N-hydroxy-AAF per 100 g of body weight resulted in the death of approximately 60 percent of the rats as a consequence of massive periportal necrosis in the liver (Fig. 2) (*14*). The toxicity of *N*-hydroxy-AAF was decreased by prior treatment of the rats with *p*-hydroxyacetanilide, but concurrent administration of sulfate ion partially overcame this protective effect (Fig. 2). An apparent increase in the toxicity of *N*-hydroxy-AAF on administration of sulfate ion as compared to chloride ion was also noted in the absence of prior treatment with *p*-hydroxyacetanilide. The protection afforded by *p*-hydroxyacetanilide is consistent with the decreased toxicity and carcinogenicity of AAF observed by Yamamoto *et al.* (*15*) in rats fed large amounts of acetanilide. *p*-Hydroxyacetanilide is a major metabolite of acetanilide in several mammals (*16*).

The increased binding of fluorenyl derivatives from *N*-hydroxy-AAF in vivo to hepatic protein, ribosomal RNA, and DNA, and the apparent increase in the toxicity of *N*-hydroxy-AAF when rats are supplemented with sulfate ion thus suggest that the highly reactive ester AAF-*N*-sulfate is formed from this carcinogen in vivo (Fig. 1). These data and the correlations between factors that influence the hepatocarcinogenicity of 2-aminofluorene derivatives and the sulfotransferase activity for *N*-hydroxy-AAF in the rat (*6*) support the view that AAF-*N*-sulfate is an ultimate reactive carcinogenic metabolite of *N*-hydroxy-AAF in the rat liver. It is not known which of the interactions between the ultimate carcinogenic form, or forms, of *N*-hydroxy-AAF and tissue constituent, or constituents, are critical in the conversion of normal hepatic cells to neoplastic cells. The amounts of the fluorene derivatives bound to protein and nucleic acids in the livers of animals administered AAF or *N*-hydroxy-AAF have generally paralleled susceptibility to hepatic carcinogenesis (*17*). However, the roles of specific proteins and nucleic acids in this carcinogenic process remain to be determined.

The subcutaneous tissue of rats has been employed in relatively direct tests of the carcinogenicity of possible metabolites of *N*-hydroxy-AAF, and the synthetic lipid-soluble esters *N*-acetoxy-AAF and *N*-benzoyloxy-AAF are more carcinogenic than *N*-hydroxy-AAF at this site (*1*). However, subcutaneous injections of AAF-*N*-sulfate have produced only a few tumors (*1*); the short half-life in water and the ionic nature of this reactive ester probably prevent adequate dosage of the cells. The potential biological activity of AAF-*N*-sulfate is evident from its high activity as a mutagen in vitro for a bacterial transforming DNA (*4*).

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18. Supported by NIH grants CRTY-5002 and CA-0175 and the Jane Coffin Childs Memorial Fund for Medical Research.

10 July 1969; revised 7 October 1969

Immunoglobulin Production: Method for Quantitatively Detecting Variant Myeloma Cells

Abstract. *Mouse myeloma cells in continuous culture were cloned in soft agar. The clones were assayed for their ability to synthesize heavy and light chains of gamma globulin by immunoprecipitation directly in the agar. A minor population secreting only light chains was identified. The two cell types were recloned and a low incidence of conversion of 7S to light-chain production was demonstrated. This technique can be used to isolate rare variants of cells secreting specific macromolecules.*

Plasma cell tumors that produce immunoglobulin can be induced in Balb/c mice and maintained by animal passage (*1*). Some of these tumors are defective in immunoglobulin production. Variants have been described which synthesize partially assembled molecules, unassembled heavy and light chains, or light chains only; other variants do not synthesize detectable immunoglobulin polypeptide chains (*1, 2*). These defects in synthesis and assembly are usually present when the tumor is first examined, although some have arisen during animal passage (*3*) or in tissue culture lines which had originally synthesized complete molecules (*2*). We now present a method for quantitatively detecting variants in cultured cell lines and describe the frequency and characteristics of those variants that produce light chains in an MPC 11 cell line.

MPC 11 is a tumor originally obtained from Dr. J. Fahey, which produces γG_{2b} immunoglobulin. The types of γ-globulin molecules produced and their synthesis, assembly, and secretion by this tumor have been described (*4*). The tumor has been adapted to grow as a suspension culture in spinner bottles. These cells have grown continuously for almost 2 years in Dulbecco's modified Eagle medium (Grand Island Biological Co.) supplemented with nonessential amino acids and horse serum (20 percent). The line produces predominantly 7*S* γ-globulin but also secretes small amounts of half molecules, light chain dimers, and free light chains (*5*).

Cells which no longer secrete molecules containing heavy chains were detected by first cloning the cultured cells in soft agar by a slight modification of a known technique (*6*). Feeder layers

Table 1. Percentage of colonies with no precipitate when antiserum to H chains and antiserum to H and L chains are used.

Antiserum to chain	Cloning efficiency (%)	Total colonies	Without precipitate (%)
H	37.4	374	11.8
H	43.8	219	11.4
H + L	36.3	360	0.0
H + L	32.9	600	.0

of mouse 3T3 fibroblast cells were grown to confluence in petri dishes (60 by 15 mm; Falcon) and then overlaid with 5 ml of complete growth medium containing 0.22 percent agarose. After the agar was solidified, the cultured myeloma cells were suspended in 1 ml of the same agarose-containing medium and pipetted onto the base layer. Cloning efficiency was between 30 and 45 percent, and it was independent of the number of cells plated in the range tested (125 to 1000 cells per dish).

After 10 days, the colonies were approximately 0.5 mm in diameter and contained several thousand cells. Such colonies could be removed from the plate with a sterile Pasteur pipette under microscopic observation and either grown up to mass culture, or immediately replated in agar, or injected into animals to form tumors.

The type of γ-globulin polypeptide chain each colony secreted was determined directly on the plate by overlaying an additional 1 ml of agar-containing medium in which the horse serum was replaced by rabbit antiserum. In Fig. 1, an antiserum overlay containing antibody specific for γG heavy (H) chains was added 4 days after plating, and the colonies were examined 3 days later. A distinct precipitate surrounded many of the colonies (Fig. 1A), obscuring the cells. A few colonies showed little or no precipitate (Fig. 1B). This technique was used to analyze the MPC 11 cell line after it had been in continuous culture for 1½ years. Twelve percent of the colonies showed no precipitate with antiserum to H chains (Table 1). When colonies were overlaid with antiserum which reacted with both H and L (light) chains, all of the colonies were surrounded by precipitate (Table 1). Colony growth was not affected by the presence of the antiserum. Short-term experiments showed that the antiserum did not kill the cells even in the presence of complement and had no effect on the synthesis of protein, RNA, or DNA.

It seemed likely that the colonies which stained with antiserum specific for H chains were secreting molecules containing H chains. Those which did not react with antiserum to H chains, but which reacted with the antiserum specific for both H and L chains were probably secreting L chain molecules only. In order to characterize the colonies further, seven of each type were recovered from the agar and grown up to mass culture. A portion of each of the cultures was examined by the Ouchterlony double-diffusion technique with the same antiserums used for the overlay. The pattern of reactivity was identical to that seen with the colonies in agar.

Cultures were grown up from two colonies of each type, and the intracellular material was analyzed after 30 minutes of incubation with C^{14}-valine, threonine, and leucine (Fig. 2). The method of gel electrophoresis used (*7*) resolves molecules according to size (*8*) and has been used to characterize the types of γ-globulin molecules produced by the parent MPC 11 tumor (*4*) and the cultured cell line (*5*). In addition, the material secreted by cultures derived from five colonies of each type was also analyzed on SDS-acrylamide gels. All colonies of each type gave similar results, and the gel patterns from one colony which reacted with the antiserum to H chains and one colony which did not react are shown in Fig. 2. The intracellular material from the colonies which reacted with the antiserum to H chains showed distinct peaks of radioactivity (Fig. 2A), which on coelectrophoresis migrated with tritium-labeled complete 7*S* γ-globulin (fraction 14), half molecules (fraction 29), light-chain dimers (fraction 41), and free light chains (fraction 52) of the parent tumor (*5*). The intracellular γ-globulin molecules were immunologically precipitable (Fig. 2B) and were also secreted (Fig. 2C).

Table 2. Determination of the homogeneity of colonies with antiserum to H chains. L, cells that secrete light chains only; 7*S*, cells that secrete molecules containing H chains.

Clone number	Cell type	Colonies with precipitate	Colonies without precipitate
1	L	0	1364
3	L	0	2580
17	L	0	3052
9	7*S*	2860	2

Colonies of the type shown in Fig. 1B did not contain 7*S* γ-globulin or half molecules either intracellularly or in their secretion. They did, however, secrete light chains and dimers (Fig. 2, D–F). The "light chain dimer" peak of the secretion is completely converted to free light chains by treatment with 2-mercaptoethanol. These results suggested that, at least in the instances so far examined, the direct immunological evaluation of agar colonies proved useful for detecting a minor population

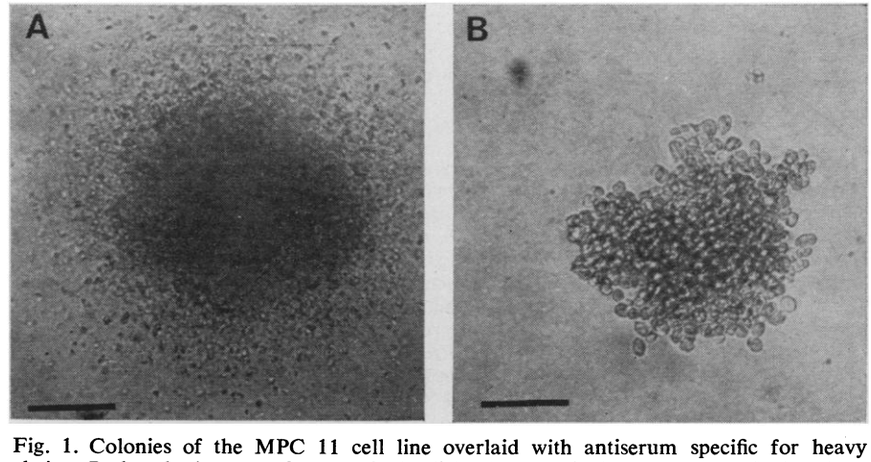

Fig. 1. Colonies of the MPC 11 cell line overlaid with antiserum specific for heavy chains. Both colonies were from a single dish photographed on day 7. (A) A colony secreting molecules containing heavy chains. (B) A colony secreting light chains. Scale is 0.1 mm. The dark masses in part A are the secreted molecules containing heavy chains after they have been precipitated by antiserum to H chains in the agar overlay. Immunoprecipitates in Ouchterlony plates have the same appearance when examined microscopically.

of cells that secrete predominantly light chains.

To examine the homogeneity of the colonies as they occurred in the soft agar and to determine whether the conversion of cells that produce 7*S* to those that produce L chains, or vice versa, was a frequent event, colonies of each type were recloned three times and then examined by the agar cloning method for secretion of H and L chains. No colonies secreting molecules containing H chains were observed among 7000 colonies derived from three different clones secreting L chains. Of the 7*S* colonies examined, only two out of 2900 had become producers of L chains (Table 2).

Studies on the regulation of protein synthesis in mammalian cells would be greatly facilitated if, as with microorganisms, the desired variants could be induced and detected. Our method allows the detection, determination of incidence, and subsequent recovery in cultured cell lines of minor populations that are secreting a variant protein. Clones of a mouse myeloma line which secrete only light chains, instead of both heavy and light chains, have been identified. The incidence of this variant in an established culture has been determined, and some of the individual clones have been further characterized.

In addition, a recloned line producing 7*S* protein has been shown to convert to the variant that produces light chains. This technique of direct immunological analysis of individual colonies provides a useful method of determining the incidence of the predominant molecules being secreted by the cells. However, if there were colonies secreting relatively small amounts of molecules containing heavy chains, they would probably be scored as secreters of light chains unless examined further. Intracellular events must also be independently evaluated. For example, some of the MPC 11 clones that secrete light chains synthesize heavy chains which do not assemble with light chains and are either rapidly destroyed or at least not secreted (*9*). A similar variant has been described by Schubert and Cohen (*10*). This technique may enable one to examine the somatic cell genetics of immunoglobulin synthesis.

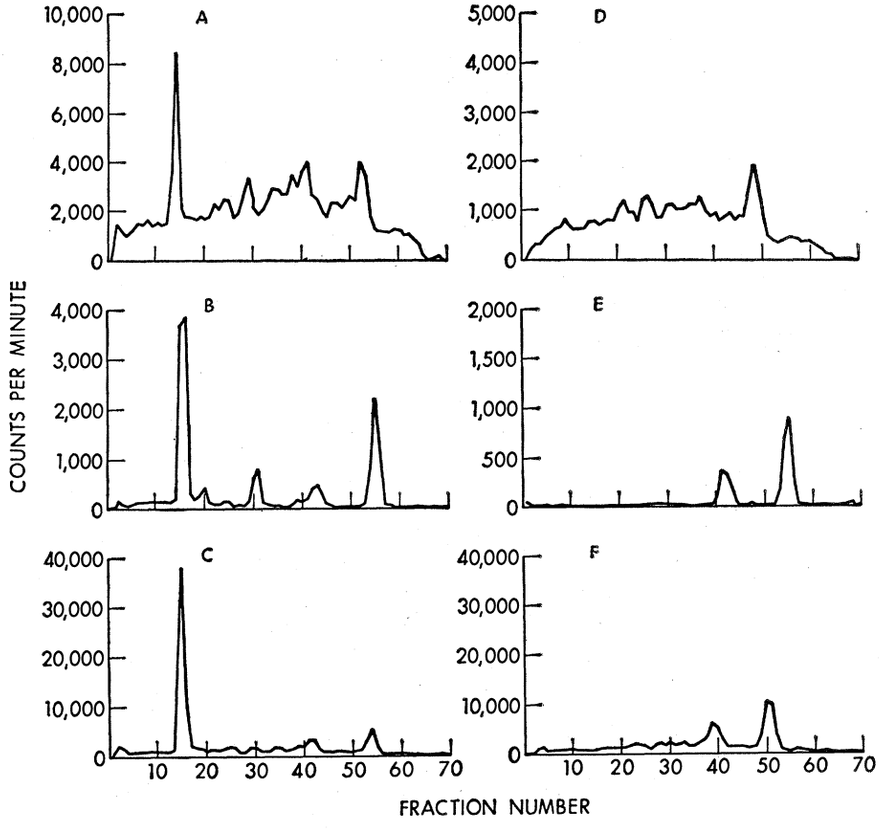

Fig. 2. Gel electropherogram of a colony producing 7*S* γ-globulin and one secreting only light chains. Cells (8×10^6) were incubated in 1.3 ml of media containing 1/20 the normal amount of unlabeled valine, threonine, and leucine, and 9 μc each of C^{14}-valine, -threonine, and -leucine. After 30 minutes at 37°C, half of the sample was removed, cytoplasm was prepared (*4, 11*), and one portion was treated with 2 percent SDS for 1 minute at 100°C in the presence of 0.03*M* recrystallized iodoacetamide. The other portion was subjected to immunoprecipitation in the region of antibody excess with antiserum which reacts with both L and H chains (*5*). After 16 hours at 4°C, the precipitate was washed twice, resuspended in SDS and iodoacetamide, and dissociated for 1 minute at 100°C. Samples were prepared for electrophoresis and analyzed in acrylamide gels that contained SDS. After 3 hours of incubation, the other half of the culture was removed, and the secreted material was treated in the same way as the cytoplasm (*6*). The cytoplasm shown in part A was mixed with H^3-labeled material secreted from the parent MPC 11 tumor, which served as a marker for the γ-globulin molecules. Migration was from left (cathode) to right (anode). (A) Cytoplasm; (B) immunologically precipitated cytoplasm; (C) secretion of cells grown up from a colony such as that shown in Fig. 1A. (D) Cytoplasm; (E) immunologically precipitated cytoplasm; (F) secretion of cells grown up from a colony such as that shown in Fig. 1B.

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12. Supported by grants from the NIH (AI 5231, AI 4153), NSF (GB-6364, GB-4751), ACS (E-379). P.C. is a medical scientist trainee supported by NIH grant 5T5 GM 1674-05; R.L. holds a research training fellowship from the International Agency for Research on Cancer; M.D.S. is the recipient of a career development award. We thank Dr. D. Pluznik for assistance with the agar cloning technique.

8 August 1969; revised 7 October 1969