at reducing the immunogenicity of a nonhuman antibody was accomplished by producing a chimera, the variable domains of which were from the nonhuman molecule whereas the constant domains were from a human antibody (28, 29). Such chimerae do possess the same antigen-binding properties as the original molecules, but the nonhuman variable domains usually elicit adverse immune reactions by the host. A major improvement in reducing the immunogenicity of chimeric molecules is achieved by transplanting only the CDRs (5).

Three-dimensional structures obtained by X-ray crystallography are now available for the antigen-binding regions of many different antibodies from different species and with a variety of ligand-binding specificities, many in complex with specific ligand (30). These structures allow us to make some generalizations: (1)  $V_H$  and also  $V_L$  domains have very similar three-dimensional structures regardless of species origin, (2) the framework regions are essentially superposable so that the variable domains might differ in structure only in their CDRs, (3) CDRs, which have the same number of residues and possess certain critical residues, usually have the same loop conformations (31), and (4) the combining site of an antibody is mainly formed by CDR residues, with the occasional involvement of one or two framework residues.

These results strongly suggest that transplantation of only the CDRs and possibly of some framework residues also can achieve a successful transfer of antigen-binding properties. The implications for humanization by CDR grafting (5) are obvious, especially because the CDRs are frequently shared among antibodies from different species (27, 32) and, thus, can be expected to be the least immunogenic of the various parts of an antibody molecule.

Indeed, the successful humanization of numerous nonhuman (usually rodent) antibodies has been accomplished by CDR grafting, although the retention of some nonhuman framework residues also is often required (6–9, 11); those framework residues presumably influence the structure of the CDR loops.

As more structural data become available, however, it is becoming obvi-

ous that not all of the CDR residues are involved in the interaction with the ligand. The antibody residues that have been observed to be in actual contact with antigen, the specificity-determining residues (SDRs), are presented in Table I. It is found that, even in the most extensive interaction with antigen [in the case of antibody NC41 binding to influenza virus neuraminidase (33)], only 22 residues from the antibody participate in the binding. The SDRs, therefore, represent only a small fraction of the CDRs, which could be composed of anywhere from 46 to 100 amino acids [from the authors' survey of human antibody sequences (data not shown)]. The immunogenicity of a humanized antibody can be further reduced by transplanting only those parts of the CDRs that contain the SDRs, and those "abbreviated" CDRs have been defined (34). But, even better, immunogenicity can be reduced to the barest minimum if only the SDRs are transferred to a human scaffold (34).

With SDR transfer, it may be necessary to transplant a few critical framework residues also, but those would be fewer than with CDR grafting. During CDR grafting, all the CDR residues are transplanted, including those that are inward-pointing and thus cannot directly contact the antigen. Therefore, in CDR grafting, the framework residues that contact those inward-pointing CDR residues in the nonhuman antibody may need to be preserved also in the humanized molecule in order to produce the same combining-site structure. In contrast, SDR transfer involves transplantation of residues that are in the main outward-pointing and exposed to solvent. Because there is preservation of interior volume in the variable domains (35, 36), there is then no need to preserve the CDR-contacting framework residues. Nonetheless, some of the CDR loops are deformable and their disposition may be determined by contacts with the framework. Indeed, there are certain framework residues, e.g., residue 71 in the light chain (37) and residue 71 also in the heavy chain (38), that make important contacts with the CDRs and that should probably be preserved in the humanized molecule even when transplanting only the SDRs.

Particularly critical are the framework residues involved in the association of the  $V_L$  and  $V_H$  domains, because the quaternary structure of the antigen-binding region of the antibody can modulate antigen-binding properties (39). Further, the N termini of both light and heavy chains of an antibody are in the periphery of the combining site (40), so that one or both could be involved in antigen binding; both N-terminal residues should be preserved in the humanized version.

If the three-dimensional structure of the antibody-antigen complex is available, the SDRs as well as the critical framework residues can be readily identified. Such structures are available for only a few antibodies. Nevertheless, an attempt can be made to identify the SDRs from the results for other complexes (Tables I and II) and from the analysis of sequences. Indeed, the

TABLE I  
V<sub>L</sub> RESIDUES IN CONTACT WITH THE LIGAND IN MURINE ANTIBODY-LIGAND COMPLEXES OF KNOWN THREE-DIMENSIONAL STRUCTURE<sup>a</sup>

	10	20	27abcdef	30	40	50	CDR2	60	70	80	CDR3	95ab	100	PDB Code
HVHBL-5														
D1.3														3HFL
HVHBL-10														1VFB
D44.1														3HFB
D11.15														1MLC_1
F9.13.7														1JBT
NC41														1NCA_1
NC10														1NCA
ES.2														1NWB
409.5.3														1DVF
N10														1IAT
Je142														1NSN
														1JEL
R45-45-11														1IKF
50.1														1GGI_1
59.1														1ACV
C3														1PPT
TE33														1WPT
B1312														2IGF
17/9														1IFH
26/9														1FRG



TABLE II  
 $V_H$  RESIDUES IN CONTACT WITH THE LIGAND IN MURINE ANTIBODY-LIGAND COMPLEXES OF  
 KNOWN THREE-DIMENSIONAL STRUCTURE<sup>a</sup>

	10	20	30	CDR1 35ab	40	52abc	CDR2 60
HyHEL-10			T	SDY		Y.S	--YS.S.Y
D1.3				gY			.W--gD
HyHEL-5				.W		.W	E.L--SgSTN
D44.1			s	TYW	E		E.L--SgS.Y
D11.15				S.W			.Y--D.Y
Je142			T	TyA			L.SP--SS.Y
NC41			t	NY			.N--N
F9.13.7			t	S.W			E.D--SD.Y.N
NC10							.Y--gN.DtS
E5.2				k	.H		.D--AN.N.Q
409.5.3	EV			.FN	N		.R.NS
N10			Y	S			Y.T--YS.T
TE33			t	tYg			W.NT--Y
B13I2				r.A			.iSS--g.SY.F
17/9							T.SN--g.gY.Y
26/9							T.SN--gggY.Y
C3				.L			V.N--S.g.D
R45-45-11				d.Y	Y		F.N--
50.1						.W	H.F--wD.D.R
59.1				.n	C		R.C--YE.S
Se155-4				.W	H		A.--F
BR96				DYY	Y		Y..Q
Je1103							
BV04-01				tnA			R.RS.N
48G7				.Y	H		
1F7				.hN	N		N
17E8				.H		.V	.W
CNJ206				.H		.V	.W
28B4				.Y	N		F.R..K
McPC603				.Y			.R
40-50				.H			L.W
CHA255				.T			T.L--F.F
NC6.8				.W	E		E.--R.N
88C6/12				.L	H		R.D--K
DB3				.g	N		W
26-10				.Y	N		Y
NI69				.W	H		R
4-4-20				d	W		Y
AN02							

<sup>a</sup>See footnote to Table I.

SDRs are found in the main to coincide with positions that display high sequence and structural variability (34). The residues that are probably involved in the  $V_L:V_H$  association can be guessed from the results for other antibody structures (Tables III and IV).

The most suitable human variable domains onto which the SDRs are transplanted should be those having as many of the critical framework residues as possible. For obvious reasons, it is probably best to use germ-line

TABLE II  
CONTINUED

70	82abc	90	CDR3 100abcdefghi	110	PDB Code	LIGAND
			W.....		3HFM	Lysozyme
			.RDYR.....		1VFB	Lysozyme
			GNV.....		3HFL	Lysozyme
			gdg.....		1MLC_1	Lysozyme
			D.NY.....		1JHL	Lysozyme
			.MgE.Y.....		1JEL	HPr
			.EDNF.SL.....		1NCA	Neuraminidase
			...gTS.....		1FBI_1	Lysozyme
			...Y.YD.....		1NMB	Neuraminidase
			.VIYYQgR.....		1DVF	D1.3 Fv
			...LFYY.....		1IAI	730.1.4 Fab
			.N.....		1NSN	Staph. nuclease
			RSW.....		1TET	Ch. toxin peptide
			Y...PF.....		2IGF	Mhr peptide
			ReR..E.g.....		1IFH	Hemagg. peptide
			R.R..E.g.....		1FRG	Hemagg. peptide
			DfYDYD.....		1FFT	Poliovirus peptide
			H.L..T.ygNYP.W.....		1IKF	Cyclosporin A
			EgY.....I.....		1GGI_1	HIV-1 peptide
			..HM..T.....		1ACY	HIV-1 peptide
			.gHg.....		1MFA	trisaccharide
			glD.gaW.....		1CLZ	Lewis Y sacch.
			.R.....		1MRD	inosine diphosph.
			DqTgtaW.....		1CBV	trinucleotide
			YYg.....		1GAF	TSA
		r	R.d..Y.F.....		1FIG	TSA
		K.	SYyg.....W.....		1EAP	TSA
			gdYY.....W.....		1KNO_1	TSA
			W.....		1KEL	TSA
			N.....W.....		2MCP	phosphocholine
			F.F..YY.Y----V.....		1IEG	ouabain
			HR.....		1IND	Indium-EDTA
			.YSsM.....		2CCR	NC174 sweetener
			YayC.....		1YUH_1	nitrophenyl hapten
			gdY..W.F.....		1DBB	progesterone
			S...WaM.....		1IGJ_1	digoxin
			Y.Y...S.....		1NGP	nitrophenyl hapten
			SYyg.....		1FLR	fluorescein
			.WP.....		1BAF	spin-label hapten

sequences (V and J). Fortunately, the human heavy and light chain loci have now been completely mapped (41-43) so that the functional human germ-line antibody genes are known.

The case of CDR3-H (the third complementarity-determining region of the heavy chain) merits special consideration because it is the result of V-D-J recombination and cannot be in any way predicted from germ-line gene segments. However, CDR3-H sequences from a large number of rearranged VH domains are available and can be used as templates.

TABLE III  
 $V_L$  RESIDUES INVOLVED IN THE  $V_L:V_H$  CONTACT IN MURINE ANTIGEN-BINDING REGIONS OF  
 KNOWN THREE-DIMENSIONAL STRUCTURE<sup>a</sup>

	CDR1				CDR2		
	10	20	27abcdef 30	40	50		
HyHEL-5	D			Y.Q	SP.R	Y D	
J539			S.H	Y.Q	SP.P	Y E . . . A.	
17-IA			Y.H	F.Q	SP.L	Y	
AN02			Y	Y.Q	gSSP.L	Y	
17E8	D			Y.H	P.L	H . . . LP	
D1.3	D		Y	Y.Q	SP.L	Y	
HyHEL-10			H	Y.Q	SP	. . . K	
NC41			V	Y.Q	SP.L	Y . . . HI	
NC10			Y.N	Y.Q	g.V.L	Y . . . H.	
D11.15			A	Y.E	TN.L	Y . . . Q.	
R19.9			Y.N	Y.Q	TVKL	Y Y . . . H.	
36-71			F.N	Y.Q	g.I.L	Y F . . .	
YsT9-1				Y.Q	g.V.L	Y . . . H.	
B72.3			N1A	Y.Q	KSP.L	Y a . . .	
CW206	Q		Y.S	L.Q	g.I.R	. . .	
F9.13.7	D		Y.N	Y.K	gTV.L	Y Y . R.H.	
R45-45-11			Y1N	Y.Q	g.V.L	F Y . R.R.	
D44.1			H	Y.Q	SP.L	K Y . . .	
MOPC21	N	K	Y.S	Y.Q	SP.L	Y . . . Y.	
OPG2			N.H	Y.Q	SP.L	Y . . .	
730.1.4			TA.A	Y.Q	SP.L	Y S . Y.Y.	
48G7	D			L.Q	g.I.R	Y . . . H.	
E5.2			Y.N	Y.Q	DgTV.L	Y Y . R.H.	
GHI002			N	Y.Q	g.V.L	Y Y . . .	
409.5.3			SN.H	Y.Q	SP.P	Y g . N.A.	
1F7			Y.H	Y	P.L	Y S . . .	
50.1				Y.Q	PP.L	. . . I.	
59.1			F.H	Y.Q	PP.V	Y I . . . E.	
N10				Y.Q	PP	. . .	
40-50			Y.H.H	Y.Q	PP.L	Y L . . .	
Je142		L	Y.E	Y.Q	SP.L	Y . . . F.	
B13I2			L	Y.E	Y.Q	SP.L	Y K . . . F.
TE33			Y.E	Y.Q	SP.L	Y . . . F.	
BV04-01				Y.Q	qSP.L	Y . . . F.	
DB3			H	Y.H	Y.Q	SP.L	Y K . . . F.
26-10	D	L	Y.N	Y.Q	SP.L	Y K . . . F.	
4-4-20			Y.R	Y.Q	SP.V	Y K . . . F.	
NC6.8	E		Y.H	Y.Q	qSPKL	Y . . . F.	
Je1103				Y.Q	SP.L	Y . . . F.	
C3			Y.H	Y.Q	SP.L	Y K . . . F.	
R6.5	D		nY.H	Y.Q	qAP.L	Y K . . . F.	
L5MK16			H	Y.K	SP.L	Y . . . F.	
BR96			Y.E	Y.Q	SP.L	Y K . . . F.	
mAb735			H.Y.Y	Y.Q	SPKP	. . . FS	
28B4			Y.E	Y.Q	qSP.L	Y . . . F.	
1583				L.Q	SP.R	. . .	
17/9			Y.T	Y.Q	PP.V	Y W . . .	
26/9			K.F.T	Y.Q	PP.L	Y W . . . E.	
McPC603			N.KnF.A	Y.Q	PP.L	Y g . . .	
8F5				Y.Q	SP.L	Y . . . ES	
N1G9			Y.N	V.E	H.F.g.ig	g . NrAP	
88C6/12			Y.N	V.E	R.Ftg.ig	. . . n . P	
Se155-4			H.N	V.E	H.F.g.g	D . . . P	
CHA255			Y.N	V.E	H.Ftg.g	g . N.AP	
HC19			Y.N	V.E	H.F	. . . g . N . . .	

<sup>a</sup>See footnote to Table I.

TABLE III  
CONTINUED

		CDR2			CDR3		
40	50	60	70	80	90	95ab	100
.Y.Q...	SP.R.Y.D...				.Y.	.W.N--P.	Fg...
.Y.Q...	SP.P.Y.E...A.				.Y.	Q.W.YPL--I.	F...
.F.Q...	SP.L.Y				.Y.	.R.YP--I.	F...
.Y.Q...	gSSP.L.Y				.Y.	.Y.P-I.	F.V...
.Y.H...	P.L.H...LP				.Y.	.L--R.	F.gg...
.Y.Q...	SP.L.Y				.Y.	Q.F.TP--R.	F.g...
.Y.Q...	SP...K				M.F.	.WP--Y.	F...
.Y.Q...	SP.L.Y...HI				.Y.	Q.H.PP--W.	F...
.Y.Q...	g.V.L.Y...H.				.F.	Q.D.LP--F.	F...
.Y.E...	TN.L.Y...Q.				.I.	Q.H.YP--W.	F...
.Y.Q...	TVKL.Y.Y...H.				.F.	Q.gstLP--R.	F.g...
.Y.Q...	g.I.L.Y.F...				.F.	.g.LP--R.	F...
.Y.Q...	g.V.L.Y...H.				.I.	.LP--F.	Fg...
.Y.Q...	KSP.L.Y a...				.Y.	.F.TP--Y.	F...
L.Q...	g.I.R...				.Y.	YA.Sp--Y.	F...
.Y.K...	gTV.L.Y.Y.R.H.				.F.	Q...LP--Y.	Fgg...
.Y.Q...	g.V.L.F.Y.R.R.				.F.	Q...IP--P.	F.g...
.Y.Q...	SP.L.K.Y				.F.	Q.S.WP--R.	F...
.Y.Q...	SP.L.Y...Y.				.H.	.g.YP--Y.	Fg...
.Y.Q...	SP.L.Y				M.F.	Q.Sn.WP--L.	F...
.Y.Q...	SP.L.Y.S.Y.Y.				.Y.	H.H.Tp--F.	F...
L.Q...	g.I.R.Y...H.				.Y.	.Y.YP--R.	F...
.Y.Q...	DgTV.L.Y.Y.R.H.				.F.	Q.gn.LP--W.	F.g...
.Y.Q...	g.V.L.Y.Y				.F.	Q...LP--Y.	F...
.Y.Q...	SP.P.Y.g.N.A.					.W.YP--Y.	F...
.Y...	P.L.Y.S...				.Y.	Q...YP--L.	Fga...
.Y.Q...	PP.L...				.Y.	Q...DP--L.	F...
.Y.Q...	PP.V.Y.I...E.				.Y.	Q.N.DP--P.	F...
.Y.Q...	PP...				T.Y.	.IP--Y.	F.g...
.Y.Q...	PP.L.Y.L				.Y.	.S.YP--L.	F...
.Y.Q...	SP.L.Y...F.				V.Y.	F.g.VP--Y.	F.gg...
.Y.Q...	SP.L.Y.K...F.				.Y.	F.g...P--P.	F...
.Y.Q...	SP.L.Y...F.				.Y.	F...IP--F.	F...
.Y.Q...	qSP.L.Y...F.				.F.	.S...P--L.	F.A...
.Y.Q...	SP.L.Y.K...F.				.F.	.S...P--P.	F.g...
.Y.Q...	SP.L.Y.K...F.				.F.	.T.VP--P.	F.g...
.Y.Q...	SP.V.Y.K...F.				V.F.	.VP--W.	F.gg...
.Y.Q...	qSPKL.Y...F.				.F.	.VP--YT	F...
.Y.Q...	SP.L.Y...F.				V.F.	.P--R.	F.g...
.Y.Q...	SP.L.Y.K...F.				.F.	.S.VP--Y.	F...
.Y.Q...	gAP.L.Y.K...F.				.F.	.S.VP--L.	F...
.Y.K...	SP.L.Y...F.				.F.	.S.VP--F.	F...
.Y.Q...	SP.L.Y.K...F.				.Y.	F.g...P--F.	F.S...
.Y.Q...	SPKP...FS				.F.	F.g.VP--Y.	F...
.Y.Q...	qSP.L.Y...F.				.Y.	F...--R.	F.g...
L.Q...	SP.R...				.Y.	.W.Fp--R.	F...
.Y.Q...	PP.V.Y.W...				.Y.	.D.NP--L.	F...
.Y.Q...	PP.L.Y.W...E.				I.Y.	Q.D.HP--L.	F...
.Y.Q...	PP.L.Y.g...				.Y.	Q.D.YP--L.	FgA...
.Y.Q...	SP.L.Y...ES				.Y.	.Y.P--L.	F...
.V.E...	H.F.g.ig.g.NrAP				.F.	.W.NH--W.	F...
.V.E...	R.Ftg.ig...n.P				.F.	A.W.sNH--W.	F...
.V.E...	H.F.g.g.D...P				.F.	A.W.NH--W.	F...
.V.E...	H.Ftg.g.g.N.AP				R.F.	.W.NL--W.	F...
.V.E...	H.F...g.g.N...				.F.	A.W.NH--W.	F...

TABLE IV  
 $V_H$  RESIDUES INVOLVED IN THE  $V_L:V_H$  CONTACT IN MURINE ANTIGEN-BINDING REGIONS OF  
 KNOWN THREE-DIMENSIONAL STRUCTURE<sup>a</sup>

	CDR1				CDR2	
	10	20	30	35ab	52abc	60
HyHEL-10				I.K.	N.L.Ymg	Y.
D1.3				N--	V.Q.	gL.W.
40-50				H--	F.Q.	L.W.
HC19				H--	V.Q.	gL.W.
CHA255					V.Q.	K.L.W.
B72.3				H--	Q.	eggLeW.
48G7				Y.	V.Q.	gL.W.
Jel103					V.Q.	Q.L.W.
D44.1				E--	V.Q.	L.W.
NC6.8				E--	E.	L.W.
HyHEL-5				E--	V.Q.	gL.W.
TE33					V.Q.	F.W.
mAb735				H--	V.Q.	L.W.
88C6/12				H--	I.Q.	L.W.
Jel142				H--	V.Q.	K.L.W.
D11.15				N--	V.Q.	L.W.
J539				W.	V.Q.	kgL.W.
Se155-4				H--	I.Q.	L.W.
8F5				H--	V.Q.	gL.W.
17-1A					V.Q.	ggL.W.
B13I2					V.Q.	K.L.W.
DB3					V.E.	ELKW.
26-10					V.Q.	K.L.Y.
1F7					I.	SL.W.
R6.5					V.E.	k.L.W.
17E8				H--	V.Q.	OgL.W.
BR96					V.Q.	KRL.W.
NI69				H--	V.Q.	g.L.W.
C3				Q--	I.Q.	gL.W.
17/9					V.Q.	K.LeW.
26/9					V.H.	KRL.W.
36-71					V.Q.	g.L.W.
NC41					V.Q.	LkW.
730.1.4					V.Q.	L.W.
NC10				Y--	V.Q.	L.W.
E5.2					Q.	gL.W.
F9.13.7				H--	V.Q.	gL.W.
L5MK16					V.Q.	gLeW.
R19.9				N--	V.Q.	gLeW.
OPG2					V.Q.	K.L.W.
MOPC21				H--	Q.	EKgL.W.
R45-45-11					Q.	kRL.W.
4-4-20				W.N--	V.Q.	K.L.W.
Yst9-1					V.Q.	AL.W.
409.5.3				W.S--	V.Q.	LeW.
BV04-01					V.Q.	K.L.W.
CNJ206				H--	V.Q.	gLeW.
McPC603				Y.E--	Q.	RL.W.
NI0					I.Q.	N.L.W.
AN02					I.Q.	NKL.W.
50.1					I.Q.	gL.W.
59.1					I.Q.	gL.W.
GH1002					Q.	L.W.
28B4					A.Q.	ALeW.
1583				H--	Q.	gL.W.

<sup>a</sup>See footnote to Table I.

TABLE IV  
CONTINUED

70	82abc	90	CDR3 100abcdefghi	110	PDB Code
		Y	WDg	Wg	3HFM
		Y	E.YRL	D.Wgq	1FDL
		Y	R.yDYAV	D.WgQ	1IBG
		M.Y	FYYAM	W	1GIG
		L.F	RF	V.W	1IND
		F.K	YY	WgQ	1BBJ
		Y	Yg	I.W	1GAF
		Y	L.gY	D.Wg	1MRC
		Y	dgNY	g.Wgq	1MLC_1
		Y	SSM	DY.Wgq	2CGR
		Y	gmY	D.Wg	2HFL
		F	R.wYF	D.Wgt	1TET
		F	KFAM	DY.Wgq	1PLG
		V.Y	CRPM	D.W.Qg	1YUH_1
		Y	eQYF	D.Wg	1JEL
		D	YgAM	D.Wg	1JHL
		Y	L.yYgYN	a.WgQ	2FBJ
		V.Y	HgYYg	DY.W	1MFA
		Y	yDm	D.Wg	1BBD
		F	YpYA	Wg	1FOR
		Y	D.FYF	D.Wg	2IGF
		F	NWYF	D.W.a	1DBB
		Y	NKWAM	D.Wg	1IGJ_1
		Y	gNYgF	T.W	1FIG
		Y	LLLSF	DY.Wg	1RMF
		Y	Y.gSsY	D.W	1EAP
		Y	L.DgAWF	AY.Wgq	1CLZ
		Y	Y.SSYF	d.W	1NGP
		F	DVgF	DY.Wg	1FPT
		Y	R.dENGF	Wg	1IFH
		Y	R.dEKGF	A.Wg	1FRG
		F	Y.gSyKF	D.W	6FAB
		F	FgSLS	DY.Wg	1NCA
		F	D.YE.YYAM	dY.Wgq	1IAI
		Y	YDggF	DY.W	1NMB
		Y	YqgRgAM	D.W.Q	1DVF
		Y	Y.sYgVL	D.W.Q	1FBI_1
		Y	WYVL	D.Wg	1LMK_1
		F	S.lAvYYF	D.Wgq	2F19
		Y	YYAM	D.Wgq	1OPG
		Y	W.PYYAM	DY.Wg	1IGC
		Y	YdT Y.VWfAd	W	1IKF
		Y	S.ygm	DY.Wg	1FLR
		Y	YgP	A.W.q	1MAM
		Y.V	R.YyaV	D.W.Q	1IAI
		Y	Q.TAWF	AY.Wgq	1CBV
		Y	YY.r	A.W	1KNO_1
		Y	N.STWYF	d.Wg	2MCP
		Y		WgQ	1NSN
		F	WPL	A.WgQ	1BAF
		Y	Y	I.W	1GGI_1
		Y	E.eTYF	d.W	1ACY
		F	AgF	d.W	1GHF
		Y	W.sYAM	D.Wgq	1KEM
		Y	g	D.Wgq	1NLD

## A. Sample Design of a "Humanization" Protocol by SDR Transfer

Humanization by SDR transfer is illustrated here for the case of the murine antibody, B72.3, which binds to the tumor-associated antigen, TAG-72 (3). The three-dimensional structure of the antigen-binding region of B72.3 has been determined by X-ray crystallography (44), although only in the uncomplexed form. The residues involved in the  $V_L:V_H$  contact in B72.3 are included in Tables III and IV.

Based on the results for other antibody:antigen complexes (Table I), the residues in B72.3 likely to be involved in antigen binding are residues 30, 32, and 34 in CDR1, the framework residue 49, residues 50 and 53 in CDR2, and residues 89, 91, 92, and 94–96 in CDR3 in the light chain; and the framework residue 30, the residues 31, 33, and 35 in CDR1, residues 50 and 52–57 in CDR2, and residues 95–97 and 101 in CDR3 of the heavy chain.

The residues that contribute side-chain interactions to the  $V_L:V_H$  contact in B72.3 are 32, 34, 36, 38, 42–44, 46, 49, 87, 90, 94–96, 98, and 103 in the light chain (Table III) and 35, 39, 45, 47, 50, 58, 60, 61, 91, 93, 96, 97, 103, and 105 in the heavy chain (Table IV; see also Table V).

In addition to the putative antigen-contacting residues, those residues involved in the quarternary interaction between the variable domains, the N-terminal residues, and the residue at position 71 in both light and heavy chains should be preserved during humanization. This requirement is used in the identification of the human sequences that would best serve as templates for humanization.

A comparison with known human germ-line  $V_k$  sequences reveals that B72.3  $V_L$  is most similar to VKI-ZI (45), among several, in the critical framework residues. The  $J_L$  segment of B72.3 is most similar to the human  $J_k$ -2. The sequences are compared to Table VI. Also indicated in Table VI are the residues that are probably involved in the interaction with the antigen and the framework residues that are most probably critical to the preservation of the structure of the antigen-binding site. A protocol for the humanization of B72.3  $V_L$  by the method of SDR transfer is included in Table VI.

A comparison with known human germ-line  $V_H$  sequence reveals that B72.3  $V_H$  is most similar to DP-7 (41) in the critical framework residues. The  $J_H$  segment of B72.3 is most similar to the human  $J_H$ -1 and its CDR3-H has the same number of residues as that of antibody JM 0-131 (46). The sequences are shown in Table VII. The protocol that we propose for the 'humanization' of B72.3  $V_H$  by the method of SDR-transfer is included in Table VII.

The CDR residues of B72.3 that are transferred to the human templates number 16 using the method of SDR transfer. This contrasts with the 54

TABLE V  
ENVIRONMENT OF SIDE CHAINS<sup>a</sup>

Distance range (Å)	Number of atoms in that distance range		
	Nonpolar	Polar (main chain)	Polar (side chain)
<b>Of Tyr-407 in IgG1 Fc</b>			
$r < 3.5$	5	2	1
$3.5 < r < 4.0$	28	2	1
$4.0 < r < 4.5$	38	7	1
$4.5 < r < 5.0$	<u>42</u>	<u>8</u>	<u>0</u>
Total	113	19	3
<b>Of Phe-506 in IgE Fc</b>			
$r < 3.5$	12	0	0
$3.5 < r < 4.0$	23	2	1
$4.0 < r < 4.5$	33	4	1
$4.5 < r < 5.0$	<u>47</u>	<u>9</u>	<u>2</u>
Total	115	15	4

<sup>a</sup>These values are derived from the crystal structure of human IgG1 Fc (PDB Entry 1FC1) and from the model of human IgE Fc (PDB Entry 2IGE).

that would have been transferred if the method of CDR grafting were employed. A substantial reduction is therefore achieved in the number of non-human residues that will be present in the humanized molecule with a consequent reduction in its immunogenicity. This number may be reduced even further if the structure of the B72.3 antibody:antigen complex becomes available and the actual CDR residues that make contact with antigen become known. We believe that humanization by SDR transfer will reduce immunogenicity to the barest minimum.

### III. Generation of Multivalent/Multispecific Antibodies

We are making use of electrostatic complementarity for the efficient generation of heterodimeric molecules. Each molecule consists of two chains, each with one or more different antigen-binding specificities, the two chains differing from each other by the presence of oppositely charged residues at a judiciously chosen position. We choose to engineer an aspartic acid or a glutamic acid residue in one chain and a lysine or an arginine in the other. The rationale behind the procedure lies in the expectation that identical chains will repel each other and that only heterodimers will form. The charged residues are introduced at a position where electrostatic forces are

TABLE VI  
HUMANIZATION OF B72.3 V<sub>L</sub> BY SDR TRANSFER<sup>a</sup>

	10	20	30	40	50	60	70	80	90
			CDR1		CDR2				CDR3..
			*	*	*	*	*	*	
x							x		
B72.3	DIQMTQSPASLSVSGELVITC	RASENIYSNLA	WYQQKQKSPQLLIVY	AATNLA	GVPSRFSGSGSGTQYSLKIDISLQSEDFGSYYC	QHF	WGT	P	
VK1-21	DIQMTQSPFSLASVGGRTITC	RASQGIENLN	WYQQKPGKPKLLIY	AASLQS	GIPSRFSDSGGADYTLTISLQPEDFAAYC	QQSD	STP		
	:	:	:	:	:	:	:	:	:
Hu B72.3	DIQMTQSPFSLASVGGRTITC	RASQGIYNNLA	WYQQKPGKPKLLIY	AASNLA	GIPSRFSDSGGADYTLTISLQPEDFAAYC	QQF	WSTP		
			100						
	•								
B72.3	YT	FGG	TKLEIK						
J <sub>K</sub> -2	YT	FGG	TKLEIK						
Hu B72.3	YT	FGG	TKLEIK						

<sup>a</sup>Proposed humanization protocol for B72.3 V<sub>L</sub> by SDR transfer. The vertical bars indicate the B72.3 residues (44) that contribute side-chain contacts in the quaternary interaction with V<sub>H</sub> and the dots indicate the residues that contribute only main-chain contacts (Table II); the asterisks indicate the residues that could be involved in the interaction with the antigen (based on the results presented in Table I); other framework residues that may influence the antigen-binding site structure are indicated by an x; the colons indicate the changes to be imposed on the closest human germ-line sequences (V<sub>K</sub> and J<sub>K</sub>) in order to generate a humanized B72.3 V<sub>L</sub> (Hu B72.3).

TABLE VII  
HUMANIZATION OF B72.3 V<sub>H</sub> BY SDR TRANSFER<sup>a</sup>

	10	20	30	40	52a	60	70	82abc	90
			CDR1		CDR2				
			*	*	*	*	*		
B72.3	QVQLQDSDAELVKPGASVKISCKASGYTFT	DHAIH	WAKQKPEQGLEWIG	YISPGNDDIKYNEKFKG	KATLTADKSSSTAYMOLNSLTSEDSAVYFCR				
DP-7	QVQLVQSGAEVKKPKGASVKVCKASGYTFT	SYIMH	WRQAPGQGLEWVG	IINPSGGSTYAQKPFQG	RVITMTRDTSTSTVYMELSSLRSEDTAVYYCAR				
			:	:	:	:	:		
Hu B72.3	QVQLVQSGAEVKKPKGASVKVCKASGYTFT	DYAMH	WARQAPGQGLEWVG	YISPGNDDIKYAQKPFQG	RVITMTADTSTSTVYMELSSLRSEDTAVYFCR				
			CDR3						
			*	*	*	*	*		
B72.3	SYIGH	WGQGTLLTVSS							
JM 0-131	VMRGI	WGQGTLLTVSS							
J <sub>H</sub> -1									
			:	:	:	:	:		
Hu B72.3	SYIGY	WGQGTLLTVSS							

<sup>a</sup>Proposed humanization protocol for B72.3 V<sub>H</sub> by SDR transfer. The vertical bars indicate the B72.3 residues (44) that are involved in the quaternary interaction with V<sub>L</sub> and the dots indicate the residues that contribute only main-chain contacts (Table IV); the asterisks indicate the residues that could be involved in the interaction with the antigen (based on the results presented in Table II); other framework residues that may influence the antigen-binding site structure are indicated by an x; the colons indicate the changes to be imposed on the closest human germ-line sequences (V<sub>H</sub> and J<sub>H</sub>) and template CDR3 in order to generate a humanized B72.3 V<sub>H</sub> (Hu B72.3).

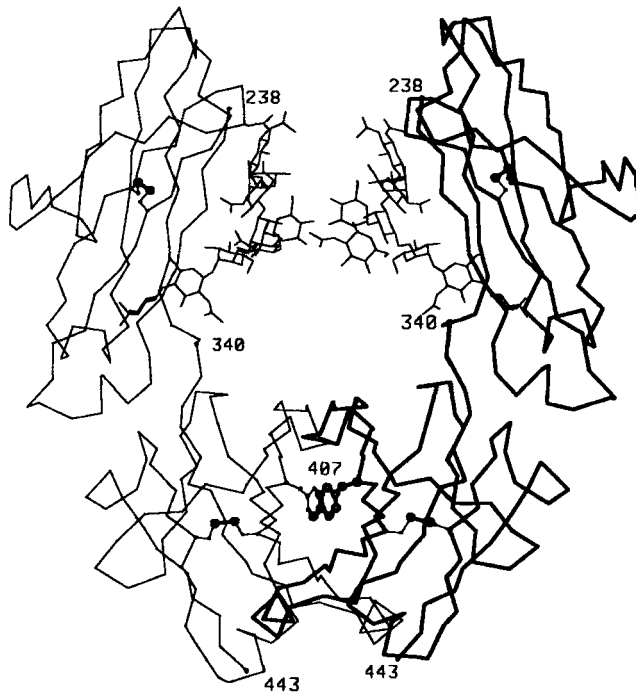


FIG. 1. Alpha-carbon trace of the human IgG1 Fc [Protein Data Bank (PDB) (32a) Entry 1FC1]. One chain of the Fc is drawn with thick lines. The start and end of the  $C_H2$  and  $C_H3$  domains are indicated with numbers. The intradomain disulfide bonds are shown as filled circles and the carbohydrate moieties between the two  $C_H2$  domains are drawn with thin lines. The side chains of the Tyr-407 of the two chains are drawn in bold.

amplified, e.g., in a region of low dielectric constant, as in the interface between domains.

Each chain in the heterodimer consists of an antigen-binding region linked to an Fc. For ease of production, each chain is expressed as a single polypeptide chain. In our constructs, the antigen-binding moiety is a single-chain Fv (47, 48) or a single-chain diabody (17), which is then linked to an Fc. Electrostatic complementarity is effected via engineered mutations in the Fc.

For antibodies of the IgG class, we are engineering the change at position 407 [Eu numbering (49); number 438 according to the convention of Kabat *et al.* (1)]; for antibodies of the IgE class, we choose to introduce the charged residues at position 506 [numbering of Bennich and von Bahr-Lindstrom (50); number 569 according to the convention of Kabat *et al.* (1)]. Subsequent formation of disulfide bonds involving the cysteines in the hinge region then stabilizes the heterodimer.

In the structure of human IgG1 Fc (51), the residue at position 407, a tyrosine, lies near the molecular dyad and is in close contact with the Tyr-407 of the opposite chain (52) (Fig. 1). The environment of Tyr-407 is mainly hydrophobic (Table V) so that the dielectric constant in that region will be low. No structure is yet available for the Fc of IgE, although models have been built (53, 54). In those models, the phenylalanine at position 506 (which is analogous to Tyr-407) is in intimate contact with the Phe-506 of the opposite chain (Fig. 2) and both are surrounded by hydrophobic structures (Table V). The environment of Phe-506 in IgE also should have a low dielectric con-

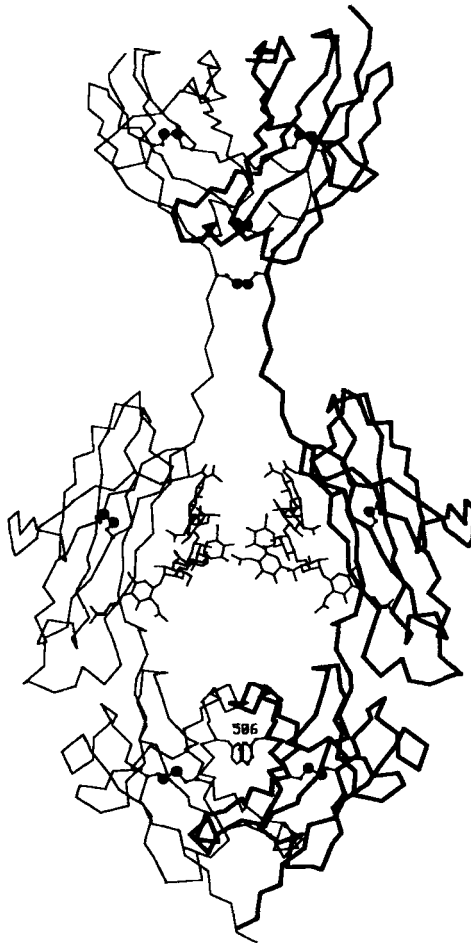


FIG. 2. Alpha-carbon trace of the putative structure of human IgE Fc (PDB Entry 2IGE) in the same orientation as in Fig. 1. The side chains of the Phe-506 are drawn in bold.

stant, possibly even lower than that of Tyr-407 in IgG. We have already shown that replacing Phe-506 with Arg does prevent dimer formation (55).

Heterodimer formation through the use of electrostatic complementarity in the Fc can be used in conjunction with other methods to produce multi-specific/multivalent molecules. For example, the antigen-binding moiety attached to the Fc could be a single-chain Fv or a diabody, or, if desired, several Fvs or diabodies linked in tandem. The molecular constructs that we are currently developing are as follows:



(i)



(ii)

In molecular construct (i), linker1 is  $(\text{Gly}_4\text{-Ser})_3$  (48) and V1 and V2 are the two variable domains ( $V_L$  and  $V_H$ ) of the antibody; this is then a single-chain Fv linked to an Fc. In molecular construct (ii), linker1 is  $(\text{Gly}_4\text{-Ser})$  and linker2 is  $(\text{Gly}_4\text{-Ser})_6$ , V1 and V3 are the variable domains of one antibody, and V2 and V4 are the variable domains of another; this is then a single-chain diabody linked to an Fc. The engineered electrostatic mutations are in the  $C_{H3}$  domains of the Fc (for IgG), or in the  $C_{H4}$  (for IgE).

The order of the variable domains is critical. For example, in molecular construct (i), the  $V_L$  could be first and its C terminus would then be linked to the N terminus of  $V_H$ ; blocking of the N terminus of  $V_H$  could result in altered ligand-binding properties (40) and the alternative linkage might have to be tried. The situation is even more critical in the case of molecular construct (ii), where, for one antibody, both  $V_L$  and  $V_H$  have their N termini blocked. The dilemma can be resolved only by trial and error.

### A. Sample Construction of a Bispecific Molecule Using Two Single-Chain Fvs Dimerized via Electrostatically Complementary Fc Regions

The generation of electrostatically complementary Fc regions is outlined in Fig. 3. Two 129-mer alternating oligonucleotides with 24-base overlaps and *XmaI* and *NsiI* sites on the 5' and 3' flanks, respectively, are synthesized (Fig. 3A). The antisense strand contains the altered codon (solid area in the figure) for the ionic amino acid, in this example, the negatively charged aspartic acid. With two 20-base end primers 1 and 2, polymerase chain reaction (PCR) is carried out and the product is treated with *XmaI/NsiI* to yield a 225-base pair fragment.

The construction of a single-chain Fv linked to the human Fc $\gamma$ 1 has been previously described (56). Here we call it sFv1Fc/Tyr to differentiate it from

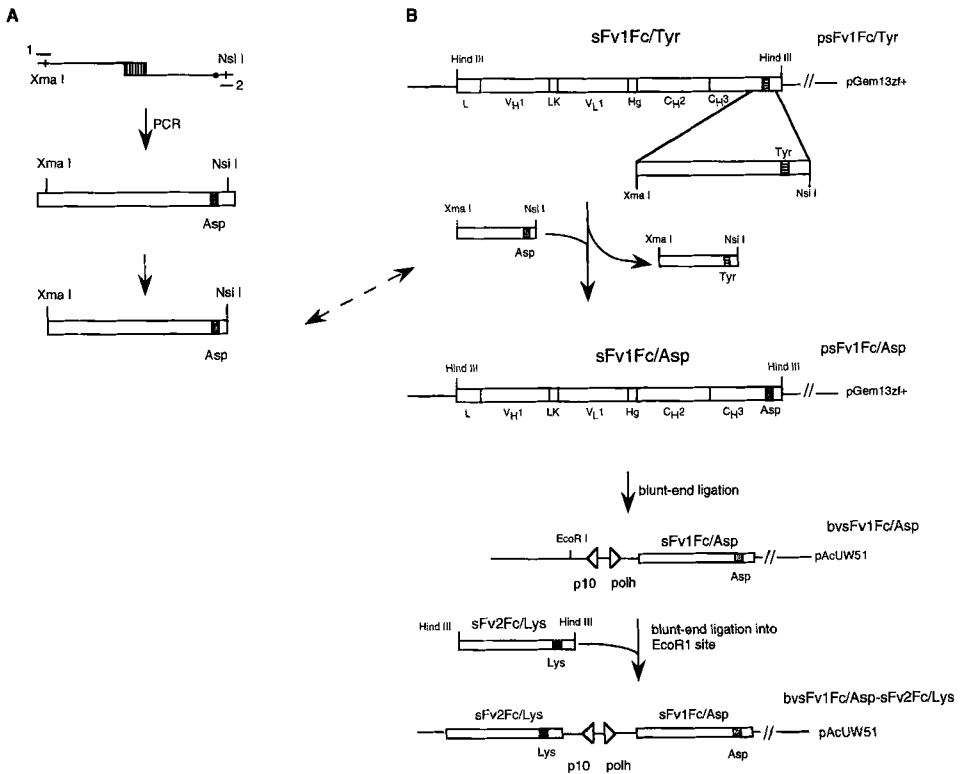


FIG. 3. Schematic flow sheet for the generation of a single gene coding for an Fv linked to an Fc modified for electrostatic complementarity. Abbreviations: L, leader peptide; LK, the (Gly<sub>4</sub>-Ser)<sub>3</sub> linker; Hg, hinge region. The open arrowheads show the p10 and polyhedrin promoters and their transcriptional directions.

the subsequent constructs in which Tyr is replaced with an ionic amino acid. This single gene is subcloned into pGem13zf+ (Promega, Madison, Wisconsin) (Fig. 3B), designated as psFv1Fc/Tyr, and is treated with *Nsi*I/*Xma*I. The *Xma*I/*Nsi*I fragment carrying the aspartic acid codon is exchanged for the original *Xma*I/*Nsi*I fragment carrying the tyrosine codon to make the psFv1Fc/Asp. The 1.5-kb *Hind*III fragment is cleaved and blunt-end ligated at the *Bam*HI site downstream of the polyhedrin promoter of the baculovirus transfer expression vector pAcUW51 (Pharmingen, San Diego, California) to form the bvsFv1Fc/Asp. A second single gene targeted to another antigen, sFv2Fc/Lys (codon for tyrosine replaced with one for a positively charged amino acid, for example, lysine), generated in the same manner as the

sFv1Fc/Asp is blunt-end ligated at the *EcoRI* site downstream of the p10 promoter of the same baculovirus expression transfer vector. The resulting dual-expression baculovirus construct is designated as bvsFv1Fc/Asp-sFv2Fc/Lys (Fig. 3B).

### B. Sample Construction of a Tetraspecific Molecule from Two Bispecific Single-Chain Diabodies Dimerized via Electrostatically Complementary Fc Regions

The assembly of the construct for a single gene encoding a single-chain bispecific diabody linked to the Fc (sdbFc) is outlined in Fig. 4. Two different templates are used: template I encodes an scFv against antigen I, and template II encodes an scFv-Fc against antigen II. The Fc region is modified as described above for electrostatic complementarity.

In each case, the 3' primer of one set and the 5' primer of the other provide the sequence overlap, and together they code for the respective linker peptides  $(\text{Gly}_4\text{-Ser})_n$  (48). Primers 1 and 8 carry the *Bam*HI site at the flanks; in primer 8, the enzyme site is preceded by a stop codon.

The purified products of the first round of PCR amplifications,  $V_L1$ ,  $V_H2$ , and  $V_H1$ , are put together in one DNA molecule by overlap extension PCR, using primers 1 and 6 (Fig. 4). The resulting product is combined with the Fc-containing PCR product and amplified with primers 1 and 8 by another round of overlap extension PCR. The gene encoding the diabody-Fc (sdb1Fc) thus generated is treated with *Bam*HI and inserted into the baculovirus expression transfer vector pAcUW51 (Pharminggen, San Diego, California) at the *Bam*HI site located downstream of the polyhedrin promoter. A second single gene encoding another diabody (targeted against two other different antigens) linked to the modified Fc (sdb2Fc) is subcloned at the *EcoRI* site downstream of the p10 gene promoter. The resulting dual-expression baculovirus construct is designated bvsdb1Fcsdb2Fc (Fig. 4).

## IV. Conclusion

We have presented a procedure that is designed to generate novel molecules that, in view of their multivalency and/or multispecificity, will have improved binding properties. We are currently applying this procedure to generate molecules with antibody-like properties, i.e., with binding sites derived from antibodies and with effector functions similar to those of antibodies in view of the use of the entire Fc region for dimer formation. Nevertheless, the procedure can be used also for the easy generation of multivalent/multispe-

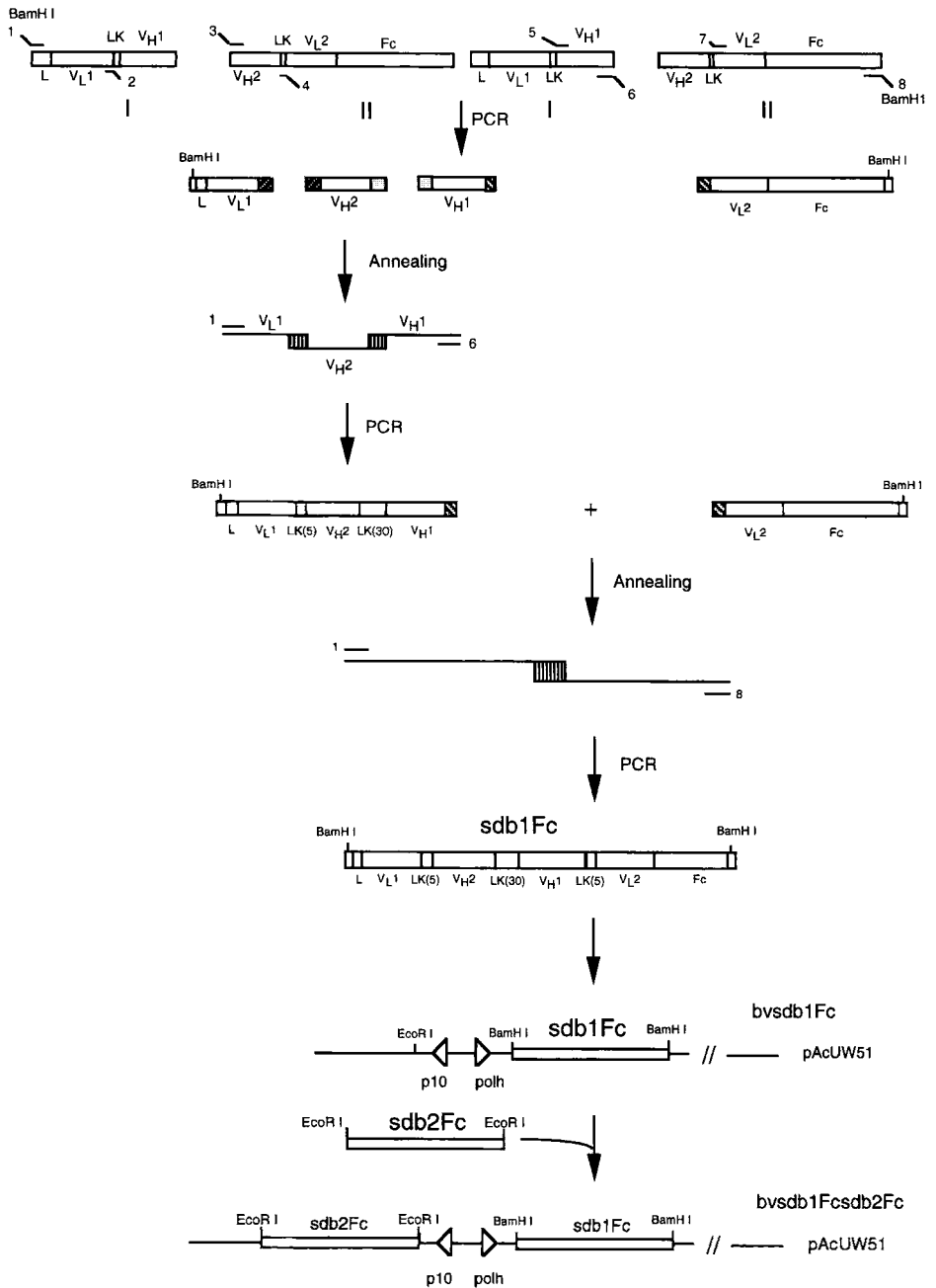


FIG. 4. Schematic representation of the generation of the construct for a single gene encoding a diabody linked to Fc. Abbreviations: L, leader peptide; LK, the (Gly<sub>4</sub>-Ser)<sub>3</sub> linker; LK(5), the (Gly<sub>4</sub>-Ser) linker; LK(30), the (Gly<sub>4</sub>-Ser)<sub>6</sub> linker. The open arrowheads show the p10 and polyhedrin promoters and their transcriptional directions.

cific molecules in which the "binding sites" in the above examples are replaced by other ligands and/or receptors. Further, because dimerization of the Fc region is accomplished via electrostatic complementarity in the C<sub>H</sub>3 only (in IgG), or in the C<sub>H</sub>4 only (in IgE), the other parts of the Fc need not be present, thereby eliminating Fc-associated effector functions that may not be desirable in certain applications.

We have also presented a humanization procedure that is designed to reduce immunogenicity to the barest minimum. Here again, although the procedure was developed to be applied to antibodies, the basic principles of the method may be useful in the humanization of other nonhuman proteins for which homologous human counterparts exist.

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