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37. Supported by the National Cancer Institute (grants CA08494 and CA19264), the National Institute for Allergy and Infectious Diseases (grant AI124009), the United States Public Health Service, and the American Cancer Society (MV2W). N.M., T.M.K., and D.S. were predoctoral trainees of USPHS training grants AI07099, CA09241, and GM7281, respectively.

23 October 1987; accepted 31 December 1987

Reshaping Human Antibodies: Grafting an Antilysozyme Activity

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The production of therapeutic human monoclonal antibodies by hybridoma technology has proved difficult, and this has prompted the "humanizing" of mouse monoclonal antibodies by recombinant DNA techniques. It was shown previously that the binding site for a small hapten could be grafted from the heavy-chain variable domain of a mouse antibody to that of a human myeloma protein by transplanting the hypervariable loops. It is now shown that a large binding site for a protein antigen (lysozyme) can also be transplanted from mouse to human heavy chain. The success of such constructions may be facilitated by an induced-fit mechanism.

FOR PASSIVE IMMUNITY OR ANTIBODY therapy in humans, monoclonal antibodies designed to eliminate toxins, viral and bacterial pathogens, or other cells would ideally be of human origin (*1*). Unfortunately it has proved difficult to make human monoclonal antibodies by hybridoma technology (*2*). Chimeric antibodies with mouse variable and human constant domains have been constructed by linking together the genes encoding each domain (*3*, *4*), and expressing the recombinant antibodies in myeloma cells. However, the mouse variable region may itself be seen as foreign (*1*). We have therefore attempted to insert the antigen-binding site of a mouse antibody, rather than the whole variable region, directly into a human antibody. In previous work, the three heavy-chain hypervariable regions [or complementarity-determining regions (CDRs)] from a mouse antibody to a hapten were transplanted onto the framework regions of the heavy-chain variable (V_H) domain of a human myeloma protein. In combination with the mouse light chain, the reshaped heavy chain bound tightly to hapten (*5*). Although it seems likely that both heavy and light chains make contacts to the hapten, the relative contribution of each chain is unknown. Nor is it clear whether the small hydrophobic hapten NP (4-hydroxy-3-nitrophenacetylaminocaproate) simply binds to a hydrophobic pocket at the base of the hypervariable loops. By contrast, the three-dimensional structure of the complex of lysozyme and the mouse antibody D1.3 has been solved (*6*), and about 690 Å^2 of the solvent-accessible surface of the antibody is buried on complex formation. Both V_H and light-chain variable (V_L) domains make extensive contacts to lysozyme, but most of the hydrogen-bonding contacts are made to the CDRs of the heavy chain. Thus, of 12 hydrogen bond interactions proposed (*6*), 9 are made to the heavy chain. We have replaced the hypervariable loops of the human NEW heavy chain (*5*) with those from the D1.3 heavy chain.

The variable domains of the mouse antibody to lysozyme were cloned and sequenced as described (*7*). To reshape the NEW heavy chain, we started from a synthetic gene in an M13 vector (Fig. 1b) containing the framework regions of human NEW and the CDRs from mouse antibody B1-8 (*5*). Long oligonucleotides with multiple mismatches with the template (*8*) were used to replace each of the hypervariable loops in turn by site-directed mutagenesis: the central mismatched portion of the primer encoded each CDR of the D1.3 heavy chain, and the 5′ and 3′ ends of the primer were complementary to the flanking framework regions. Thus after three rounds of mutagenesis, the reshaped gene (HuV_HLYS) encoded the framework regions of NEW with the hypervariable regions of D1.3 (Figs. 1c and 2). This was assembled with the heavy-chain constant region of human immunoglobulin G2 (HuIgG2) (*9*) to give the plasmid pSVgpt-HuV_HLYS-HuIgG2. The plasmid was transfected by electroporation (*10*) into the myeloma line J558L (*11*), which secretes a mouse λ light chain. Transfectants resistant to mycophenolic acid were screened for secretion of immunoglobulin by gel electrophoresis of supernatants from [^{35}S]methionine-labeled cells. The secreted product (HuV_HLYS-HuIgG2, λ) was purified on protein A–Sepharose; the λ light chain was exchanged for the D1.3 κ light chain in vitro; and the HuV_HLYS-HuIgG2, κ antibody was purified (*12*). In parallel experiments as control, the mouse D1.3 variable region (MoV_HLYS) was attached to the heavy-chain constant region of mouse IgG1 (MoIgG1) in a pSVgpt vector (pSVgpt-MoV_HLYS-MoIgG1), and antibody was expressed and reassociated as above (Fig. 1d).

Fluorescence quench was used to measure

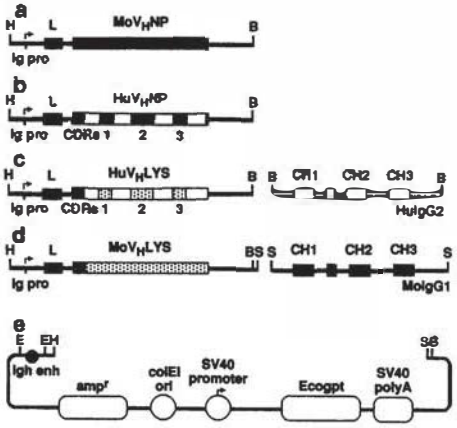

Fig. 1. Vectors for heavy-chain expression. (**a**) Mouse V_{NP} gene (*4*) and designated here as MoV_HNP, (**b**) reshaped HuV_{NP} gene (*5*) designated here as HuV_HNP, (**c**) HuV_HLYS gene with the heavy-chain constant region gene of human IgG2, (**d**) MoV_HLYS gene with the heavy-chain constant region gene of mouse IgG1, and (**e**) the backbone of the pSVgpt vector with immunoglobulin heavy-chain enhancer (Igh enh) (*4*). The V_H domains are denoted in black, stippled, or open boxes to signify sequences encoding mouse antibody to NP (B1-8), mouse antibody to lysozyme (D1.3), and human myeloma protein (NEW), respectively. The constant domains are denoted in black or open boxes to signify sequences encoding mouse or human constant domains. Ig pro, heavy-chain promoter; L, leader exon; H, Hind III; B, Bam HI; S, Sac I; E, Eco RI restriction sites. The reshaped NEW heavy chain was expressed from a vector derived from the pSVgpt-HuV_HNP vector (b). Thus the HuV_HNP gene, cloned initially in M13mp9 as a Hind III–Bam HI fragment, contains heavy-chain promoter, the CDRs of the B1-8 antibody, and the framework regions of human NEW (*5*). The CDRs of B1-8 were then replaced with those of D1.3 using long mutagenic oligonucleotides (see Fig. 2). The Hind III–Bam HI fragment, now carrying the HuV_HLYS gene (c) was excised from the M13 vector and cloned into the pSV vector (e) along with a Bam HI fragment encoding the heavy-chain constant region of human IgG2 (*9*). The construction of the pSVgpt-MoV_HLYS-MoIgG1 vector to express recombinant D1.3 is summarized in (d).

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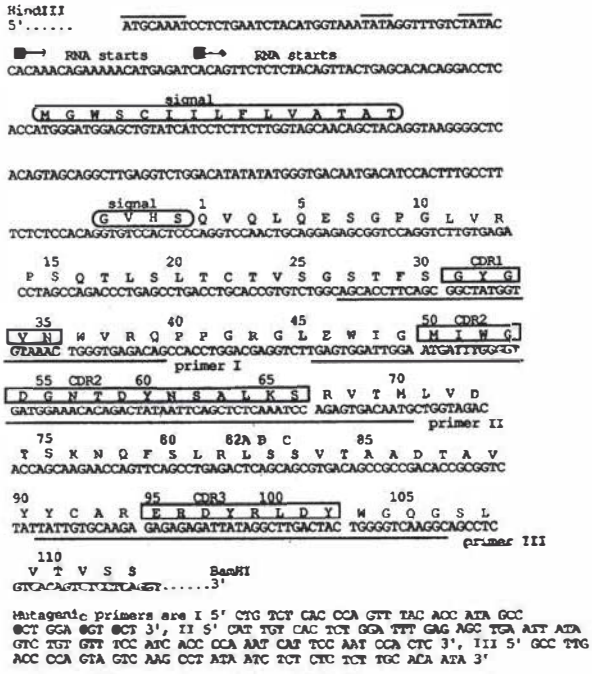

Fig. 2. Sequence of the reshaped V_H domain. The reshaped domain HuV_HLYS is based on the HuV_HNP gene (*4*), with the framework regions of human NEW alternating with the hypervariable regions of mouse D1.3 (*7*). Three oligonucleotides I, II, and III were used to replace each of the B1-8 CDRs with those from D1.3. Each oligonucleotide has 12 nucleotides at the 5′ end and 12 nucleotides at the 3′ end which are complementary to the NEW framework regions, whereas the central portion encodes CDRs 1, 2, or 3 of the D1.3 antibody. The primers are complementary to the marked portions of the coding strand of the reshaped domain. Three rounds of mutagenesis were used, transfecting into the *Escherichia coli* strain BMH71-18mutL, plating on a lawn of BMH71-18, and probing infected colonies with the mutagenic primers as in (*8*). In washing the filters, it was necessary to gradually increase the wash temperature from 65° to 75°C to distinguish the mutant from wild-type clones. The yield of mutants at each step was about 5%.

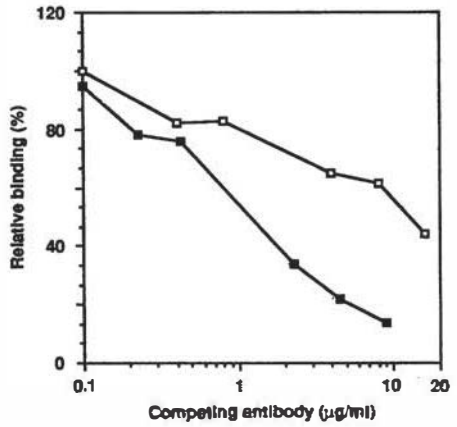

Fig. 3. Competition of reshaped and engineered mouse antibody for lysozyme. Th wells of a microtiter plate were coated with lysozyme at a concentration of 100 μg/ml in phosphate-buffered saline (PBS), and remaining binding sites on the plastic were blocked with 1% bovine serum albumin (in PBS). The binding of ^{125}I-labeled mouse antibody D1.3 (specific activity, >0.6 μCi per micrograms of D1.3) to lysozyme was competed with increasing concentrations of (■) engineered antibody (MoV_HLYS-MoIgG1, κ) or (□) reshaped antibody (HuV_HLYS-HuIgG2, κ), and the data presented are typical of two independent experiments. Antibody D1.3 and reassembled antibody D1.3 competed with the labeled D1.3 as effectively as did the engineered antibody D1.3. Radioiodination was carried out by the chloramine T method (*22*).

the binding of lysozyme to these antibodies. The binding to both antibodies was tight, with 2 mol of lysozyme molecules binding 1 mol of antibody. Our results indicate that the affinity is better than 5 n*M*, although previous work had indicated a value of 20 to 40 n*M* (*6*, *13*). The antibodies were then compared in a competition assay for lysozyme; ^{125}I-labeled mouse antibody D1.3 competing with mouse antibody or with the reshaped antibody for binding to lysozyme. The reshaped antibody competes, albeit less effectively (about tenfold less) than the mouse antibody (Fig. 3). Nevertheless, the grafting of hypervariable regions from mouse to human framework regions is sufficient to transfer the lysozyme-binding site, an extensive surface of interaction, despite the 31 of 87 residues that differ between the heavy-chain framework regions.

The results confirm that the hypervariable regions (mainly loops) fashion the antigen-binding site and that the more conserved regions (mainly β-sheet) form a structural framework (*14*). However, the result is remarkable given the several assumptions underlying the building of an antigen-binding site on a new framework region—namely, (i) the V_H and V_L domains pack together in the same way (*15*), (ii) the two β-sheets within each variable domain pack together in the same way (*16*), (iii) the antigen usually binds to the hypervariable regions, and (iv) the hypervariable regions are supported by the framework regions in a similar way (*17*). In particular, the hypervariable loops are not stand-alone structures, but make extensive contacts to the framework and to other hypervariable regions. Since the crystallographic structures of both parent antibodies are known, we could confirm that in the reshaped antibody the D1.3 CDRs could be supported by the NEW framework regions with the same (or similar) contacts as are used with the D1.3 framework regions. However, we also identified some potential problems. For example, in CDR1 of D1.3, Tyr-32 makes a van der Waals contact with the Phe-27 in FR1; in NEW this phenylalanine is replaced by serine, and this contact is therefore lost when the D1.3 CDRs are mounted on NEW framework regions. Furthermore, in antibody D1.3 the loop including the end of FR1 bulges out from the surface, whereas in NEW (and other solved structures), it is pinned back to the surface. Despite these problems, the reshaped antibody is able to bind lysozyme.

It is conceivable that the several contacts made by the FR1/CDR1 loop to lysozyme make only a small contribution to the overall energetics of binding. Problems in this region would then have only a slight effect on the affinity. It is also possible that the shape of the binding site could adjust to the binding of antigen (*18*) and therefore that small errors in assembling the CDRs on a new framework might be "self-correcting" when the antigen binds. The energetic price for such putative self-correcting induced fit would be low, given the small loss in binding affinity suggested by the competition data of Fig. 3. It is not possible to deduce the exact change in affinity from the competition data, as the competition may only reflect the relative on-rates. However, even if the difference in affinities were tenfold, this would correspond to no more than the loss of a single hydrogen bond interaction (*19*). The shape of the antigenic epitope could also adjust to the binding of antibody (*20*), although no structural change in lysozyme was detected in its complex with the mouse D1.3 antibody (*6*). Furthermore, the whole surface of interaction could reorientate slightly, perhaps by rocking on side chains (*21*), to make a set of new contacts. This is an attractive model for large surfaces making multiple interactions. Thus self-correction could occur at the level of the antibody, the antigen, or the complex.

In conclusion, the structure of antibodies may incorporate features that assist the grafting of hypervariable regions. The critical features are that framework contacts are largely conserved between V_H and V_L domains, between the sheets within a domain, and to the CDR loops. In addition, the shape of the antigen binding site or the antigenic determinant (or both) might be able to adjust to each other in an induced-fit mechanism.

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29 September 1987; accepted 8 February 1988

Peroxisomal Membrane Ghosts in Zellweger Syndrome—Aberrant Organelle Assembly

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Peroxisomes are apparently missing in Zellweger syndrome; nevertheless, some of the integral membrane proteins of the organelle are present. Their distribution was studied by immunofluorescence microscopy. In control fibroblasts, peroxisomes appeared as small dots. In Zellweger fibroblasts, the peroxisomal membrane proteins were located in unusual empty membrane structures of larger size. These results suggest that the primary defect in this disease may be in the mechanism for import of matrix proteins.

ZELLWEGER SYNDROME IS A DISEASE in which an entire organelle, the peroxisome, appears to be missing, as first recognized by Goldfischer *et al.* (*1*). The peroxisome is nearly ubiquitous in eukaryotic cells and functions in fatty acid β-oxidation, plasmalogen biosynthesis, cellular respiration (H_2O_2-fonning), gluconeogenesis, bile acid synthesis, and purine catabolism (*2*). This human genetic disorder, characterized by profound neurological impairment, metabolic disturbance, and neonatal death, has taught us much about peroxisome function (*3*) and promises to teach us more about peroxisome assembly. Some peroxisomal proteins are synthesized normally in Zellweger syndrome (*4*), but they are not assembled into peroxisomes. Many of these proteins are rapidly degraded, with the result that important soluble matrix enzymes (catalyzing β-oxidation) (*4*) and membrane-bound enzymes (catalyzing the initial steps in plasmalogen biosynthesis) (*4*, *5*) are missing or seriously deficient, thus causing severe metabolic abnormalities (*3*). On the other hand, some peroxisomal enzymes (for example, catalase) are present in normal amounts in Zellweger cells but are located free in the cytosol (*4*, *6*, *7*).

These defects correlate with the known facts of peroxisome biogenesis. All peroxisomal proteins investigated thus far are synthesized on free polyribosomes and are assembled posttranslationally into preexisting peroxisomes (*8*). If the organelle is missing (*1*), newly made proteins will just diffuse through the cytosol, unable to enter a peroxisome. Under these circumstances, it is not surprising that many are degraded.

The autosomal recessive genetics of Zellweger syndrome indicates that a single mutation is responsible for the defects. One explanation would be that the mutation prevents the assembly of the peroxisomal membrane. In this case peroxisomal integral membrane proteins (PxIMPs) (*9*) should be absent, since those that have been studied are made on free polyribosomes (*10*–*12*) and are unlikely to be stable if not integrated into a membrane. Two other possibilities were suggested by the unexpected finding that several PxIMPs are present in normal amounts in Zellweger liver (*13*). The peroxisomal membranes could be assembled in Zellweger syndrome, but the import of matrix proteins is defective. This would result in empty (or nearly empty) membrane ghosts, which would not be recognizable by electron microscopy or cytochemistry. Alternatively, the PxIMPs, in the absence of peroxisomes, might have violated the rules of protein sorting and inserted into the wrong intracellular membrane(s).

To differentiate among these possibilities, we analyzed fibroblasts with a polyspecific antiserum against rat liver PxIMPs (polyspecific anti-PxIMPs) (*12*) (Fig. 1A, lane 1). This serum detected three human PxIMPs with masses of approximately 140, 69, and 53 kD in control cells (Fig. 1A, lanes 2 to 4). These PxIMPs were also present in Zellweger fibroblasts (lane 5), and they cosedimented in equilibrium density centrifugation of Zellweger fibroblast homogenates. However, the PxIMPs were found at an abnormally low density of 1.10 g/cm^3 (instead of the usual fibroblast peroxisome density of 1.17 g/cm^3)[lanes 6 and 7; further details in (*14*)]. Several other cell membranes also sedimented in the low-density region of the gradient where the PxIMPs were found (*14*). Therefore, the fractionation data are consistent with the possibility that the PxIMPs are present in aberrant peroxisomal membrane ghosts, but they do not exclude erroneous localization in lysosomes, the endoplasmic reticulum, or some other low-density organelle. Immunofluorescence studies were carried out to resolve this uncertainty.

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