DERIVATION AND DIVERSIFICATION OF MONOCLONAL ANTIBODIES

Nobel lecture, 8 December, 1984

by

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A mouse can make ten 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 analysed, only 5-10 different antibody species are seen, representing probably a random sample of the total repertoire. So even when appropriate absorptions or allogeneic immunizations have confined the antibody heterogeneity to one single determinant the sera obtained have four disadvantages: (1) the titers are low, (2) the antibodies, while specific for a single determinant, are nevertheless heterogeneous, (3) the supply is limited, and (4) the same combination of specific antibodies is impossible to reproduce in a new animal.

The method of lymphocyte fusion developed together with C. Milstein, MRC, Cambridge, England, provides a tool to overcome these limitations (Fig. 1). Mouse myeloma tumour cells are fused to spleen cells derived from a mouse which previously had been immunized with antigen. About 50% of the hybrid cells combine the hoped for parental traits: vigorous growth in tissue culture derived from the myeloma tumour cell and antibody production coming from the splenic B cell. A relatively high proportion of the hybridoma cells secrete antibody specific to the immunizing antigen (1, 2).

The advantages of this technique are:

1) Single specificity. Each hybrid produces only one antibody.
2) Unlimited supply of antibody. The hybrids are immortal like tumour cells, can be frozen, secrete l0-50 µg/ml antibody into the culture fluid, and produce titers as high as l-10 mg antibody per ml body fluid upon injection into mice.
3) Impure antigens lead to pure antibody reagents. The monoclonal antibody by definition characterizes only one antigen of the many 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 also specific hybridomas can be derived.
5) Enrichment of specific hybridomas. Specific B cells are rare even in the spleen cell population of an immunized mouse. They are found to be enriched l0-100 fold in the corresponding hybridoma population.

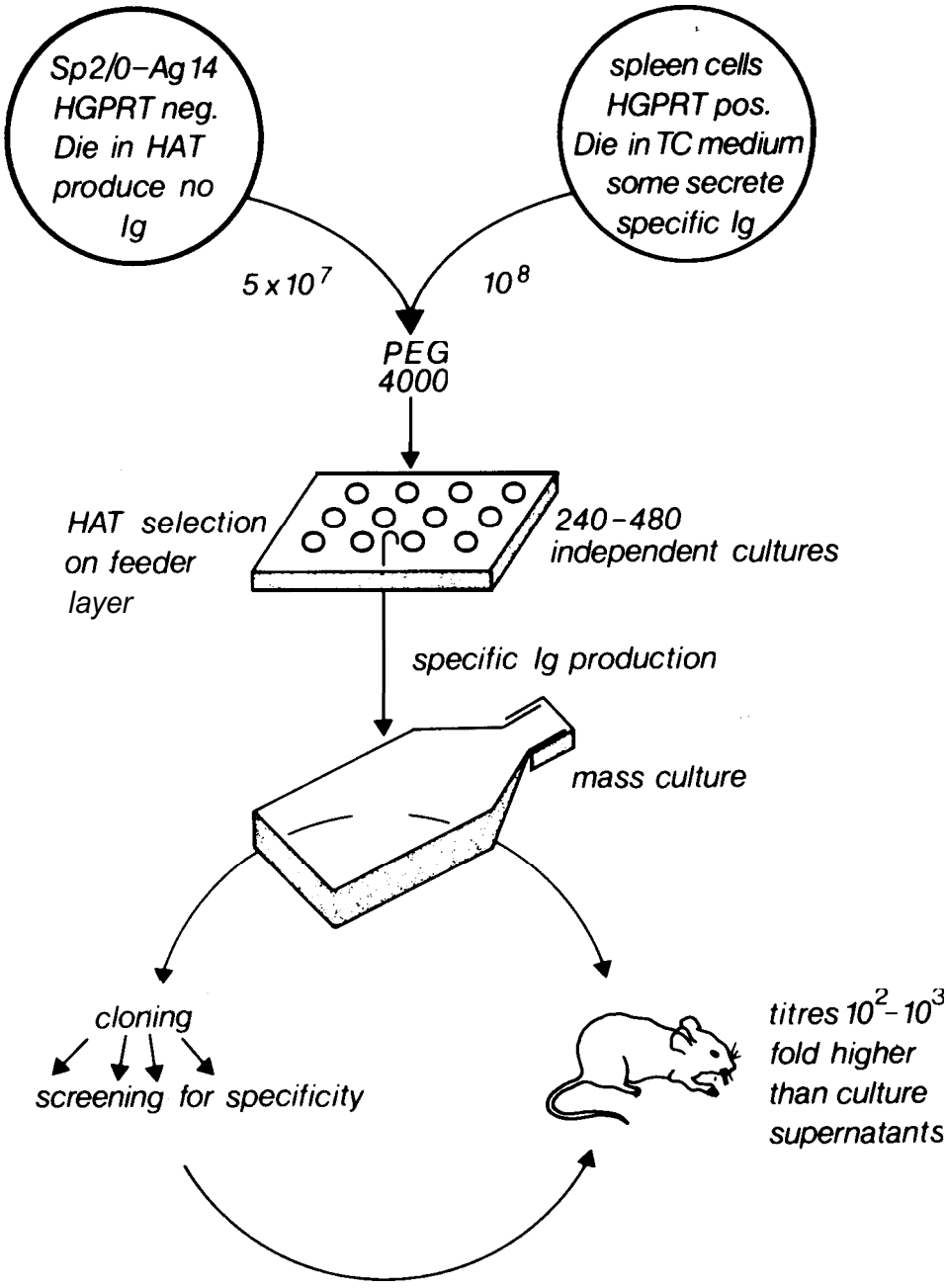


Fig. 1 – The hybridoma technique.
The fusion line Sp2/0-Ag14 comes itself from a hybridoma line and is devoid of endogeneous immunoglobulin production (61). HGPRT =hypoxanthinephosphoribosyltransferase, an enzyme necessary in DNA synthesis when the normal pathway is blocked by aminopterin. HAT = hypoxanthine, aminopterin, thymidine (1) (62,63). PEG = polyethylenglycol.

Table 1. Uses of monoclonal antibodies © 1982 John Wiley & Sons Ltd, From Genetic Engineering to Biotechnology - The Critical Transition. Edited by W. J. Whelan and Sandra Black.

Use	Application	Success, example	References
to *define* antigen	– on bacteria, viruses parasites	Classification becomes easier	3 4 5
	– on cells	11 human T cell antigens established	6
		Histocompatibility antigen typing	
		tumor antigens	8
purify	– factors, hormones	5000 fold enrichment of interferon	9
	– cell membrane	200 fold enrichment of Ia antigen of rat	10
detect and *quantify*	– in crude mixtures	human chorionic-gonadotropin in pregnancy tests	11
map	– epitope characterization	7 determinants on mouse μ chain (constant region)	12
modify	– infectivity	sporozoite of plasmodium berghei	13
	– toxicity	digoxin overdose	14
	– function	αLy2suppresses T cell killing	15
	– immunogenicity	αRhesus factor	16
select	– α idiotype	enhancement and suppression of anti-NP immune response	17
	– mutations	Histocompatibility antigen	18
		Influenza A virus	19
localize	– in organs	nervous system of leech	20
	– in body	tumor imaging in humans	21

6) Dominance of antibody secretion. B-hybridomas secrete high level of antibodies, irrespective whether or not 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. The only generalization so far, was to apply cell fusion for the rescue of T cell functions, where T hybridoma lines secreting different lymphokines, exerting killer function or helper and suppressor activities have been generated.

Some selected applications 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 (18, 19), the monoclonal antibodies have not led to applications unthought of previously. However, as pure chemicals of higher precision and unlimited supply they are replacing the conventional sera and thereby contributing to a worldwide standardisation of antibody mediated reactions.

One of the great advantages of the hybridoma lines lies not only in the

production of monoclonal antibodies but also in the availability of the machinery which produces them. One can diversify a single antibody molecule by selecting mutant molecules, by introducing new immunoglobulin heavy and light chains into hybridoma lines, by cloning and mutating its genes by "reverse genetics" techniques and reintroducing them into cell lines or into the germline of a mouse. The variants give some insights into the structure-function relationship of immunoglobulin protein, their RNA and genes. The analysis of variants have also led to a more speculative hypothesis about the interaction of H and L chains in multigene families.

Diversification of monoclonal antibodies

Many investigators have generated variant forms 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 not meant to be a review. It is focused, where possible, on experiments using one hybridoma cell line, Sp6/HLGK. The line is derived from a Balb/c mouse immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS). It secretes an immunoglobulin mu heavy chain associated with kappa light chain of anti-TNP specificity (designated HL in Sp6 and equivalent hybridomas) together with a gamma and kappa chain derived from the fusion myeloma line X63-Ag8 (designated GK in Sp6 and equivalent hybridomas) (2).

Table 2. Mutations and alterations in cell lines producing monoclonal antibodies. Reprinted with permission from Pergamon Press Ltd. © 1983 Pergamon Press.

Selection	Mutation/alteration	Frequency	Reference
loss of isotype	loss of H- or L-chain	$1/10^2$	22
	domain deletions	$1/10^3$	23, 24
	loss of λ-chain secretion due to point mutation	$>1/10^4$	25, 26
change in IEF	domain deletions frameshift, point mutation	$1/10^3$	27
cell sorter, positive selection for different isotype	change of isotype often associated with deletions	$\sim 1/10^5 - 10^7$	28, 29, 30
loss of idiotype, loss and gain of antigen binding	possible gene conversion point mutations	$10^{-2} - 10^{-3}$	31, 32, 33
loss of lytic activity	deletions, frameshifts insertions	$1/10^3 - 10^4$	34, 35, 36
reverse genetics	chimaeric antibodies		
	$V_H - C\kappa$		37, 38
	$V_{mouse} - C_{human}$		39, 40
	antibody-enzyme		41
secondary hybrids	monovalent antibodies		41 a
	two specificities		41 b
	complementation of specificity		49

Table 3. H-CHAIN TOXICITY

3H + 3L →	2H + 3L / 3H + 2L	~ 2/1
2H + 2L →	1H + 2L / 2H + 1L	~ 8/1
1H + 1L →	0H + 1L / 1H + 0L	≳ 100/1
2H + 3L		
1H + 2L	L-loss as frequent as H-loss	
0H + 1L		

Irnmunoglobulin chain loss variants lead to the heavy chain toxicity hypothesis

The first hybridomas all 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 which had lost the production of the non-specific G and K chains of the myeloma fusion partner (2, 42). It was observed that heavy chains are more easily lost than light chains. The sequence of chain loss as exemplified with Sp6 was Sp6/HLGK → Sp6/HLK → Sp6/HL and not SpG/HLGK → Sp6/HLG → Sp6/HL. This was reminiscent of the results of Coffino and Scharff (22,43), who, using the myeloma line MPCll, found that H-chain loss was observed at a rate of l-2 x $10^{10\text{-}3}$ per cell and generation but L-chain loss was not observed even at a 100 times lower rate. After H chain loss, however, L chains were lost at a rate similar to H chains.

To test the generality of the observation a series of hybridoma lines were established making three, two and one antibody and their chain loss variants were monitored (42, Table 3). L chain loss was inhibited with increasing severity only in those combinations resulting in an increasing excess of H chain production. It is concluded that heavy chains not counterbalanced by enough light chains are toxic for the cells. Excess H chain producers have a growth disadvantage and cannot be cloned from the cell population. Additional support for the H chain toxicity hypothesis came from the analysis of Sp6 variants. A subline (SpG/HLk) was derived producing a variant k chain, which combined less efficiently to the H chain (this property is symbolized by the lower k in Sp6/HLk). The SpG/HLk line lost L at a 1000 x lower frequency than the original SpG/HLK line (34, 42). 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 gene of Sp6 and reintroduced them separately or physically linked into the Ig non-producing myeloma line X63Ag8.6.5.3 (Table 4). If production of free heavy chain is toxic for the cell, we would expect that p-alone transformants are selected which produce lower amounts of μ when compared to (μ and x)-transformants. A 5 to 13 fold difference was observed between these two groups of transformants. On average, about 100 times less μ RNA or protein is found in the μ-alone transformants. This low amount is comparable to the amount of μ alone found in pre-B cells (44) and may no longer be toxic for the cells. In proteins consisting of two

Table 4. Expression of Cloned Sp6μ and χ Genes in X63A~8.6.5.3 B-Myeloma Cells

Gene	DNA Number of copies	RNA (% of Sp6 parent)		Protein (% of Sp6 parent)	
		μ	κ	μ	κ
κ	50 (41)	–	62 (10)	–	50 (4)
μ	7 (8)	1.3 (9)	–	1 (9)	–
μ + κ	5 (11)	6 (8)	4 (8)	13 (12)	13 (12)
Ratio $\frac{\mu+\kappa}{\mu}$	~1	5		13	

Data from J. McCubrey, unpublishcd. In parenthesis the number of stable transformants analysed

different subunits such as H and L in immunoglobulins, often one chain is produced in excess (L chains in Ig producing cells). Potentially toxic effects of free light but not free heavy chains will therefore be selected against. In the multigene Ig-family with about 5-10.000 H and 250 L chains, elimination of cells producing H-L pairs with too low affinities to form antibody molecules may be an important control mechanism.

Somatic mutants of H and L chains-dominate late immune responses (45, 46). 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 association of origi-

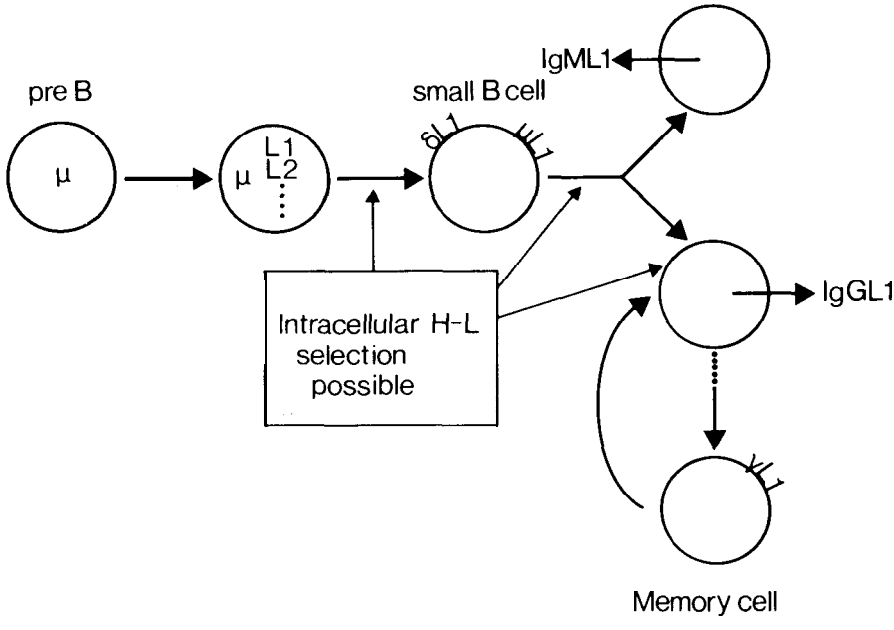


Fig. 2 - Schematic representation of B-ccl1 dcvelopment.
The points at which increased production of heavy chains or production of diffcrcnt heavy chain isotypes might become toxic for the cells arc indicated. Variable region mutations increasing heavy (H) and light (I,) chain pairing affinities may avoid toxicity of free heavy chains and be enriched.

nal H-L pairs in competition experiments (47, 48) are easily explained by this intracellular selection mechanism.

Arrays of antibodies with one specificity generated by secondary fusions

The Sp6/HK and Sp6/LK lines, (chain loss derivatives of Sp6/HLGK) are no longer producing antibodies with anti-TNP specificity (a property confined to the HL combination). Both lines were fused to unimmunized mouse spleen cells stimulated for four days with the mitogen lipopolysaccharide. The resulting hybrids were screened for restoration of the original anti-TNP specificity (49). 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 using three other pairs of hybridomas of the HK- or L-type, with similar results. The HK-lines could easily be complemented, the L-lines gave 'background' complementation. From these experiments the following conclusions were drawn (49):

1) Around 40 light chain variable region genes contribute to the light chain repertoire of the mouse.
2) Around 250 different light chains are found in unstimulated spleen cells, due to the combination of 40 V-region genes with 4 J (joining) segments and their early somatic diversification.
3) About 20-40 fold more heavy than light chains are expressed in early, unstimulated spleen lymphocytes.
4) A statistical analysis of the data leads to the generalisation, that, could one screen through all heavy chains with a given light chain one would find any given antibody specificity. Could one screen through all light chains with a given heavy chain a given antibody specificity is only found in every 20th case.

The above points have to be considered with caution, since they are based on limited numbers of hybridomas. Nevertheless, I think hybridomas have opened a new way to analyse the problem of antibody diversity, by analyzing H and L chains separately, thereby avoiding the enormous heterogeneity created by the combination of heavy and light chains in antibody molecules. The Sp6 line has been diversified into 10 different sublines, each of which makes adifferent light chain, which together with the Sp6 heavy chain gives rise to the original anti-TNP specificity. Such lines will provide insights; of heavy and light chain variable region interactions necessary to create a iven 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 for certain antigen-purification procedures may be advantageous.

Diversification by mutant selection

We have studied mutants of the Sp6 and PC 700 (IgM anti phosphorylcholine (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

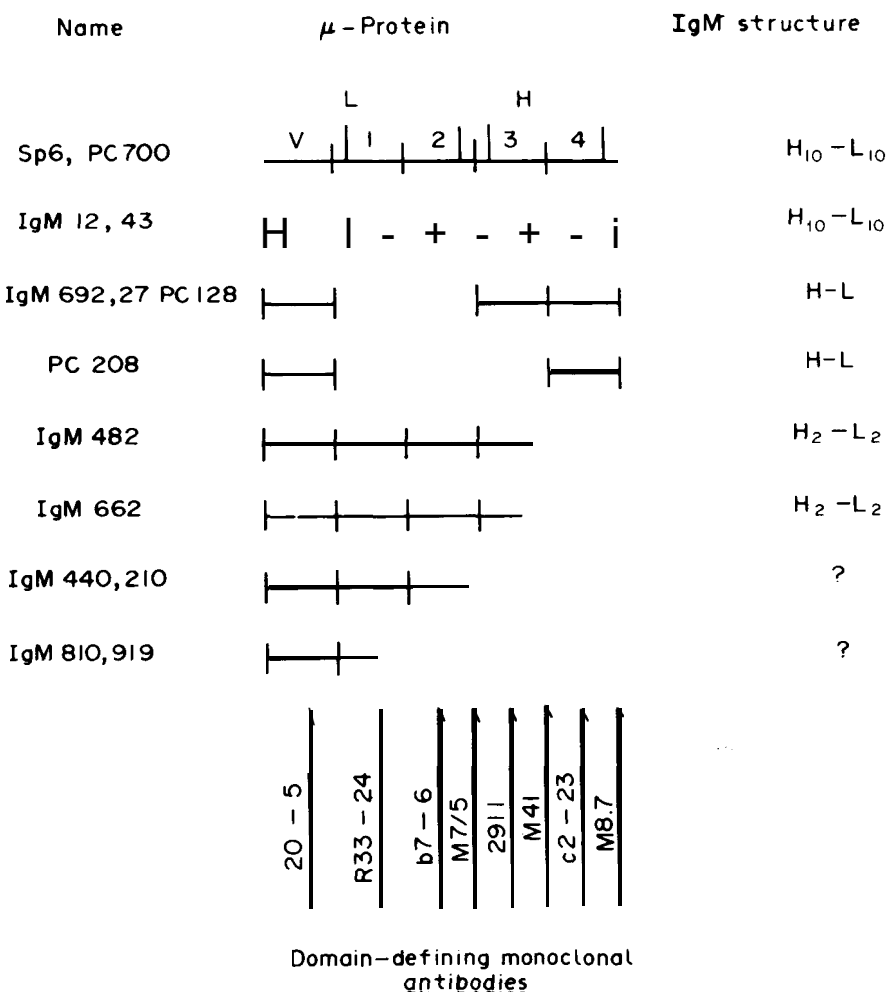


Fig. 3 - Deletions in mouse p-chains
The wild-type p-protein (Sp6 and PC7OO) is drawn in its domain structure and disulphide bridges to the light (L) or heavy (H) chains are indicated. The light chain of IgM 12 and IgM 43 is not covalently bound to μ, whereas it is in all other mutants with known IgM structure. The monoclonal rat anti-mouse b-antibodies 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. Reprinted with permission from Pergamon Press Ltd, © 1984, Pergamon Press.

incubated in the presence of complement. Cells secreting wild type IgM preferentially bind their own antibody and are killed. Cells secreting less or less lytic IgM are enriched. Three to four selection rounds lead to an almost "pure" mutant-cell population. Table 5 summarizes the mutants so far characterized for the Sp6 line. The deletion variants were used to map a panel of monoclonal antibodies (12). Fig. 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 8 defined, non-crossreacting determinants of

Table 5. Mutations affecting Sp6 immunoglobulin M.

Defect	Number	Description	Reference
not determined, possibly point-mutations	2	10×lower affinity 200×reduced lytic activity	34
insertion	2	loss of L production or reduced L production	36
deletion	4	loss of Cμ1, Cμ1,2, Cμ1,2,3,*	35
frameshift	16	loss of C-terminal portions	50

* found in the PC 700 system (52) only.

mouse μ chains were used to define new IgM variants (50), to map Clq binding to the area of binding of C2-23 monoclonal antibody (38, unpublished) and to define the quaternary structure of some of the IgM-mutants (12).

Diversification by reverse genetics

The light chain gene of Sp6 has been cloned and sequenced (36). The Sp6 heavy chain variable region gene was placed into the V_k-C_k intron using the Hind111 and XbaI restriction sites. After transfection into the X63Ag8.6.5.3 (X63/0) myeloma line (51) a V_μ-C_k chimaeric protein was revealed (Fig. 4). Fusing this line to Sp6/L (IgM 10) resulted in hybrids producing covalently linked dimers containing the V_χ-C_χ and the chimaeric V_μ-C_χ chain. Binding of the heavy-chain-dependent anti-idiotypic 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, (34)). A similar chimaeric protein with antigen binding for arsonyl was, however, described (37). Small, antigen-binding molecules lacking the heavy chain constant region determinants might be useful when only one binding site is required (to avoid modulation of cell surface antigens), when a smaller molecule with less non-specific 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 of mouse origin the construction of chimaeric antibodies using human constant regions and mouse variable regions could be a solution avoiding immunogenicity but keeping effector functions of the antibody. That this is possible has recently been shown with the Sp6 IgM (39). The mouse heavy and light chain variable regions were placed in front of human μ- and K-constant regions, respectively. Mouse-human chimaeric IgM was recovered from supernatants of a plasmid carrying myeloma line. The IgM was pentameric and functional in terms of being able to lyse TNP coupled sheep erythrocytes.

The transgenic mouse model

So far three groups have introduced rearranged immunoglobulin genes into the germline of the mouse. A μ and a χ gene alone as well as a combination of both

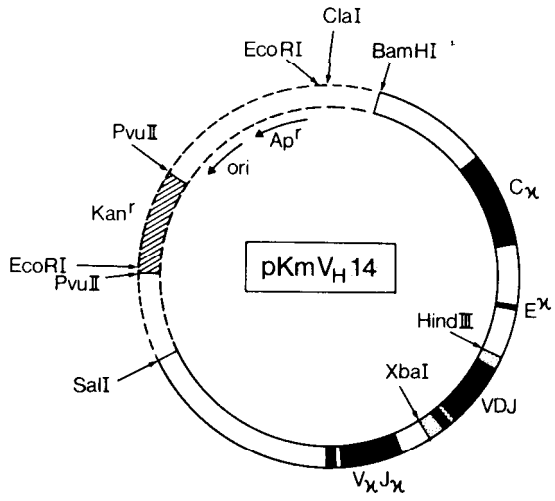
Table 6. Immunoglobulin genes introduced into mouse germline cells

Gene (specificity)	Molecules injected	Zygotes implanted	Offspring (with trans-gene)	# of copies per genome	Gene in germline	References
μ (NP)	50	284	13 (5)	17–140	3	53
$\varkappa_{21}$ (?)	440	197	11 (6)	20–200	6	54, 55, 56
μ, ϰ (TNP)	50	13	5 (1)	4	1	57

genes derived from the Sp6 line have been used (Table 6). In the Sp6 case about 50 molecules of the heavy and light chain genes both cloned into a pBR322 plasmid vector ($pRHL_{TNP}$) were injected into fertilized eggs from Swiss albino mice. From 13 implanted zygotes 5 offspring mice were obtained, one of them carrying the gene. Analysis of the germline transmission pattern indicated integration of 4 copies of the pRHL TNP plasmid into one autosomal chromosome.

Expression of trans-immunoglobulins

In all cases with germline transmission of the immunoglobulin genes also expression of the respective chains was observed. Expression of x_{21}-chains was confined to the B cell lineage excluding Abelson virus transformed pre-B cell lines (55). Expression of μ_{NP}-chains was observed in B- and in T-cells (53). Other tissues like kidney, brain, heart, lung and liver did not transcribe the genes. By fluorescence analysis the Sp6 μ6 and χ6 chains were expressed on the membrane of B- but not T-cells, although the μ6 chain (χ6 was not testable) was expressed in around one quarter of Con A-stimulated, splenic T-cells. The Sp6 immunoglobulin was found as pentameric, functional IgM in the sera of the transgenic mice where it represented around one fifth to one third of the



pKmV$_H$14 : 12.1 kb

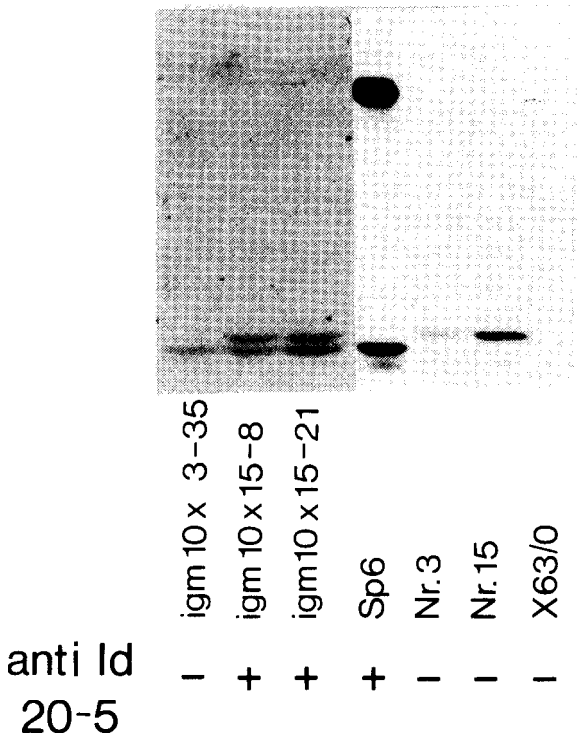


Fig. 4 - Production of chimaeric antibodies

Top: The plasmid pKmV$_H$14 carries the kanamycin-resistance gene (shaded), pBR322 sequences (dashed) and an insert of the Sp6 kappa gene from the BamHI to the Sal1 restriction sites. The Sp6 variable heavy chain gene (VDJ) was inserted into the Xbal and the Hind3 restriction sites in front of the kappa enhancer element (E^{κ}). Bottom: SDS-gel electrophoreses under reducing conditions of products formed after transfection of pKmV$_H$14 plasmid into X63/0 myeloma cells. Stably trans-

Table 7. Allelic exclusion in trans hybridomas.

κ_{21}:	11/24 Hybrids		H κ_{21} − (germline)		
ref 56	2		H − shift L		
	7		− κ_{21} L		
	2		− κ_{21} − (germline)		
	2		− κ_{21} − (rearranged)		
$\mu_6\kappa_6$:	3/11 Hybrids		μ_6 κ_6 H L		
ref (57)	8		μ_6 κ_6 − L		
*5 lines still have both H-encoding chromosomes		→10 alleles:	VDJ⁻ 1	DJ 8	germline 1

H = endogeneous heavy chain, I,= endogeneous light chain; in parenthesis light chain gene configuration.

* Heavy chain gene configuration of 5 of the 8 H-minus hybrids is shown.

chain variable region or both have not been completely rearranged. In order to explain this finding a regulatory feedback mechanism of the immunoglobulin chains on the rearrangement process was postulated (58).

To study the effect of rearranged and expressed immunoglobulin transgenes to the rearrangement process of endogeneous Ig genes, a series of B cell hybridomas were made and their endogeneous heavy and light chain expression monitored. In Table 7 our results and those obtained from transgenic mice carrying a rearranged kappa gene alone (56, 57) are summarized (results from the μ alone transgenic mice are not yet available). From 24 hybridomas derived from χ21 transgenic mice none expressed a heavy chain with two light chains. In seven cases where no heavy chain production is observed, two light chains, the endogeneous and the transgenic one, are expressed. These results are compatible with a negative feedback mechanism of H-L molecules on the light-chain gene rearranging process.

Expressing the transgenic μ_6- and Q-chains should, therefore, inhibit endogeneous light chain gene-rearrangement, which was not observed in any of the eleven hybridomas. However, the trans-κ_6- chain was expressed at one tenth the level of the endogeneous light 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 light chain gene rearrangement. This observation is well in agreement with the exceptional light chain double producers found in myeloma cell lines such as S107 where one of the kappa light chains was unable to combine with the alpha heavy chain (59), or such as in the MOPC315 line where a truncated λ_1 chain unable to bind to its heavy chain is found together with a functional λ_1 light chain (60). A high proportion (8/11) of hybridomas

formed lines No. 3 and No. 15 show a band with slightly slower mobility than the Sp6 light chain. Light-chain and pKmV$_H$14-directed production of V$_H$C$_\kappa$in (15 x igm 10) hybrids 8 and 21 is observed. Only in these hybrids is the heavy-chain-dependent idiotype 20-5 restored. Reprinted with permission from Pergamon Press Ltd, © 1984, Pergamon Press.

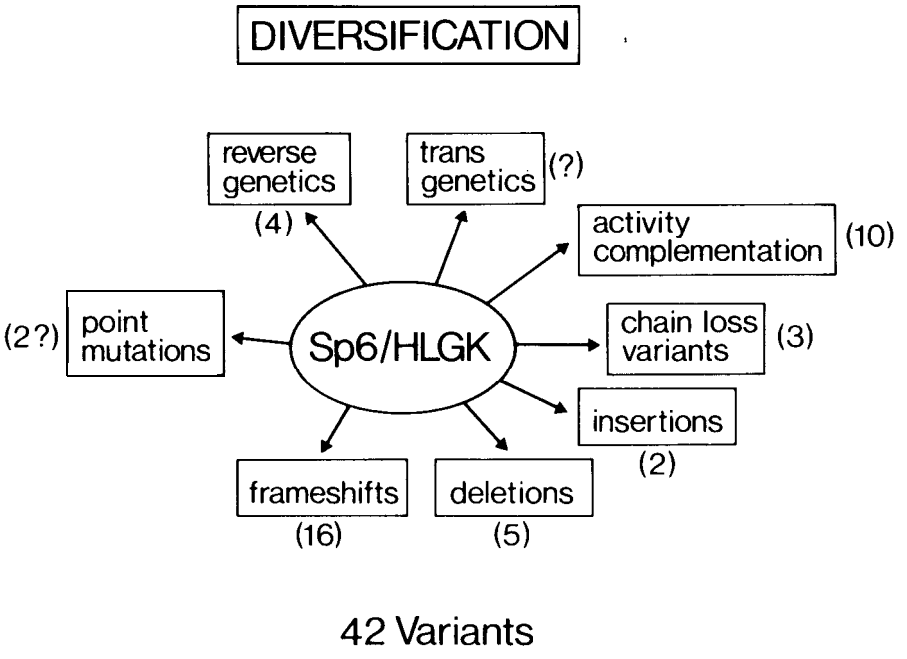


Fig. 5 - Summary of Sp6 variants.

derived from the $\mu_6\kappa_6$ expressing mouse did not express a second endogeneous heavy chain. Five of these lines still retained both heavy chain encoding chromosomes. Only in one of the ten heavy chain alleles a complete (though inactive) VDJ_H nit was found. One allele was apparently frozen in the germline configuration, and eight stopped rearrangement at the immature DJ_H state. Thus again, the result is compatiblewith a (leaky) feedbackregulation of the heavy chain gene rearrangement process by the mu chain itself. Whether also the light chains are involved in this process has to be clarified by analysing pre-B cell lines. However, other interpretations such as different cellular selection processes in transgenic versus normal mice are possible. Nevertheless, the power of the transgene approach to study immmunological phenomena is clearly demonstrated.

Let me now go back to the general theme of this paper and ask whether or not 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 immunoglobulin light and heavy chain genes at an approximate rate of 10^{-3} per base and generation (45,46). It seems possible that the introduced genes will profit from this mechanism and that we will generate a whole series of somatically mutated Sp6 IgM molecules. Figure 5 summarizes the variants obtained so far from the Sp6 line using different techniques and underlines my belief that a single monoclonal antibody will only be the starting point of a variety of man-made secondary antibodies, each manufactured to fulfill a special requirement.

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