Rate of Somatic Mutation in Immunoglobulin Production by Mouse Myeloma Cells

PHILIP COFFINO* AND MATTHEW D. SCHARFF†

Departments of Cell Biology (Division of Biological Sciences) and Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Communicated by Harry Eagle, October 23, 1970

ABSTRACT Cultures of mouse myeloma cells which secrete both heavy (H) and light (L) immunoglobulin chains were cloned in soft agar. Variants that synthesized neither chain, or only light chains, were detected by overlaying the growing colonies with antiserum specific for the individual immunoglobulin chains. The rate of conversion of heavy plus light chain producers to light chain producers was 1.1×10^{-3} per cell per generation, as determined by fluctuation analysis.

Immunoglobulin G (IgG) is a well-characterized, multi-chained protein composed of two heavy (H) and two light (L) polypeptide chains joined by disulfide bonds. It is synthesized and secreted in large amounts by plasma cells. The genetic control of immunoglobulin synthesis appears to be unusual in that two genes are thought to code for each of the polypeptide chains made by a single cell (1, 2), only one of the two alleles for the constant region gene is expressed in any given cell (3-5), and a single individual can produce immunoglobulin molecules that vary enormously in their amino acid sequence (1).

In order to study the somatic-cell genetics of immunoglobulin synthesis, we have established cells from a mouse plasma cell tumor in continuous culture (6). With the aid of feeder layers, these cells were cloned in soft agar. By overlaying the colonies with antiserum specific for H or L chains, it was possible to determine that there were minor populations that had lost the ability to produce one or both of the polypeptide chains produced by the parent tumor (7). The rate at which such variants are produced has been determined by fluctuation analysis.

METHODS

The adaptation of cells from the MPC-11 mouse plasma cell tumor to continuous culture and the maintenance of these cultures has been described (6). The cells were grown in Dulbecco's modification of Eagle's medium (Grand Island Biological Co.) supplemented with 20% inactivated horse serum, non-essential amino acids, and 4 mM glutamine. The cultures continue to produce IgG_{2b} immunoglobulin (6).

Production of antiserum

IgG was purified from the serum of mice bearing the MPC-11 tumor by DEAE-cellulose column chromatography and was

injected, in complete Freund's adjuvant, into rabbits. The antiserum obtained after primary immunization reacted only with heavy chains when tested by double diffusion in agar. Antiserum which reacted with both heavy and light chains was obtained from the same animal by repeated immunization.

Cloning and assay for variant colonies

The cultured myeloma cells were cloned by a modification of the techniques of Pluznik and Sachs (8) and Bradley and Metcalf (9). 3T3 cells, a highly contact-inhibited line of mouse fibroblasts, served as feeder layer. After they had grown to confluence in 60- × 15-mm plastic tissue culture dishes (Falcon), the medium was replaced with a 5-ml base layer consisting of 0.24% agarose (Bausch and Lomb) in growth medium. An appropriate dilution of cells in 0.2 ml of growth medium was mixed with 2 ml of the agar medium; 1 ml of this mixture was gently pipetted onto the base layer. After 10 min at room temperature, the dishes were placed in a 37°C CO₂ incubator. The cells grew in the agar with a normal doubling time and formed colonies (see Fig. 1), each of which was derived from a single cell (8). The cloning efficiency was approximately 50%. After 10 days, the colonies had grown to a diameter of about 0.5 mm and could be removed from the agar with a sterile Pasteur pipette. The cells from these colonies were grown to mass culture either in a small volume of conditioned growth medium obtained from cultures of MPC-11 cells or in dishes containing monolayers of confluent 3T3 feeder cells.

To assay for the types of immunoglobulin chains secreted by the individual colonies, an additional layer of agarose that contained 20% rabbit antiserum in place of horse serum, was added to the dishes. 1 ml of the antiserum-agar mixture was pipetted on top of the cell layer 3 days after the cells were originally seeded. The presence of the rabbit antiserum had no effect on the cloning efficiency of the cells. An antigen-antibody precipitate developed around the growing colonies within 24 hr and became progressively heavier and darker during the next 2-4 days (see Fig. 1). However, as the cells continued to grow and to secrete more immunoglobulin, the precipitate became less heavy, presumably due to the solubilization of the antigen-antibody complex by excess antigen. Precipitate was seen only around colonies secreting molecules reactive with the particular antiserum used. Colonies with precipitate and colonies without precipitate were counted, and individual colonies were removed and grown to mass culture for further analysis.

Abbreviations: SDS, sodium dodecyl sulfate; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

* Medical Scientist Trainee supported by NIH grant 5T5 GM 1674.

† Supported by a Career Development Award from NIH.

FIG. 1. Plate assay for immunoglobulin variants. The technique is presented schematically in the *top* panel. A culture of H- + L-chain producing cells was plated and then overlaid with antiserum reactive with H chains. The plates were photographed 6 days after plating and 3 days after overlaying with antiserum. The *middle* panel shows a field containing one colony without precipitate (*top*) and three colonies with precipitate. Colonies with (*left*) and without (*right*) precipitate are shown at higher magnification in the *bottom* panels. The scale unit is 0.1 mm.

Characterization of Immunoglobulins

The techniques used to characterize the newly synthesized molecules have been described in detail elsewhere (6, 7). Briefly, a suspension of cells was incubated with radioactive amino acids for 30 min, washed, and then lysed with the detergent Nonident P-40 (NP-40, Shell Chemical Co.) which leaves the nuclei intact but dissolves the cytoplasmic membranes, including the endoplasmic reticulum. The proteins in the resulting cytoplasmic lysate were either dissociated immediately with sodium dodecyl sulfate (SDS), or precipitated with H- and L-chain specific antiserum prior to dissolution in SDS. The immunoglobulin secreted into the medium was examined by incubating the cells for 3 hr with radioactive amino acids, removing the cells by centrifugation, and dissociating with SDS. All samples were then analyzed on neutral, SDS-containing, polyacrylamide gels as has been described (6, 10).

Mutagenesis

Nitrosoguanidine (NTG, K&K Laboratories, Plainview, N. Y.) was dissolved in 0.1 M sodium acetate buffer, pH 5 (11), at a concentration of 100 $\mu\text{g}/\text{ml}$, sterilized by Millipore filtration, and aliquots stored at -70°C . The solution was thawed immediately before use and added to the growth medium. An equal volume of buffer was added to the control culture. Cells were incubated in the presence of NTG for 24 hr, then in the

absence of the agent for an additional 24 hr before they were plated in agar.

Karyotypes

Karyotypes were prepared using a slight modification of the method of Bunker (12) and stained with Giemsa. Metaphase chromosome spreads were located at low power and counted at $\times 450$.

RESULTS

Myeloma cells, which had been cloned 2 months previously and which were producing both H and L chains, were examined for variants secreting only L chains. The cells were cloned in soft agar and overlaid 3-days later with antibody specific for H chains. Fig. 1 shows the microscopic appearance of such colonies 6 days after cloning. A large amount of precipitate, which appeared as black flakes under bright-field illumination, surrounded those colonies producing molecules containing H chains. Of 2400 colonies examined, 10 were not surrounded by precipitate (Table 1, line 1 and Fig. 1). In order to determine whether these negative colonies were producing L chains, or were nonproducers making neither chain, another portion of the same culture was analyzed with antiserum which reacted with both H and L chains. In this particular experiment, all 4100 colonies examined (3100 from NTG-treated cells and 1000 from untreated cells) reacted with this antiserum, indicating that they were all producing L chains (Table 1).

To characterize those colonies that had not reacted with antiserum against H chains, isolated clones were removed from the agar; each was grown to mass culture. Cells from 12 such presumptive L-chain producing clones were incubated with [^{14}C]amino acids and their total cytoplasmic protein, immunologically-precipitated cytoplasmic protein, and secretion were examined by polyacrylamide gel electrophoresis (Fig. 2). This SDS-gel technique does not disrupt disulfide bonds and resolves molecules on the basis of their size. The total cytoplasmic extract of the parent cell, which produced H + L chains, yielded a number of prominent radioactive peaks (Fig. 2A) that were specifically precipitated with antiserum against H and L chains (Fig. 2B). Some of these proteins were also secreted (Fig. 2C). Based on their size, and relative H- and L-chain content, these peaks have previously been characterized as completely-assembled 7S IgG_{2b} im-

TABLE 1. Incidence of variant cells in cloned cell populations

Type of culture	Clone number	Proportion of colonies that did not react with		
		Anti-H	Anti-(H + L)	
			-NTG	+NTG
H + L	(45, 6)	10/2400	0/1000	0/3100*
L	(45, 2)		1/1000	6/1000†
L	(78, 4)		8/3200	35/4500†
L	(45, 2)		1/500	5/1000†
L	(86, 3)		0/1800	0/2400†
L	(86, 4)		0/956	0/1018†

* NTG was used at concentrations ranging from 0.25–2.0 $\mu\text{g}/\text{ml}$.

† 0.5 $\mu\text{g}/\text{ml}$ of NTG was used. This dose reduced the cloning efficiency by 30%.

FIG. 2. Characterization of the immunoglobulin produced by different types of clones. Clones were recovered from the agar and grown to mass culture as described in **Methods**. About 8×10^6 cells were incubated with [^{14}C]valine, threonine, and leucine as described (6). After 30 min, part of the culture was removed, cytoplasm was prepared and either treated with SDS and analyzed directly (A, D, G) or immunologically precipitated and the immune precipitates were treated with SDS and analyzed (B, E, H). Another part of the culture was incubated for 3 hr with the radioactive precursors and the cells were then removed by centrifugation. The secreted material in the medium was dissociated with SDS and analyzed on 20-cm, 5% acrylamide gels containing 0.1 M Na_2HPO_4 buffer (pH 7.0) and 1% SDS (10). Migration is from left to right. A, B, and C represent the total cytoplasm, immunologically-precipitated cytoplasm, and secretion of the parent H- + L-chain-producing cells. D, E, and F represent similar samples of an L-chain producing alone, and G, H, and I are the gels from a nonproducer.

munoglobulin (H_2L_2), half molecules (HL), light-chain dimers (L_2), and free light chains (L) (6). When the presumptive L-chain-producing colonies were examined, only L-chain dimers and free L chains were detected (Fig. 2D–F). Treatment with mercaptoethanol converted the L-chain dimers into free L chains.

Although no nonproducing cells were detected in the parent H- + L-chain-producing culture, different numbers of such nonproducers were detected in clones of L-producing cells (Table 1, lines 2–6). When such clones were exposed to NTG, the incidence of nonproducing colonies increased 3-fold (Table 1). However, when clones which did not contain any spontaneous nonproducers were similarly mutagenized, no nonproducing clones were seen (Table 1).

One of the spontaneously occurring nonproducers and two of those isolated after NTG treatment have been examined in detail. Cells were incubated with radioactive precursors as described above and their total cytoplasm (Fig. 2G), immunologically-precipitated cytoplasm (Fig. 2H), and secretion (Fig. 2I) were examined on SDS gels. No detectable immunoglobulin chains were present.

Since immunoglobulins are thought to be synthesized on the rough endoplasmic reticulum (13, 14), the nonproducers were examined in the electron microscope for membrane-associated ribosomes. They were indistinguishable from the parent H- +

L-chain producers in that both contained a well-developed endoplasmic reticulum with attached ribosomes, a Golgi apparatus, and abundant virus-like A particles. The cell doubling time of the H- + L-chain producing culture, the light-chain producer, and the nonproducer were about the same. All three cell types were aneuploid, with chromosome numbers ranging between 60–70. None of the clones examined were agglutinable with a preparation of Sendai virus (8000 HAU/ml) that produced extensive fusion in HeLa cells.

The findings described above suggested that cells producing H + L chains could convert to light-chain producers, and then to nonproducers, in a stepwise manner. The mutation rates of each of these steps were determined directly by fluctuation analysis (15). Cells from a recently cloned H- + L-chain producer were recloned in agar. Each of a number of the individual colonies were recovered, single-cell suspensions made, and cells from each colony were plated on fresh agar dishes. Each of these daughter colonies was then examined for L-chain producers (Table 2). Replicate plates of cells taken from a mass culture, producing H and L chains, served as a control (Table 3). The variability in percentage of light-chain producing cells in the 14 clones examined in Table 2 was not seen in the control (Table 3). As was first shown by Luria and Delbrück (15), this indicates that the mutants arose spontaneously and were not induced by the assay method. This conclusion was con-

TABLE 2. *Fluctuation analysis to demonstrate randomness of mutation and determine mutation rate*

Plate no.	Total colonies	No. of light-chain-producing colonies	No. of light-chain-producing colonies per 11,800
1	928	12	153
2	466	0	0
3	769	5	77
4	782	11	166
5	738	10	160
6	429	0	0
7	611	2	39
8	761	4	62
9	664	8	142
10	1236	8	76
11	1130	42	439
12	972	6	73
13	859	5	69
14	526	3	67

8-day-colonies of uniform size were picked from agar plates and put into 0.4 ml of complete growth medium. Colonies of this size contain about 11,800 cells (mean of five colonies). A single-cell suspension was formed by gentle agitation and 0.2 ml of the suspension was then added to 2 ml of agar medium. 1 ml of this mixture, containing about 2300 cells, was then plated on a single dish containing 5 ml of the usual base layer. The average number of colonies per dish was 777, giving a cloning efficiency of 34%. This is close to the 53% cloning efficiency found in the replicate plates (Table 3). The difference is probably due to cell loss or cell death associated with the manipulations required for recovery and resuspension of the colony. No correlation was found between total colonies per dish and the incidence of light-chain-producing colonies. The mutation rate was calculated by finding the median number (75) of light-chain producing-colonies per 11,800, determining m , the most probable number of independent mutations, using Table 3 of Lea and Coulson (16), and dividing by 11,800.

The data from 40 colonies analyzed in four experiments were pooled and a mutation rate of 1.1×10^{-3} per cell per generation determined. The hypothesis that all 40 independent samples arose from the same population, with the true proportion of mutants estimated by the proportion in the total samples, was rejected by the χ^2 test ($\chi^2 = 322$, $DF = 39$, $P < 0.0005$).

The goodness-of-fit of the distribution of mutants to that predicted by the mathematical model of Lea and Coulson (16) with $m = 13$ was tested and the fit was found to be consistent with that model ($\chi^2 = 0.9$, $DF = 3$, $P = 0.80$).

firmed by using the χ^2 test to compare the data presented in Table 2 with the values expected on the assumption of random mutation (see caption, Table 2). Four similar fluctuation analyses were performed sequentially and the mutation rate was calculated for each by the median method of Lea and Coulson (16) (see caption, Table 2). The mutation rate in the individual experiments was 0.8 – 1.5×10^{-3} . The data from all four experiments were pooled, and a mutation rate of 1.1×10^{-3} was calculated for the conversion of H- + L-chain production to L-chain production.

In similar experiments to determine the rate of conversion of L-chain-producing cells to nonproducers, we found it difficult to examine enough cells to detect nonproducers. A maximum mutation rate of about 1.7×10^{-4} per cell per generation is indicated by the absence of any nonproducers among 5825 L-

chain-producing colonies examined. No reversion to light-chain production was detected in 8000 nonproducing colonies derived from a single clone, nor have any revertants to heavy-chain production been detected in 26,000 L-chain-producing clones.

DISCUSSION

During the serial passage of mouse myeloma tumors or cultures, variants occasionally arise that have lost the ability to produce H or both H and L chains (17–19). In the case of variants that produce only light chains, the light chain has been compared with that produced by the parent to establish lineage (19, 20). Since nonproducing variants may not have the characteristic morphology of plasma cells, their origin is even more difficult to establish, but marker chromosomes have been used for this purpose (19).

The plate method described here makes it possible to isolate rare variants of unambiguous origin and simultaneously to determine their incidence with precision. Such quantitation is necessary for genetic analysis. As here shown, the MPC-11 parent cell line mutates to L-chain production at a rate of 1.1×10^{-3} per cell per generation, while the conversion of L-chain producers to nonproducers occurs at a much slower—as yet undetermined—rate. It is of interest that this difference is consistent with the finding that in human myelomas about 90% of the tumors produce H and L chains, while 12% produce L chains only and a fraction of 1% are nonproducers (21). The two mutations appear to be stepwise, and both are spontaneous, inheritable, and stable. The mechanisms responsible for these conversions are not yet known; they could be either genetic or epigenetic, due to changes in structural or control loci, and could involve point mutations, deletions, chromosomal loss, or several other genomic lesions. Since the MPC-11 cells are heterogeneous with respect to chromosome number, and since it is difficult to distinguish individual mouse chromosomes morphologically, chromosome loss in the variants cannot be excluded.

The rate of 1.1×10^{-3} per cell per generation for conversion to light-chain production is higher than mutation rates that

TABLE 3. *The incidence of light-chain-producing colonies in the control*

Plate no.	Total colonies	No. of light-chain-producing colonies	No. of light-chain-producing colonies per 11,800
1	540	8	175
2	758	12	187
3	841	12	168
4	671	4	70
5	672	6	105
6	618	6	115
7	695	10	170
8	727	7	114

1300 cells per dish from a cloned culture of 7S-producing cells were plated from a mass culture (control for fluctuation analysis) in eight dishes. The number of L-chain-producing colonies and the total number of colonies on each dish were counted. Overall cloning efficiency was 53%. The hypothesis that all eight independent samples arose from the same population, with the true proportion of mutants estimated by the proportion in the total samples, was not rejected by the χ^2 test ($\chi^2 = 5.2$, $DF = 7$, $P = 0.7$).

have been measured for other somatic cell markers by fluctuation analysis. For instance, Breslow and Goldsby (22) found that Chinese hamster fibroblasts lost their ability to transport thymidine at the rate of 2.6×10^{-4} per cell per generation, while Chu *et al.* (23) showed that Chinese hamster lung-cells developed resistance to 8-azaguanine at a rate of 1.5×10^{-8} per cell per generation. Other reported mutation rates for mammalian cells fall between these values (24, 25). Burnet (26) has speculated that the mechanism responsible for generating the diversity in amino acid sequence in normal immunoglobulins may continue to operate in differentiated antibody-producing cells. The mutations that we have observed may represent primary-sequence changes that cause failure of chain initiation or premature termination. However, the high rate may be the sum of a number of lower mutation rates, each of which alone would result in cessation of H-chain synthesis.

Since reversions of point mutations usually occur at rates which are orders of magnitude lower than the forward mutation rate, our failure to find reversions may reflect our inability at this time to detect extremely rare events, and does not necessarily argue against point mutation as the genetic change. The 3-fold increase in nonproducers in the presence of nitro-soguanidine also does not suggest a specific mechanism, since the drug is known to have a number of different effects, such as chromosomal breakage (27) and point mutation.

With the techniques described in this paper it should be possible to detect other sorts of variations, such as the synthesis of heavy-chain fragments (28). Thus, it may be possible to generate a whole spectrum of defects in immunoglobulin synthesis from a single clone of mouse myeloma cells.

We thank Dr. Herman P. Friedman of the IBM Systems Research Institute for performing the statistical analysis and Mrs. Ellyn Fischberg and Mrs. Linda Stern for their expert technical assistance.

This work was supported by grants from the National Science Foundation, American Cancer Society, and National Institutes of Health (AI-4153, AI-5231), and was presented in preliminary form to the American Association for Clinical Investigation (*J. Clin. Invest.*, **49**, 19a, 1970).

1. Hood, L., and D. W. Talmage, *Science*, **168**, 325 (1970).
2. Cohn, M., in *Nucleic Acids in Immunology*, eds. Plescia and Braun (Springer, New York/Heidelberg/Göttingen, 1968), p. 671.
3. Pernis, B., G. Chiappino, A. S. Kelus, and P. G. H. Gell, *J. Exp. Med.*, **122**, 853 (1965).
4. Martensson, L., *Vox Sang.*, **11**, 521 (1966).
5. Cebra, J. J., *Bacteriol. Rev.*, **33**, 159 (1969).
6. Laskov, R., and M. Scharff, *J. Exp. Med.*, **131**, 515 (1970).
7. Coffino, P., R. Laskov, and M. Scharff, *Science*, **167**, 186 (1970).
8. Pluznik, D. H., and L. Sachs, *J. Cell. Comp. Physiol.*, **66**, 319 (1965).
9. Bradley, T. R., and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.*, **44**, 287 (1966).
10. Maizel, J. V., Jr., *Science*, **151**, 988 (1966).
11. Cerdá-Olmedo, E., and P. C. Hanawalt, *Biachim. Biophys. Acta.*, **142**, 450 (1967).
12. Bunker, M. C., *Can. J. Genet. Cytol.*, **7**, 78 (1965).
13. Vassali, P., B. Lisowska-Bernstein, M. E. Lamm, and B. Benacerraf, *Proc. Nat. Acad. Sci. USA*, **58**, 2422 (1967).
14. Mach, B., H. Koblet, and D. Gross, *Proc. Nat. Acad. Sci. USA*, **59**, 446 (1968).
15. Luria, S. E., and M. Delbrück, *Genetics*, **28**, 491 (1943).
16. Lea, D. E., and C. H. Coulson, *J. Genet.*, **49**, 264 (1949).
17. Potter, M., and E. Kuff, *J. Mol. Biol.*, **9**, 537 (1964).
18. Cohn, M., *Cold Spring Harbor Symp. Quant. Biol.*, **32**, 211 (1967).
19. Schubert, D., and K. Horibata, *J. Mol. Biol.*, **38**, 263 (1968).
20. Lieberman, R., F. Mushinski, and M. Potter, *Science*, **159**, 1355 (1968).
21. Zawadzki, Z. A., and G. A. Edwards, *Amer. J. Clin. Pathol.*, **48**, 418 (1967).
22. Breslow, R. E., and R. A. Goldsby, *Exp. Cell Res.*, **55**, 339 (1969).
23. Chu, E. H. Y., P. Brimer, K. B. Jacobson, and E. V. Merriam, *Genetics*, **62**, 359 (1969).
24. Lieberman, I., and P. Ove, *Proc. Nat. Acad. Sci. USA*, **45**, 872 (1959).
25. Szybalski, W., *Exp. Cell Res.*, **18**, 588 (1959).
26. Burnet, M., *Cellular Immunology*, (Melbourne U. Press, 1969) p. 136.
27. Kao, F.-T., and T. T. Puck, *J. Cell Physiol.*, **74**, 245 (1969).
28. Franklin, E. C., J. Lowenstein, B. Bigelow, and M. Meltzer, *Amer. J. Med.*, **37**, 332 (1964).