Mutations in Immunoglobulin-Producing Mouse Myeloma Cells

Abstract. *Three mouse myeloma cell lines were cloned in soft agar and screened by an antiserum overlay method for variants defective in secretion of the myeloma protein. Variants that had lost the capacity to synthesize heavy chains arose spontaneously at a high rate of about 10^{-3} per cell per generation. Such variants lost the capacity to produce light chains at a similarly high rate. After cells were treated with the acridine half mustard ICR-191, variants occurred with an even higher incidence, and some of these synthesized heavy chains differing from that of the parent.*

The MPC-11 cultured cell line of mouse myeloma cells spontaneously generates stable variants with altered capacity to produce immunoglobulins. With the use of a plate assay to detect and count such variants (*1*), fluctuation analysis revealed that a cloned population of these cells lost the capacity to produce heavy chains at a rate of 1.1×10^{-3} per cell per generation (*2*). This extremely high rate was important not only from the viewpoint of somatic cell genetics, but also because of the possibility that antibody diversity is generated somatically (*3*).

We report here that we have determined the rate at which two other mouse myeloma cell lines lost the capacity to produce heavy chains and the rate at which cells of an MPC-11 light chain producer converted to nonproducers. The effects of several mutagenizing agents on the incidence of variants have also been studied.

The cell lines used were (i) clones of the parent MPC-11 cell line, which secretes immunoglobulin G_{2b} (IgG_{2b}) and was originally adapted to culture from a BALB/c plasmacytoma (*4*); (ii) a spontaneously occurring variant secreting only light chains, which was derived from the MPC-11 cell line (*5*); (iii) the P3 cell line, which was adapted to culture from the BALB/c MOPC-21 tumor producing immunoglobulin G_1 (IgG_1) (*6*); and (iv) the C1 cell line, which was adapted to culture from the C3H X5563 myeloma tumor producing immunoglobulin G_{2a} (IgG_{2a}) (*6*). All cells were grown in suspension culture in Dulbecco's modification of Eagle's medium (Grand Island Biological) supplemented with glutamine, nonessential amino acids, penicillin, streptomycin, and 20 percent heat-inactivated horse serum.

Cultured myeloma cells were cloned at high efficiency in soft agar (*5*). To determine if individual clones were secreting molecules containing heavy chains, dishes with clones 3 to 4 days old were overlaid with agar containing antiserum specific for heavy chains. The clones neither surrounded nor partially obscured by immunological precipitate were scored as variants. Such variants might (i) secrete heavy chains in diminished quantity; (ii) secrete heavy chains that lack the original antigenic determinants; (iii) synthesize but not secrete heavy chains; (iv) no longer synthesize heavy chains (that is, become light chain producers); or (v) synthesize or secrete neither chain (that is, become nonproducers). These possibilities were examined by recovering variants from the agar, growing them to mass culture, and then incubating the cells with radioactive amino acids. The total cytoplasmic proteins, the cytoplasmic proteins specifically precipitable with antibody against heavy and light chains, and the secreted proteins were examined by electrophoresis on acrylamide gels containing sodium dodecyl sulfate (SDS). This technique separates molecules mainly by size.

In order to find variants which had lost the capacity to secrete both heavy and light chains, clones producing light chains were overlaid with agar containing antiserum reactive with both heavy and light chains. Representatives of those clones scored as presumptive nonproducing variants were further analyzed on SDS-acrylamide gels.

The rate of appearance of variants was determined by fluctuation analysis (*7*) (Table 1). When the incidence of variants in several independent clones was compared with that in replicate plates of the parent population, it was found that the generation of variants was spontaneous; that is, variation was not induced by the selection technique (*7*).

As described previously (*2*), the parent MPC-11 cells, which produce heavy plus light chains, lost the capacity to produce heavy chains and became light chain producers at a high rate (Table 1). Twenty of these MPC-11 light chain–producing variants were recovered and studied by SDS gel electrophoresis. None contained detectable amounts of heavy chains intracellularly. This technique could detect 1 to 2 percent of the immunoglobulin synthesized by the parent cell line (*8*). Cells of one of the light chain–producing MPC-11 variants converted to nonproducers at a rate of 4.0×10^{-4} per cell per generation (Table 1). No revertants were found among 26,000 light chain–producing clones examined by the agar plate assay (*2*) or among 100,000 nonproducing cells examined by immunofluorescence.

Cells of P3, a second IgG-producing cell line, converted to light chain producers at a rate of 1.0×10^{-3} per cell per generation (Table 1). Several light chain producers were recovered, and none synthesized detectable amounts of heavy chains. C1, a third cell line, lost the capacity to produce heavy chains at a rate of 2.0×10^{-3} per cell per

Table 1. Fluctuation analysis to demonstrate randomness of "mutation" and determine "mutation rates." The "mutation rate" per cell per generation was calculated by the median method of Lea and Coulson (*21*) from three fluctuation analyses for conversion of MPC-11 producing heavy plus light chains (H + L) to variants producing light chains only (L); one analysis for conversion of MPC-11 L to nonproducers (NP); four analyses for conversion of P3 H + L to L; and four analyses for conversion of C1 H + L to NP. The Lea-Coulson method does not correct for the considerable variations in plating efficiency in these experiments. However, similar estimates were obtained by a statistical analysis which took this feature into account. In this analysis, the method of maximum likelihood was applied to the marginal distribution of the number of mutants, derived from the empirical distribution of the total colony counts; No., number of clones; d.f., degrees of freedom.

Cell line	Variants	Replicate plates				Independent clones				"Mutation rate" per cell per generation (*21*)
		No.	χ^2	d.f.	*P*	No.	χ^2	d.f.	*P*	
MPC-11 (IgG_{2b})	H + L→L	5,522	5.2	7	.64	6,388	100.4	13	<.001	1.1×10^{-3}
MPC-11 (IgG_{2b})	L→NP	5,660	7.0	6	.32	10,871	25.1	8	.0025	0.4×10^{-3}
P3 (IgG_1)	H + L→L	3,960	3.0	7	.88	2,796	29.3	7	<.001	1.0×10^{-3}
C1 (IgG_{2a})	H + L→NP	2,500	4.8	5	.45	9,790	60.5	7	<.001	2.0×10^{-3}

generation (Table 1). However, 18 presumptive C1 light chain producers contained no detectable heavy or light chains. C1 therefore differed from P3 and MPC-11 in that it simultaneously lost the capacity to produce both immunoglobulin chains. Schubert *et al.* (*9*) also reported the appearance of nonproducing variants in the X5563 tumor from which C1 was derived. While fluctuation analysis has not been done for other cell lines, we observed similar incidences of light chain–producing variants with the IgG_1-producing MOPC-31C and the IgA-producing MOPC-315 cell lines.

These observations indicated great instability in the production of immunoglobulins by the cultured mouse myeloma cells. To determine if variants for other traits were also generated at a high rate, the MPC-11 cell line was examined for clones resistant to bromodeoxyuridine (30 μg/ml), 6-thioguanine (3 μg/ml), puromycin (12 μg/ml), 2-deoxyglucose (5 m*M*), and 2,6-diaminopurine (10 μg/ml). The incidence of resistance to these drugs was much lower than the incidence of variants in immunoglobulin production, since no resistant clones were detected when 10^6 cells were cloned in the presence of each drug. Reconstruction experiments showed that one resistant cell among 10^5 to 10^6 sensitive cells could have been detected. The incidence of bromodeoxyuridine- or 6-thioguanine-resistant cells was equally low in the P3 and C1 cell lines. We concluded that the instability of immunoglobulin production did not reflect a general genetic instability in the cultured myeloma cells.

In an attempt to understand the mechanism responsible for this instability, the effect of mutagenic agents on the MPC-11 cell line was examined (Table 2). Neither x-irradiation (data not shown) nor ethylmethanesulfonate, an alkylating agent, increased the incidence of variants. A dose of nitrosoguanidine that killed 10 percent of the cells caused a statistically significant twofold increase in the incidence of light chain–producing variants. However, when larger doses were used, the incidence of variants returned to control levels. A similar dose dependency for nitrosoguanidine mutagenesis has been reported in bacteria (*10*), and it has been suggested that repair processes are induced with higher doses. Nitrosoguanidine also increased the rate of conversion of light chain producers to nonproducers.

The acridine half mustard ICR-191 (*11*), an effective inducer of frame-shift mutations in microorganisms (*12*), was also studied. When a clone producing heavy plus light chains was treated with the drug, a dose-dependent increase in the incidence of light chain–producing variants was observed (Table 2); these variants comprised up to 6 percent of the surviving cells. Cells of a light chain–producing clone converted to nonproducers at a similarly high rate when treated with ICR-191. In other experiments with a recently isolated clone, the frequency of spontaneous variants in the control was considerably lower (0.1 percent), and a 20-fold increase in the incidence of variants was detected after mutagenesis.

Sixteen of the light chain producers induced by ICR-191 were recovered from the agar, grown to mass culture, and examined by incubation with radioactive amino acids to characterize the secreted and intracellular immunoglobulin. Twelve resembled spontaneously occurring light chain producers in that heavy chains could not be detected intracellularly. However, in four clones, easily detectable amounts of heavy chains were found in cells. Upon reduction of the disulfide bridges, the intracellular heavy chains in two of the clones were found to be smaller than those of the parental clone not treated with mutagen. In the third clone, the heavy chain was the same size as the parental heavy chain; in the fourth, the heavy chain was larger than the parent. Comparative peptide mapping revealed differences between each of the variant heavy chains and the parent.

These experiments showed that cultured mouse myeloma cells spontaneously lost the capacity to produce immunoglobulin polypeptide chains at an unusually high rate. We and others observed an unexpected instability of some immunoglobulin-producing mouse myeloma tumors during serial transplantation in vivo (*9, 13*). Hobbs reported the conversion of immunoglobulin-producing human myelomas to light chain producers and to nonproducers after chemotherapy (*14*).

Both the spontaneous rates and the incidence of variants induced by ICR-191 are the highest reported for cultured mammalian cells. Published spontaneous mutation rates range from 2.6×10^{-4} per cell per generation for loss of thymidine transport in Chinese hamster fibroblasts (*15*) to 1.5×10^{-8} per cell per generation for 8-azaguanine resistance in Chinese hamster lung cells (*16*).

Either genetic or epigenetic phenomena could be responsible for the instability of immunoglobulin production. It is unlikely that the functionally haploid state (*17*) of the immunoglobulin genes is responsible for the high incidence of variants, since 6-thioguanine resistance is thought to be X-linked and occurs infrequently in these cells. An

Table 2. Effect of mutagenic agents on the incidence of variants of immunoglobulin-producing MPC-11 cells. To detect the incidence of variants after mutagenesis, the cells were treated with the mutagen for 24 hours, and then washed and resuspended in fresh medium for 24 hours before plating to allow segregation to occur. Three days after the cells were plated, the dishes were overlaid with an antiserum to mouse heavy chains or to heavy and light chains, and stained and unstained colonies were counted 3 days later. Cell survival is the number of viable colonies present after 10 days expressed as a percentage of the number of colonies formed by MPC-11 cells not treated with mutagen; EMS, ethylmethanesulfonate; NTG, nitrosoguanidine.

Mutagen	Dose (μg/ml)	Cell survival (%)	Marker	Incidence of variants		*P*
				(No./total)	(%)	
EMS	0	100	H + L→L	13/2168	0.60	
	50	85		13/1594	0.81	.45
	100	45		10/1288	0.80	.55
	200	13		4/721	0.55	.79
NTG	0	100	H + L→L	29/4993	0.58	
	0.25	90		46/3828	1.21	.0017*
	0.5	68		33/4332	0.76	.30
	1	33		14/2404	0.58	.84
	2	8		10/1977	0.51	.68
NTG	0	100	L→NP	8/3191	0.25	
	0.5	68		35/4452	0.79	.002*
ICR-191	0	100	H + L→L	18/2104	0.86	
	1	60		56/3635	1.54	.016*
	2	25		110/3404	3.24	< .001*
	4	<1		15/229	6.55	< .001*
ICR-191	0	100	L→NP	55/4714	1.17	
	2	25		115/3067	3.75	< .001*
	4	<1		35/538	6.51	< .001*

* Statistically significant.

epigenetic mechanism could explain the high incidence of variants as well as our inability to detect any spontaneous revertants. The relative effectiveness of ICR-191 compared to ethylmethanesulfonate might also suggest an epigenetic event, since acridines can induce nongenetic changes, such as those causing petite mutants in yeast, at high rates (*18*). Harris (*19*) and Metzger-Freed (*20*) suggested that many of the variants arising from cultured cells may reflect changes in phenotypic expression of the sort that occurs during differentiation.

On the other hand, the variants described here could equally well be mutants arising from an alteration of chromosomal DNA. The incidence of light chain–producing and nonproducing variants is increased by nitrosoguanidine as well as by ICR-191. Most importantly, some of the variants induced by ICR-191 contain heavy chains that are abnormal in size and have apparent changes in their primary structure. A detailed structural analysis of the variant heavy chains may reveal the type of genetic mechanism responsible. For example, the ICR-191 variants producing small chains might be frame-shift mutants with a premature termination caused by a nonsense codon.

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22. Supported by grants from the Medical Research Council of Canada, the National Institutes of Health (AI 5231 and AI 10702), the National Science Foundation (GB 3369), and the American Cancer Society. R.B. is a scholar of the Medical Research Council; M.D.S. is the recipient of an NIH career development award; B.K.B. is a fellow of the New York Heart Association; and P.C. was a medical scientist trainee supported by NIH grant 5T5 GM 1674-05. We thank H. J. Creech for supplying ICR-191, J. J. Hsieh and D. B. W. Reed for their help in the statistical calculations, and J.-L. Preud'homme for his search for revertants by the immunofluorescence technique.

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9 May 1973; revised 2 July 1973

Overeating and Obesity from Damage to a Noradrenergic System in the Brain

Abstract. *A discrete, ascending fiber system that supplies the hypothalamus with most of its noradrenergic terminals was destroyed at midbrain level, both electrolytically and with local injections of 6-hydroxydopamine, a destructive agent specific for catecholaminergic neurons. The result was hyperphagia leading to obesity. Fluorescence histochemical analysis showed that loss of noradrenergic terminals in ventral bundle termination areas such as the hypothalamus was necessary for hyperphagia. Damage to dorsal bundle or dopaminergic projections was not. Prior treatment with desmethylimipramine to selectively block uptake of 6-hydroxydopamine into noradrenergic neurons prevented both hyperphagia and loss of norepinephrine fluorescence. The lesions that produced hyperphagia also reduced the potency of* d-*amphetamine as an appetite suppressant. It is concluded that this noradrenergic bundle normally mediates suppression of feeding, thereby influences body weight, and serves as a substrate for* d-*amphetamine-induced loss of appetite.*

Recent studies have suggested a role for endogenous catecholamines in the inhibitory control of food intake. Pharmacological evidence suggests that amphetamines and related drugs that suppress food intake act by potentiation of adrenergic or dopaminergic transmission in the brain (*1*). Various experiments have pointed to the hypothalamus as the anatomical locus for at least part of this effect in rats (*2, 3*). Although dopaminergic terminals are virtually nonexistent in the hypothalamus, this region does contain high concentrations of noradrenergic terminals (*4*). Studies with fluorescence histochemistry have demonstrated that most or all hypothalamic noradrenergic terminals derive from long fibers ascending from hindbrain cell bodies (*5*); the majority of these fibers form a discrete bundle as they course through the ventrolateral tegmentum of the mesencephalon (ventral noradrenergic bundle) (*4, 6*). These facts suggested that the ventral noradrenergic bundle with its terminal plexus in the hypothalamus serves as a substrate for amphetamine-induced inhibition of feeding and, moreover, that it may function in normal regulation as a satiety system. If so, selective destruction of this nerve bundle should produce a satiety deficit, that is, disinhibited feeding. It should also render amphetamine less effective as an anoretic.

To determine if feeding is disinhibited, we destroyed the ventral noradrenergic bundle by two different methods in adult Sherman female rats. In one group this pathway was disrupted by discrete electrolytic lesions made at midbrain level (*7*). In another group 6-hydroxydopamine (6-OH-DA), which selectively destroys catecholaminergic neurons (*8, 9*), was injected directly into the ventral noradrenergic bundle (*10*). A third group received local 6-OH-DA injections into the ventral bundle at a more posterior midbrain locus (*11*). Control groups underwent treatment identical to the experimental groups but without passage of lesion current or addition of 6-OH-DA to the injection vehicle. Animals had free access to Purina laboratory pellets and water; food and water intake and body weight were measured every 48 hours.

All 43 animals given lesions or 6-OH-DA injections increased their food