tination test (*6*) in all ten rabbits and first appeared 6 to 30 days after inoculation. Titers for individual rabbits varied from 1:20 to 1:640 during infection; all animals were nonreactive before inoculation. Antibody was also detected in fluid from both the infected and the noninoculated chambers of the same animal.

A biopsy taken 3 weeks after infection revealed massive intracellular and extracellular infiltration of gonococci into the tissue and blood vessels surrounding the infected chamber (Fig. 1). A blood culture taken at the same time was negative for *N. gonorrhoeae*.

A modification of the technique for rabbits was used to infect guinea pigs, hamsters, rats, and mice. Coil-shaped subcutaneous culture chambers were prepared from 0.5-mm stainless steel wire. After implantation the chambers were inoculated with 0.2 ml of liquid growth media containing *N. gonorrhoeae* (VDRL strain 2686). Both dexamethasone-treated and nontreated animals were infected, and although dexamethasone may promote establishment of infection, it is not required (Table 1).

The results presented here extend the principle of Miller's research (*1*) concerning gonococcal infection of the rabbit eye. The gonococci in our experiments persisted within the trunk of the rabbit, in a place easily accessible for withdrawing specimens for culture and microscopic examination. The ability to repeatedly sample gonococci growing in vivo opens the possibility of studying the in vivo interaction of bacteria with antibody, complement, leucocytes, and other host factors. These studies will facilitate research on serologic tests, strain typing, and vaccine development. Finally, the techniques reported here should also permit in vivo study of the antigonococcal effects of antibiotics and other chemotherapeutic and prophylactic substances.

Table 1. Experimental *N. gonorrhoeae* infection in guinea pigs, hamsters, rats, and mice; CFU, colony-forming units of *N. gonorrhoeae* inoculated per animal; Dex., dexamethasone.

Inoculum (CFU)	Dex. (1 mg)	Number of animals: Inoculated	Number of animals: Positive cultures 5 days after inoculation
	Guinea pigs		
10^7	+	4	4
10^7	−	4	0
	Hamsters		
10^7	+	4	3
10^7	−	7	6
	Rats		
10^7	+	5	1
10^7	−	5	0
	Mice		
10^8	−	4	4
10^6	−	4	4
10^4	−	4	3
10^2	−	4	3
10^1	−	4	0

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Variation in Tyrosine Aminotransferase Induction in HTC Cell Clones

Abstract. *Examination of tyrosine aminotransferase induction in many HTC cell subclones revealed a wide and unstable distribution of inducibility. The instability of this phenotype cannot easily be explained by classical mutation rates. These observations may be important in interpreting certain cell fusion experiments.*

Understanding gene expression and its control in higher organisms will require the development of a model that can explain the high rates of "mutation" often described in eukaryotes (*1*). We examined the inducibility of the enzyme tyrosine aminotransferase (TAT) in several clones obtained from the HTC cell line [originally cultured from Morris hepatoma 7288C (*2*)] and we found considerable variation in the extent of inducibility, with increases over basal TAT ranging from less than 100 percent to greater than 3000 percent. On recloning two widely differing clones, we found that the subclones again showed considerable variation in inducibility. In contrast, inducibility in the wild type HTC (HTC+) has been relatively stable for more than 7 years. There is no known selective pressure for or against TAT expression, nor have we been able to devise one. Therefore, to explain the apparent paradox between the two sets of observations we have to assume similar rates of change from high to low inducibility and from low to high inducibility.

The phenotypic variation described here, while not readily explained in terms of classic genetics, cannot be overlooked. It may also be occurring in other cell lines in culture. This possibility should be taken into account when quasigenetic studies are done in tissue culture systems. Simply establishing a clonal cell line does not guarantee uniformity of expression of a particular enzyme within that line, as the data in this report demonstrate.

Two clones from HTC+, one with low inducibility and one with high, were first selected. Each was recloned by plating a single-cell suspension in sterile plastic trays containing 96 wells, with an average of 0.2 cell per well. Poisson analysis indicated that not more than 10 percent of the subsequent colonies arose from two cells. Forty colonies were isolated and analyzed for TAT inducibility, and a clone with high inducibility [C3-30(H)] and a clone with low inducibility [C2-10(L)] were picked and recloned as before. For comparison, HTC+ cells were freshly cloned as well. To minimize physiologic variability in enzyme content, studies for TAT induction in the final clones were done as follows. An identical number of cells of each clone from each parent were simultaneously plated, grown for 24 hours (log phase), and induced for 24 hours with $2 \times 10^{-7} M$ dexamethasone phosphate. Then the cells were collected, broken with a probe sonicator, and assayed for TAT by a slight modification of the Diamondstone method (*3*). To further insure that the enzyme activities seen were comparable between groups, the parental lines (high and low inducibility and wild type) were identically analyzed along with each group.

The distribution of clones with respect to TAT inducibility (Fig. 1) shows that although there is a clustering

around the appropriate parent there is also considerable overlap. The clones from the HTC+ parent are distributed across the entire range of inducibility of the "low" and "high" subclones. These results are expressed as the ratio of specific enzyme activity in induced cells to that in noninduced cells. When this degree of induction is plotted against either basal (Fig. 2) or induced specific enzyme activity, no correlation is seen. That is, the degree of induction does not seem to correlate with either the initial or the induced TAT activity.

All colonies grew vigorously, with doubling times approximately the same as that for HTC+ (24 hours), and clones with low inducibility were not simply colonies with suboptimal growth. Although induction was carried out under growth conditions, the short period of induction relative to the doubling time and the expression of enzyme content as specific activity obviate the need to express enzyme activity as a differential of growth, which is commonly done for bacterial studies.

Similar cloning studies have been carried out on 72 clones from a noninducible variant of HTC+ (*4*) and on 67 clones from an HTC+ subline that is resistant to actinomycin D (*5*). In each case, the same sort of instability of TAT inducibility described above was observed, although the clustering around the modal value was much tighter for the noninducible variant.

Comparisons between the distribution patterns and inducibility among subclones from C2-10(L) and C3-30(H) suggest that we are dealing with two distinct although overlapping populations. The heterogeneity of the subclones indicates that neither the C2-10(L) population nor the C3-30(H) population is homogeneous with respect to the TAT inducibility character, although the former seems to be more homogeneous, as shown by the tighter distribution pattern of its subclones. The parent HTC+, on the other hand, is composed of a heterogeneous population (Fig. 1).

The fact that a cell population derived from a single cell is not homogeneous with respect to TAT inducibility indicates that this trait is unstable. The presence of clones of high inducibility among C2-10(L) sublines and of clones of low inducibility among C3-30(H) sublines indicates that change does occur from high inducibility to low inducibility and vice versa.

At present there is no completely

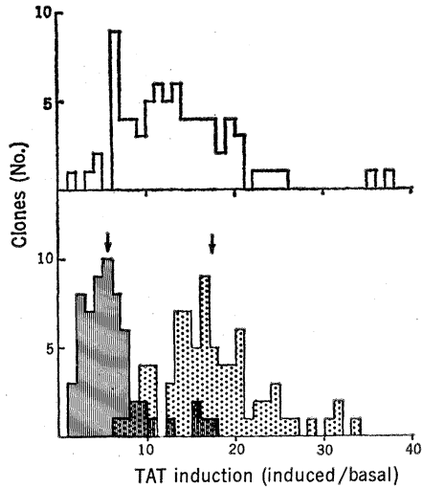

Fig. 1. Distribution of HTC subclones relative to their TAT inducibility by dexamethasone phosphate. Enzyme induction is expressed as the ratio of specific activity in induced cells to that in noninduced cells. Distribution of HTC+ subclones is given at the top, and distributions of C2-10(L) subclones (shaded area) and of C3-30(H) subclones (dotted area) are shown at the bottom. The arrows indicate the degree of induction of C2-10(L) and C3-30(H) clones.

proven or testable model that can fully explain the steroid-mediated induction of TAT—or, for that matter, of any steroid-induced enzyme. The data for TAT suggest that control of the enzyme induction requires a specific cytoplasmic steroid-binding protein (*6*), the transport of this protein with steroid to the nucleus, RNA transcription (*2, 7*), and some sort of control by a post-

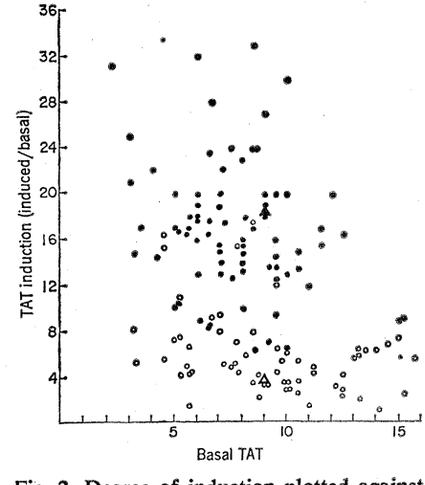

Fig. 2. Degree of induction plotted against specific activity of noninduced TAT to determine whether there was a correlation. Each point represents one subclone from Fig. 1; ○, C2-10(L) subclones; △, C2-10(L) clone; ●, C3-30(H) subclones; ▲, C3-30(H) clone.

transcriptional element (*8*). In addition, under some circumstances control of degradation of the enzyme may be required (*9*). Thus, five or six specific genes may be involved in TAT expression and induction. In the number of generations needed to grow out each clone (20 to 30), a normal mutation rate of around 10^{-6} per haploid genome at six independent sites still seems inadequate to explain the variability seen. Such a mutation rate would produce only about one variant in 10^{10} cells ($6 \times 10^{-6} \times 10^{-6} \times 30 \approx 10^{-10}$). We conclude, therefore, that the instability observed must be due to other factors, such as extraordinarily high rates of mutation, chromosomal rearrangements, or hereditary nonchromosomal factors. The cells studied here are malignant and heteroploid. Whether the observed variability is related to these properties or is general for eukaryotic cells in culture remains to be seen. Nevertheless, it is a fact that must be considered in dealing with a genetic model of eukaryotic cells in culture.

In addition, it is important to be aware of the possibility of such variation when interpreting results from some somatic cell hybridization experiments. It has been observed frequently that a particular differentiated function may not be expressed in hybrid clones. When only a few clones are examined, it is possible that parental variants lacking the desired function in the first place were inadvertently selected for fusion. Our results should also be considered in interpreting restoration of a temporarily lost function in the hybrid. For example, TAT induction was found to be partially restored, after some chromosomal segregation, in a hybrid between a Buffalo rat liver epithelial cell line and a Reuber hepatoma subclonal line (*10*). In the hepatoma parent, the basal specific activity of the enzyme was ~ 20 and increased to ~ 700 after induction [units given in (*10*)]. The initial hybrid had noninducible enzyme with specific activity of ~ 0.7. Later, one hybrid clone lost several chromosomes, and the enzyme could be induced from ~ 0.5 (basal) to ~ 4.5. Thus it appeared that only about 1 percent of wild type inducibility returned. Our data suggest the possibility that the original fusion took place with a low-inducibility variant from the wild type clone, so that actually the recovery seen is 100 percent. Others (*11*), measuring other cellular functions in

hybrids, have obtained data that could be explained by rapid phenotypic variation. Without an appropriate clonal analysis of the parental lines, one cannot evaluate this possibility.

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A New, Long-Lasting Competitive Inhibitor of Angiotensin

Abstract. *An analog of angiotensin II, [Sar¹,Ile⁸]-angiotensin II, has a potent and long-lasting competitive antagonistic effect against angiotensin II when tested for its myotropic action on the isolated rabbit aorta and for its effect on blood pressure in anesthetized cats and dogs. Compared to [Ile⁸]-angiotensin II, the new analog has equal antagonistic potency on the isolated system but a much greater potency in vivo. It is assumed that sarcosine in position 1 protects the peptide against enzymatic degradation and enhances its half-life. This study demonstrates that the modification in both positions 1 and 8 are important for the in vivo antagonistic potencies of angiotensin analogs.*

Several analogs of angiotensin II (A II) with substitutions in position 8, which were synthesized in this laboratory (*1, 2*), competitively inhibit some of the pharmacological effects of the parent peptide. One of these analogs, [Ala⁸]-A II (Ala, alanine) competitively blocks the myotropic effect of both angiotensin I (A I) and A II, but in doses from 1 to 10 μg/kg it does not antagonize the pressor response to A II in anesthetized cats (*3*). This analog, however, has an inhibitory effect against A II in anesthetized rats (*4*). The other analog, [Ile⁸]-A II (Ile, isoleucine) is a more potent competitive inhibitor of the parent peptide both in vivo and in vitro (*5*). We also reported that A II with aminophenylisobutyric acid (Apib) substituted in position 8 ([Apib⁸]-A II) is a short-acting competitive inhibitor of A II both in vivo and in vitro in several pharmacological preparations (*6*).

Since all these peptides are 8-substituted analogs of the parent peptide, it is possible that they are inactivated by the same enzymes that metabolize A II. This may be an explanation of the failure of [Ala⁸]-A II to block the pressor effect of the parent peptide in anesthetized cats (*3*). One of the enzymes that degrade A II is an aminopeptidase that acts on the NH_2-terminal amino acid (*7*). Replacement of this group by sarcosine (Sar) produced a longer-acting blocking peptide; [Sar¹,-Ala⁸]-A II is an effective competitive inhibitor of A II in rat and dog (*8*). Sarcosine, in the NH_2-terminal position, possibly protects the peptide from aminopeptidase degradation and allows it to reach the receptor site and block the effect of A II. The analog [Sar¹,-Ile⁸]-A II (molecular weight, 1003.63) has been synthesized in this laboratory (*2*). This report contains initial results of the pharmacological studies of this compound.

In vitro experiments were performed on isolated, spirally cut rabbit aortic strips in Krebs solution (*9*). The log dose-response curves of A II with and without [Sar¹,Ile⁸]-A II were determined. At the same time, the dose ratios of agonist were estimated when different concentrations of [Sar¹,Ile⁸]-A II were present. From these ratios, log K_2 values were calculated for each experiment from the equation (*10*):

$$\log K_2 = \log \frac{\text{dose ratio} - 1}{[\text{Sar}^1, \text{Ile}^8]\text{-A II}} \quad (1)$$

where the denominator is the molar concentration of the analog. Although the log K_2 value is equal to the pA_2 value when two drugs antagonize each other in a competitive manner (*11*), the pA_2 value of [Sar¹,Ile⁸]-A II was also calculated separately for each experiment. The pA_2 value of [Sar¹,Ile⁸]-A II was found to be 9.33 ± 0.2 (standard error of mean) in eight experiments. The calculated ratios in the presence of different molar concentrations of [Sar¹,Ile⁸]-A II and log K_2 values are summarized in Table 1. Log K_2 values were equal at different concentrations of [Sar¹,Ile⁸]-A II; also, the pA_2 value was not significantly different from the log K_2 values. These criteria are satisfactory for competitive antagonism (*10*). Also, the log dose-response curve of A II was a straight line, shifted to the right and parallel to the control, when [Sar¹,Ile⁸]-A II was added to the muscle bath (Fig. 1).

The antagonistic effect of continuous infusion of [Sar¹,Ile⁸]-A II was also tested on the blood pressure of cats

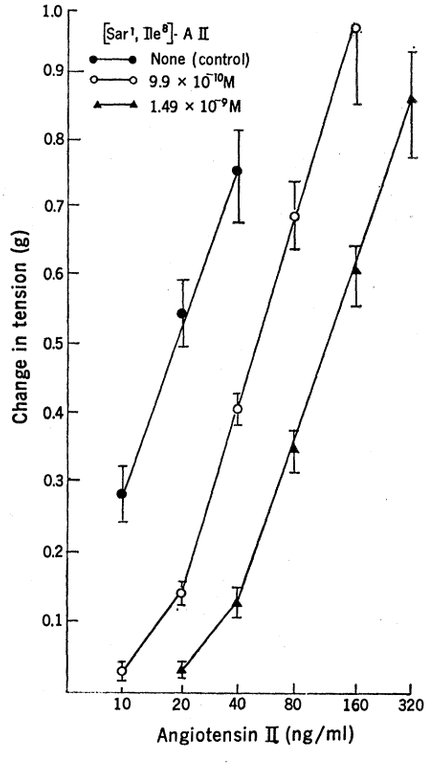

Fig. 1. Log dose-response curve of A II before and after addition of [Sar¹,Ile⁸]-A II. The ordinate shows changes in tension induced by A II on the rabbit aorta. The line to the left was derived from the mean values for three different doses of A II (32 experiments for each dose). When [Sar¹,Ile⁸]-A II ($9.9 \times 10^{-10}M$) was added to the muscle bath the dose-response curve for A II was shifted to the right and was parallel to the control (mean, eight experiments). When the concentration of [Sar¹,Ile⁸]-A II was increased (five experiments) to $1.49 \times 10^{-9}M$ the A II dose-response curve was shifted further to the right. The vertical bars indicate standard error of mean.