Derivation and Diversification of Monoclonal Antibodies

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A MOUSE CAN MAKE 10 MILLION DIFFERENT ANTIBODIES, each synthesized by its own B lymphocytes. About 1000 different antibodies are able to recognize one single antigenic determinant. When a conventional mouse response to such a determinant is analyzed, only five to ten different antibody species are seen, representing probably a random sample of the total repertoire. So even when appropriate adsorptions or allogeneic immunizations have confined the antibody heterogeneity to a single determinant, the sera obtained have four disadvantages: (i) the titers are low; (ii) the antibodies, although specific for a single determinant, are nevertheless heterogeneous; (iii) the supply is limited; and (iv) it is impossible to reproduce the same combination of specific antibodies in a new animal.

The method of lymphocyte fusion that I developed together with C. Milstein of the Medical Research Council, Cambridge, England, provides a tool to overcome these limitations (Fig. 1). Mouse myeloma tumor cells are fused to spleen cells derived from a mouse that has previously been immunized with antigen. About 50 percent of the hybrid cells combine the desired parental traits: (i) vigorous growth in tissue culture derived from the myeloma tumor cell and (ii) antibody production from the splenic B cell. A relatively high proportion of the hybridoma cells secrete antibody specific to the immunizing antigen (*1, 2*).

This technique has several advantages.

1) It has specificity in that each hybrid produces only one antibody.

2) The technique gives an unlimited supply of antibody. The hybrids are immortal like tumor cells, can be frozen, secrete 10 to 50 μg of antibody per milliliter of culture fluid, and, upon injection into mice, produce titers as high as 1 to 10 mg of antibody per milliliter of body fluid.

3) Impure antigens lead to pure antibody reagents. The monoclonal antibody, by definition, characterizes only one antigen of the many that may be injected into the mouse.

4) All specificities can be rescued. The empirical observation seems to be that, if an immune response can be elicited in the mouse, specific hybridomas can also be derived.

5) Enrichment of specific hybridomas occurs. Specific B cells are rare even in the spleen cell population of an immunized mouse. However, there is an enrichment of specific B cells (10 to 100 times) in the corresponding hybridoma population.

6) There is a high level of antibody secretion. B hybridomas secrete high levels of antibodies, irrespective of whether the normal B cell was a high producer.

7) Antibodies become manipulable. The hybridoma cell lines can be mutated to produce antibodies not found in nature.

8) It is a general method. Until now, it has only been possible to apply cell fusion for the rescue of T-cell functions. In these cases T-hybridoma lines secreting different lymphokines, exerting killer function or helper and suppressor activities, have been generated.

Some selected applications of the fusion technique are summarized in Table 1.

In the past, conventional sera have been used to define, purify, detect, quantify, map, modify, select, and localize antigen. Thus, with few exceptions (*3, 4*), the monoclonal antibodies have not led to new applications. However, as pure chemicals of higher precision and unlimited supply, they are replacing the conventional sera and thereby contributing to a worldwide standardization of antibody-mediated reactions.

With the use of hybridoma lines one can diversify a single antibody molecule by selecting mutant molecules, by adding heavy (H) and light (L) immunoglobulin (Ig) chains, and by cloning and mutating its genes with the use of "reverse genetic" techniques (see below) and reintroducing them into cell lines or into the germline of a mouse. The variants give some insights into the structure-function relations of Ig proteins, their RNA, and genes. The analysis of variants of monoclonal antibodies has also led to more speculative hypotheses about the interaction of H and L chains in multigene families.

Diversification of Monoclonal Antibodies

Many investigators have generated variants of monoclonal antibodies (Table 2) with perhaps more interesting properties than the ones I will describe here. But this is a somewhat personal account of this development and is not meant to be a review. It is focused, where possible, on experiments in which one hybridoma cell line, Sp6/HLGK, was used. The line is derived from a BALB/c mouse immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS). It secretes an immunoglobulin μ H chain associated with a κ L chain of anti-TNP specificity (designated HL in Sp6 and equivalent hybridomas) together with a γ and κ chain derived from the fusion myeloma line X63-Ag8 (designated GK in Sp6 and equivalent hybridomas) (*2*).

The H-Chain Toxicity Hypothesis

The first hybridomas produced two immunoglobulins (the specific lymphocyte-derived one and the myeloma-derived one) and were therefore, like Sp6, of the HLGK type. To obtain pure, specific antibodies, sublines were selected that had lost the production of the nonspecific G and K chains of the myeloma fusion partner (*2, 5*). It was observed that H chains are more easily lost than L chains. The

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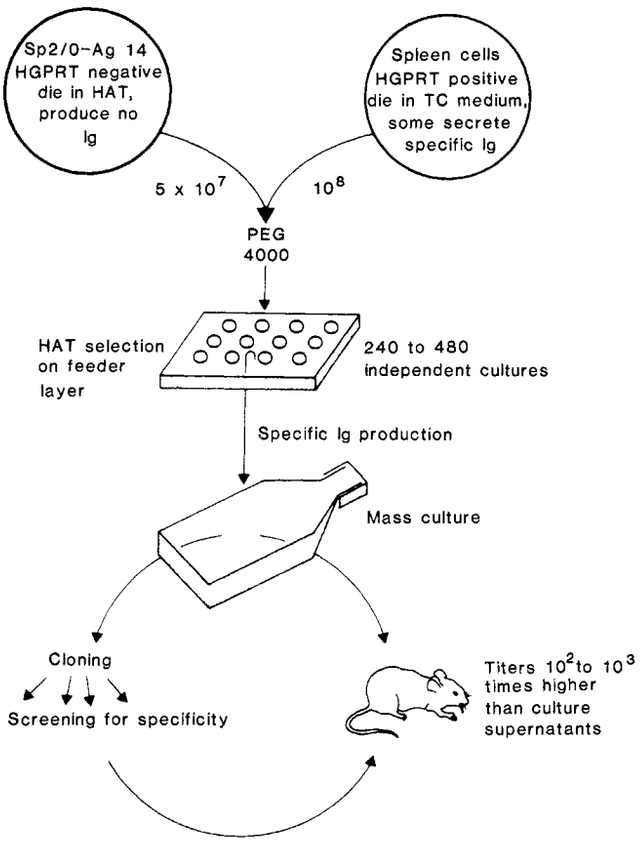

Fig. 1. The hybridoma technique. The fusion line Sp2/0-Ag14 comes from a hybridoma line and is devoid of endogenous Ig production (*61*): HGPRT, hypoxanthinephosphoribosyltransferase, an enzyme necessary for DNA synthesis, when the normal pathway is blocked by aminopterin; HAT, hypoxanthine, aminopterin, thymidine (*62, 63*); PEG, polyethylene glycol.

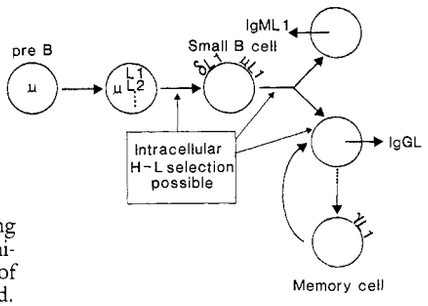

Fig. 2. Schematic representation of B-cell development. The points at which increased production of H chains or production of different H-chain isotypes might become toxic for the cells are indicated. Variable-region mutations increasing H- and L-chain pairing affinities may avoid the toxicity of free H chains and be enriched.

sequence of chain loss as exemplified with Sp6 was Sp6/HLGK → Sp6/HLK → Sp6/HL and not Sp6/HLGK → Sp6/HLG → Sp6/HL. This was reminiscent of the results of Baumal *et al.* (*6*), who, using the myeloma line MPC11, found that H-chain loss was observed at a rate of 1×10^{-3} to 2×10^{-3} per cell and generation but L-chain loss was not observed even at a rate that was 100 times lower. After H-chain loss, however, L chains were lost at a rate similar to the H chains.

To test the generality of the observation, a series of hybridoma lines was established making three, two, or one antibody, and their chain-loss variants were monitored (*5*) (Table 3). L-chain loss was increasingly inhibited only in those combinations that produced an increasing excess of H chains. We concluded that H chains not counterbalanced by enough L chains are toxic for the cells. Excess H-chain producers have a growth disadvantage and cannot be cloned out from the cell population.

Additional support for the H-chain toxicity hypothesis came from the analysis of Sp6 variants. A subline (Sp6/HLk) was derived producing a variant K chain that combined less efficiently with the H chain (this property is symbolized by the lowercase k in Sp6/HLk). The Sp6/HLk line lost L chains at a frequency that was 0.001 of the original Sp6/HLK line (*5, 7*). We concluded that the variant k could not substitute for the parental K chain to avoid toxic accumulation of free H chain. We have cloned the H- and L-chain genes of Sp6 and reintroduced them either separately or physically linked together into the Ig-nonproducing myeloma line X63Ag8.6.5.3. If production of free H chains is toxic for the cell, we would expect that transformants containing only μ genes are selected which produce lower amounts of μ genes when compared to (μ + κ) gene transformants. A five- to tenfold difference was observed between these two groups of transformants. The numbers given in Table 4 show the reduction in Ig protein or messenger RNA with cloned genes as compared to the production of the Sp6/HL parental line. On average, about 0.01 as much μ RNA or

Table 1. Uses of monoclonal antibodies.

Use	Application	Example	Reference
To define antigen	On bacteria	Classification becomes easier	(*28*)
	On viruses		(*29*)
	On parasites		(*30*)
	On cells	Eleven human T-cell antigens established	(*31*)
		Histocompatibility antigen typing	(*32*)
		Tumor antigens	(*33*)
To purify	Factors, hormones	Enrichment of interferon (500-fold)	(*34*)
	Cell membrane	Enrichment of Ia antigen of rat (200-fold)	(*35*)
To detect and quantify	In crude mixtures	Human chorionic gonadotropin in pregnancy tests	(*36*)
To map	Epitope characterization	Seven determinants on mouse μ chain	(*14*)
To modify	Infectivity	Sporozoite of *Plasmodium berghei*	(*37*)
	Toxicity	Digoxin overdose	(*38*)
	Function	αLy2 suppresses T-cell killing	(*39*)
	Immunogenicity	αRhesus factor	(*40*)
To select	α Idiotype	Enhancement and suppression of anti-NP immune response	(*41*)
	Mutations	Histocompatibility antigens	(*3*)
		Influenza A virus	(*4*)
To localize	In organs	Nervous system of leech	(*42*)
	In body	Tumor imaging in humans	(*43*)

Table 2. Mutations and alterations in cell lines producing monoclonal antibodies.

Selection	Mutation or alteration	Frequency	Reference
Loss of isotype	Loss of H or L chain	$1/10^2$	(*44*)
	Domain deletions	$1/10^3$	(*45, 46*)
	Loss of λ-chain secretion due to point mutation	$>1/10^4$	(*47, 48*)
Change in IEF	Domain deletions, frameshift, point mutation	$1/10^3$	(*49*)
Cell sorter, positive selection for different isotype	Change of isotype often associated with deletions	$\sim 1/10^5$–10^7	(*50–52*)
Loss of idiotype, loss and gain of antigen binding	Possible gene conversion point mutations	10^{-2}–10^{-3}	(*53–55*)
Loss of lytic activity	Deletions, frameshifts, insertions	$1/10^3$–10^4	(*7, 17, 56*)
Reverse genetics	Chimeric antibodies:		
	V_H-C_κ		(*16, 19*)
	V_{mouse}-C_{human}		(*20, 57*)
	Antibody-enzyme		(*58*)
Secondary hybrids	Monovalent antibodies		(*5*)
	Two specificities		(*6*)
	Complementation of specificity		(*8*)

protein is found in the transformants containing only μ genes. This low amount is comparable to the amount of μ alone found in pre-B cells (*8*) and may no longer be toxic for the cells. In proteins consisting of two different subunits such as H and L, one chain is often produced in excess (L chains in Ig-producing cells). Potentially toxic effects of free L but not free H chains will therefore be selected against. In the multigene Ig family with about 5,000 to 10,000 H and 250 L chains, elimination of cells producing H-L pairs with affinities too low to form antibody molecules may be an important control mechanism.

Somatic mutants of H and L chains dominate late immune responses (*9, 10*). It is conceivable that mutations occurring in the variable regions of low-affinity H and L pairs enhance their pairing affinity. Such cells avoid elimination caused by free H-chain toxicity and contribute to the somatic diversification of antibody molecules. Scaling up Ig production or switching Ig classes may induce new rounds of selections (Fig. 2). Preferential associations of original H-L pairs in competition experiments (*11, 12*) are easily explained in terms of this intracellular selection mechanism.

Antibodies with One Specificity Generated by Secondary Fusions

The Sp6/HK and Sp6/LK lines (chain-loss derivatives of Sp6/HLGK) no longer produce antibodies with anti-TNP specificity (a property confined to the HL combination). Both lines were fused to unimmunized mouse spleen cells that had been stimulated for 4 days with mitogenic LPS derivative. The resulting hybrids were screened for restoration of the original anti-TNP specificity (*13*). In about every 100th hybridoma, the anti-TNP activity of the Sp6/HK line was restored. In about every 2000th hybridoma, the anti-TNP activity of the Sp6/LK line was restored, which was indistinguishable from values obtained with the line Sp2/0, which does not contribute any Ig chain. The generality of this observation was confirmed by the similar results obtained when three other pairs of hybridomas of the HK or L type were used. The HK lines could easily be complemented, and the L lines gave "background" complementation. From these experiments we drew the following conclusions (*13*).

1) About 40 L-chain, variable (V)-region genes contribute to the L-chain repertoire of the mouse.

2) About 250 different L chains are found in unstimulated spleen cells, a result of the combination of 40 V-region genes with four J (joining) segments and their early somatic diversification.

3) About 20 to 40 times more H than L chains are expressed in early, unstimulated spleen lymphocytes.

4) A statistical analysis of the data leads to the generalization that, could one screen through all H chains with a given L chain, one would find any given antibody specificity. Could one screen through all L chains with a given H chain, a given antibody specificity would be found only in every 20th case.

The above points must be considered with caution, since they are based on limited numbers of hybridomas. Nevertheless, I believe that hybridomas have provided us with a new way to analyze the problem of antibody diversity, by analyzing H and L chains separately and thereby avoiding the enormous heterogeneity created by the combination of H and L chains in antibody molecules. Ten different sublines, each of which makes a different L chain, have been isolated from Sp6; the Sp6 L chain, together with Sp6 H chain, gives rise to the original anti-TNP specificity. Such cell lines can be used to provide insights into H- and L-chain V-region interactions necessary to create a given antigenic combining site. A more practical aspect of such secondary arrays of antibodies with one specificity would be the isolation of low-affinity antibodies, which may be advantageous for certain antigen-purification procedures.

Diversification by Mutant Selection

We have studied mutants of the Sp6 and PC700 [IgM antiphosphorylcholine (PC)] line. The selection method was simple. The hapten TNP or PC was covalently attached to the membranes of the cells. The cells were diluted and incubated in the presence of complement. Cells secreting wild-type IgM preferentially bind their own antibody and are killed. There is an enrichment for cells secreting lower amounts of IgM or less lytic IgM. Three to four selection rounds lead to an almost "pure" mutant-cell population.

Table 3. Inhibition of L-chain loss in proportion to excess of H chains.

3H + 3L	→	$\frac{2H + 3L}{3H + 2L}$	~	$\frac{2}{1}$
2H + 2L	→	$\frac{1H + 2L}{2H + 1L}$	~	$\frac{8}{1}$
1H + 1L	→	$\frac{0H + 1L}{1H + 0L}$	≳	$\frac{100}{1}$
2H + 3L 1H + 2L 0H + 1L	L-chain loss as frequent as H-chain loss			

Table 4 summarizes the mutants so far characterized for the Sp6 line. The deletion variants were used to map a panel of monoclonal antibodies (*14*). Figure 3 shows the IgM structure of some of the deletion variants and the mapping of monoclonal antibodies to each of the five μ domains. The monoclonal antibodies against eight defined, noncross-reacting determinants of mouse μ chains were used to define new IgM variants (*15*), to map complement lq (Clq) binding to the area of binding of C2-23 monoclonal antibody (*16*), and to define the quaternary structure of some of the IgM mutants (*14*).

Table 4. Mutations affecting Sp6 IgM.

Defect	Number	Description	Reference
Not determined, possibly point mutations	2	Lower affinity, lytic activity reduced 200 times	(*7*)
Insertion	2	Loss of L production or reduced L production	(*17*)
Deletion	4	Loss of C1μ, C2μ, C3μ*	(*56*)
Frameshift	16	Loss of C-terminal portions	(*15*)

*Found in the PC700 system (*59*) only.

Diversification by Reverse Genetics

The L-chain gene of Sp6 has been cloned and sequenced (*17*). The Sp6 H-chain V-region gene was placed into the V_κ-C_κ intron, using the Hind III and Xba I restriction sites. After transfection into the X63Ag8.6.5.3 (X63/0) myeloma line (*18*), a V_μ-C_κ chimeric protein was revealed (Fig. 4). Fusing this line to Sp6/L (IgM 10) resulted in hybrids producing covalently linked dimers containing the V_κ-C_κ and the chimeric V_μ-C_κ chain. Binding of the H chain–dependent anti-idiotypic (anti-Id) antibody 20-5 was restored in these dimers. We could not measure antigen-binding activity, because of the low anti-TNP affinity of the Sp6-IgM [$10^{-4}M$ (*7*)]. A similar chimeric protein with antigen binding for arsonyl was, however, described (*19*). Small, antigen-binding molecules lacking the H-chain (C-region) determinants might be useful when only one binding site is required (to avoid modulation of cell surface antigens) and when a smaller molecule with less nonspecific interactions (via Fc-receptors), and possibly a higher elimination rate, is needed.

Such molecules might also be less immunogenic. Since most therapeutically interesting antibodies are derived from the mouse, the construction of chimeric antibodies with human C regions and mouse V regions could be a solution that avoids immunogenicity but retains effector functions of the antibody. That this is possible has recently been shown with the Sp6 IgM (*20*). The mouse H- and L-chain V regions were placed in front of human μ- and κ-constant regions, respectively. Mouse-human chimeric IgM was recovered from supernatants of a plasmid-carrying myeloma line. The IgM was pentameric and functional in that it was able to lyse TNP-coupled sheep erythrocytes.

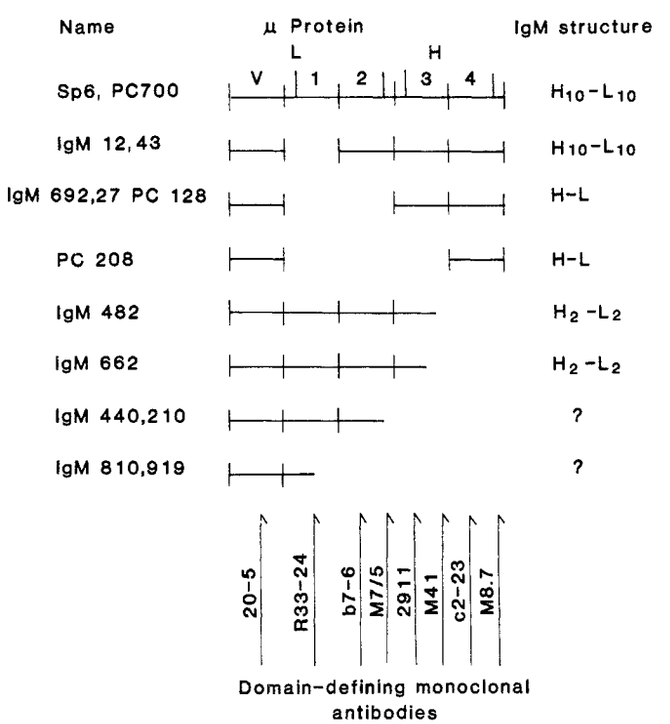

Fig. 3. Deletions in mouse μ chains. The wild-type μ protein (Sp6 and PC700) is drawn in its domain structure, and disulfide bridges to the L or H chains are indicated. The L chain of IgM 12 and IgM 43 is not covalently bound to μ, whereas it is in all other mutants with known IgM structure. The monoclonal rat antibodies to mouse μ were selected from about 20 antibodies and define independent binding sites. The line 20-5 makes a mouse anti-idiotypic antibody reacting with Sp6 but not PC700 IgM.

The Transgenic Mouse Model

So far three groups have introduced rearranged Ig genes into the germline of the mouse. A μ gene alone, a κ gene alone, and a combination of both genes derived from the Sp6 line, have been used (Table 5). In the Sp6 case, about 50 molecules of the H- and L-chain genes, both cloned into a pBR322 plasmid vector ($pRHL_{TNP}$), were injected into fertilized eggs from Swiss albino mice. From 13 implanted zygotes five offspring mice were obtained, one of them carrying the gene. Analysis of the germline transmission pattern indicated integration of four copies of the $pRHL_{TNP}$ plasmid into one autosomal chromosome.

Expression of Transimmunoglobulins

In all cases with germline transmission of the Ig genes, expression of the respective chains was also observed. Expression of κ_{21} chains was confined to the B-cell lineage, excluding Abelson murine sarcoma virus–transformed pre-B cell lines (*21*). Expression of μ_{NP} chains was observed in B and T cells (*22*). Other tissues such as kidney, brain, heart, lung, and liver did not transcribe the genes. Fluorescence analysis indicated that the Sp6 μ_6 and κ_6 chains were expressed on the membrane of B but not T cells, although the μ_6 chain (κ_6 was not testable) was expressed in about one quarter of the splenic T cells stimulated with concanavalin A. The Sp6 Ig was found as pentameric, functional IgM in the sera of the transgenic

Table 5. Immunoglobulin genes introduced into mouse germline cells.

Gene specificity	Molecules injected	Zygotes implanted	Offspring (with transgene)	Number of copies	Gene in germline	Reference
μ (NP)	50	284	13 (5)	17–140	3	(*22*)
κ_{21} (?)	440	197	11 (6)	20–100	6	(*21, 24, 60*)
μ, κ (TNP)	50	13	5 (1)	4	1	(*25*)

mice, where it repesented about one-fifth to one-third of the total mouse IgM. It will be interesting to see whether such massive production of one specificity induces changes in idiotypic network interactions. Introducing antibodies with anti–self-specificities into the germline of the mouse may give some insight on how self-tolerance is achieved at the B-cell level. With the genes introduced so far, another immunological question, the one of allelic exclusion, could be analyzed: How is it possible that B cells produce H and L chains from only one of the two homologous chromosomes, a prerequisite of effective clonal selection?

Influence of Transgenic μ and κ Chains on Allelic Exclusion

In order to create a functional H-chain gene, three spatially separated DNA segments, the V region, the diversity (D) segment, and the joining (J_H) segment, must juxtapose. To create a functional L-chain gene, a V_L and a J_L segment must join. In roughly one quarter of the B cells, allelic exclusion is achieved by having functionally rearranged VDJ_H^+ and VJ_L^+ units in one allele and nonfunctionally rearranged VDJ_H^- and VJ_L^- units in the other allele, which, as a result of mistakes during the joining process, are unable to produce the respective chains. In about 75 percent of the B cells, either the H- or the L-chain V region, or both, have not been completely rearranged. In order to explain this finding, a regulatory feedback mechanism of the Ig chains on the rearrangement process was postulated (*23*).

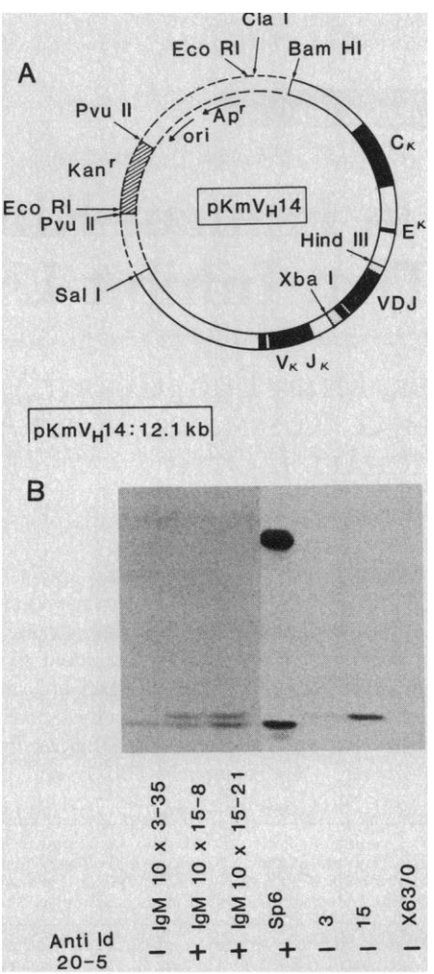

Fig. 4. Production of chimeric antibodies. (A) The plasmid $pKmV_H14$ carries the kanamycin-resistance gene (shaded), pBR322 sequences (dashed), and an insert of the Sp6 κ gene from the Bam HI to the Sal I restriction sites. The Sp6 variable H-chain gene (VDJ) was inserted into the Xba I and the Hind III restriction sites in front of the κ enhancer element ($E^κ$). (B) Sodium dodecyl sulfate–gel electrophoresis under reducing conditions of products formed after the transfection of $pKmV_H14$ plasmid into X63/0 myeloma cells. Stably transformed lines 3 and 15 show a band with slightly slower mobility than the Sp6 L chain (right). L-chain and $pKmV_H14$-directed production of $V_HC_κ$ in (X63/0 – 15 × IgM 10) hybrids 8 and 21 is observed. Only in these hybrids is the H chain–dependent idiotype 20-5 restored.

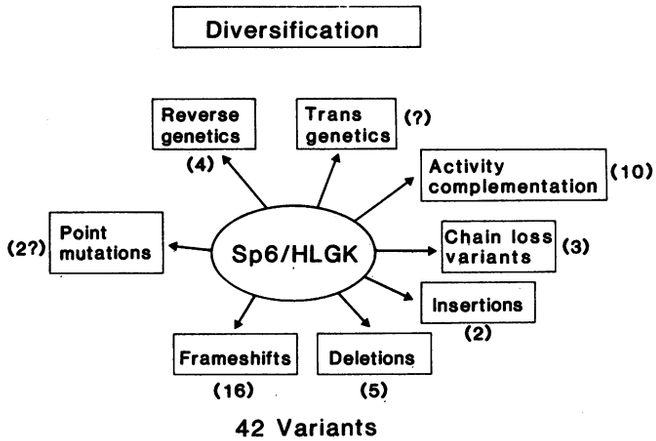

Fig. 5. Summary of Sp6 variants.

To study the effect of rearranged and expressed Ig transgenes on the rearrangement of endogenous Ig genes, a series of B-cell hybridomas were made and their endogenous H- and L-chain expressions monitored. Of 24 hybridomas derived from $κ_{21}$ transgenic mice, none expressed an H chain with two L chains (*24, 25*). In seven cases where no H-chain production was observed, two L chains, the endogenous one and the transgenic one, were expressed. These results are compatible with a negative feedback mechanism of H-L molecules on the L-chain gene-rearranging process.

Expressing the transgenic $μ_6$ and $κ_6$ chains should therefore inhibit endogenous L-chain gene rearrangement, but this was not observed in any of the 11 hybridomas. However, the trans-$κ_6$ chain was expressed at one-tenth the level of the endogenous L chains, whereas the $μ_6$ chain was made in excess. Thus, low amounts of $μ_6$ $κ_6$ molecules in an excess of free $μ_6$ chains are not a feedback signal to stop L-chain gene rearrangement. This observation is in agreement with the results obtained from the exceptional L-chain double producers found in myeloma cell lines such as S107, where one of the κ L chains was unable to combine with the α H chain (*26*), and in the MOPC315 line where a truncated $λ_1$ chain that was unable to bind to its H chain was found together with a functional $λ_2$ L chain (*27*). A high proportion (8 of 11) of hybridomas derived from the $μ_6κ_6$-expressing mouse did not express a second endogenous H chain. Five of these lines still retained both H-chain encoding chromosomes. In only one of the ten H-chain alleles was a complete (though inactive) VDJ_H^- unit found. One allele apparently was frozen in the germline configuration, and eight stopped rearrangement at the immature DJ_H state. The result is thus compatible with a (leaky) feedback regulation of the H-chain gene-rearrangement process by the μ chain itself. Whether the L chains are also involved in this process must be determined by analysis of pre-B cell lines. Other interpretations, however, such as different cellular selection processes in transgenic versus normal mice, are possible. Nevertheless, the power of the transgene approach to study immunological phenomena is clearly demonstrated.

Let me now return to the general theme of this article and ask whether diversification of our Sp6 antibody could be achieved by having introduced its genes into the mouse. At the moment one can only speculate. A somatic mutation mechanism operates on Ig L- and H-chain genes at an approximate rate of 10^{-3} per base and generation (*9, 10*). It seems possible that the introduced genes will profit from this mechanism and that we will generate a series of somatically mutated Sp6 IgM molecules. Figure 5 summarizes the variants obtained so far from the Sp6 line by the use of different techniques and underlines my belief that a single monoclonal

antibody will only be the starting point for a variety of man-made secondary antibodies, each manufactured to satisfy a special requirement.

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Research Articles

The Site of Attachment in Human Rhinovirus 14 for Antiviral Agents That Inhibit Uncoating

THOMAS J. SMITH, MARCIA J. KREMER, MING LUO, GERRIT VRIEND,* EDWARD ARNOLD, GREG KAMER, MICHAEL G. ROSSMANN, MARK A. MCKINLAY, GUY D. DIANA, MICHAEL J. OTTO

WIN 51711 and WIN 52084 are structurally related, antiviral compounds that inhibit the replication of rhino (common cold) viruses and related picornaviruses. They prevent the *p*H-mediated uncoating of the viral RNA. The compounds consist of a 3-methylisoxazole group that inserts itself into the hydrophobic interior of the VP1 β-barrel, a connecting seven-membered aliphatic chain, and a 4-oxazolinylphenoxy group (OP) that covers the entrance to an ion channel in the floor of the "canyon." Viral disassembly may be inhibited by preventing the collapse of the VP1 hydrophobic pocket or by blocking the flow of ions into the virus interior.

THE COMMON COLD, POLIOMYELITIS, HEPATITIS A, AND foot-and-mouth disease are among a large group of diseases whose cause can be attributed to various members of the picornavirus family. These viruses are among the smallest RNA-containing animal viruses with a molecular weight of approximately 8.5×10^6, of which roughly 30 percent is RNA. Their external diameter is approximately 300 Å and their protein shells have

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