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4. Temperature was maintained with a Nicholson Precision Instruments model C-300 air stream incubator, available from Carl Zeiss, Inc., New York.
5. This step, utilizing a CBS Laboratories model Mark 3 series 8000 enhancer, was required to retain high-frequency information for video tape recording.
6. This step, utilizing a Consolidated Video Systems model 500 time base corrector, was required to provide a stable signal to the electron beam recorder.
7. The transfer to 16-mm film was made with a 3M Corp., Mincom Division, electron beam recorder. A 16-mm motion picture depicting all of the events described in this report is available for distribution from the authors.
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Selection of a Variant Lymphoma Cell Deficient in Adenylate Cyclase

Abstract. *Isoproterenol, a stimulator of adenylate cyclase, was used to select a stable variant clone of mouse lymphosarcoma cells deficient in the enzyme. The inability of four different stimulators to activate cyclic adenosine monophosphate synthesis in the variant, in contrast to its wild-type parent, implies that in normal cells one type of adenylate cyclase molecule can respond to different activators.*

The earliest detectable effect of many hormones on their target tissues is the activation of adenylate cyclase, which catalyzes the formation of adenosine 3′,5′-monophosphate (cyclic AMP) from adenosine triphosphate. Because the enzyme is rather labile, bound to membranes, and has resisted purification, most knowledge of this crucial step in hormone action derives from experiments with particulate fractions of broken cells (*1*). Cyclic AMP synthesis in such preparations is often stimulated by more than one hormone, and apparently always by fluoride ion as well.

Since maximally effective concentrations of two or more different agents usually fail to produce additive enzyme stimulation (*2*), it is believed that only one kind of adenylate cyclase molecule is required for a cell to respond to multiple stimuli; alternatively, an individual cell might conceivably possess a panoply of different enzymes, each specifically responsive to a different hormone. Such a question cannot be answered rigorously without extensive purification and separation of adenylate cyclase or cyclases and other components of membranes, followed by their functional recombination in vitro —a daunting task.

We report here a genetic approach to this question, which takes advantage of a cultured cell line that is killed by prolonged elevation of intracellular cyclic AMP. We have selected stable variant clones of cells that are resistant to the cytolytic effect of isoproterenol, a stimulator of adenylate cyclase. In one such clone, in contrast to the parental cell line, cyclic AMP formation cannot be stimulated by isoproterenol or three other stimulators, including fluoride. The simplest explanation of this result, a single mutation involving adenylate cyclase, implies that in normal cells one type of enzyme molecule can respond to different stimulators of cyclic AMP synthesis.

S49 mouse lymphosarcoma cells in culture are killed by exposure for 48 to 72 hours to N^6,2′-*O*-dibutyryl (DB) cyclic AMP or to agents that stimulate the synthesis of endogenous cyclic AMP (*3*). In order to select and isolate the adenylate cyclase–deficient variant cell line, wild-type S49 cells were cloned in soft agar overlying a fibroblast feeder layer (*4, 5*). Cloning efficiency in the absence of cytocidal drugs was 50 to 90 percent, but was reduced to 0.15 percent when the agar

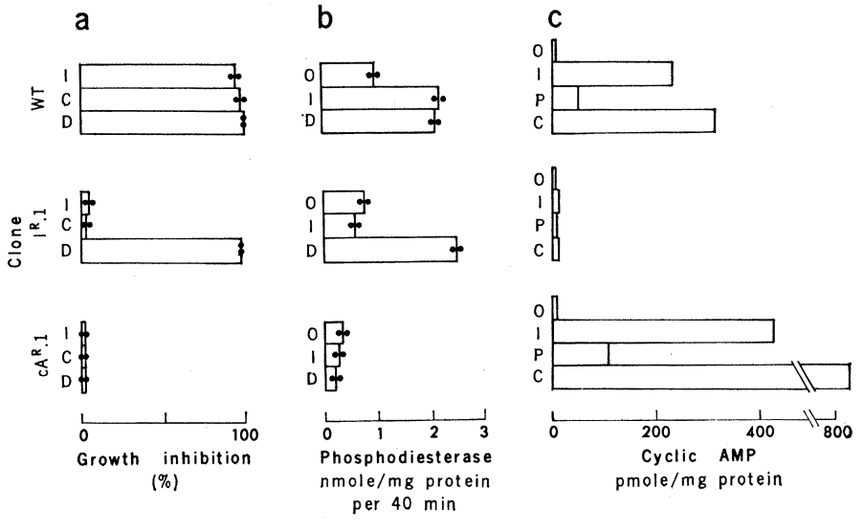

Fig. 1. Comparison of the phenotype of wild-type cells (WT, top) with those of isoproterenol resistant (I^R.1, middle) and cyclic AMP resistant clones (cA^R.1, bottom). (a) Growth inhibition by isoproterenol (I) (1.0 μM), cholera toxin (C) (30 ng/ml), or DB cyclic AMP (D) (0.2 mM). Cells were suspended at a concentration of 2×10^5 ml^{-1} in growth medium containing effectors plus RO 20-1724 (30 μM), and viable cells (those excluding trypan blue) were counted 72 hours later. Results are expressed as the percentage inhibition of growth by the effector (in duplicate incubations) as compared with cell growth in control incubations containing RO 20-1724 alone, in which cells multiplied to densities of 2.1×10^6 to 2.4×10^6 ml^{-1}. (b) Induction of cyclic AMP phosphodiesterase. Logarithmically growing cells were untreated (O) or given isoproterenol (I) (1.0 μM), or DB cyclic AMP (D) (0.2 mM) for 4.5 hours, centrifuged, sonicated, and assayed for phosphodiesterase activity as described (*3, 8, 17*) at a substrate (cyclic AMP) concentration of 1.0 μM. Each bar represents the mean of duplicate incubations. (c) Cyclic AMP accumulation in logarithmically growing untreated cells (O) or cells exposed to isoproterenol (I) (1.0 μM), PGE_1 (P) (0.1 μM), or cholera toxin (C) (30 ng/ml). Cells were incubated with cholera toxin for 90 minutes and with the other agents for 15 minutes before being centrifuged and assayed for cyclic AMP as described (*3, 8*) by the competition binding assay of Gilman (*11*). Purified cholera toxin, prepared by Finkelstein and Lospalluto (*18*), was obtained from Dr. John Seal, NIH Standards. Prostaglandin E_1 was a gift from Dr. John Pike of the Upjohn Company. Other agents were obtained commercially.

contained 10 μM isoproterenol and 60 μM RO 20-1724, a potent inhibitor of cyclic AMP phosphodiesterase (*6*). Thirty-five colonies that survived the selection procedure were grown in suspension culture and again exposed to the same drugs. Thirteen of these appeared fully resistant to isoproterenol-induced cytolysis.

The phenotype of one isoproterenol resistant clone (I^R.1) was compared in detail with that of the parental wild-type (WT) cell (Fig. 1) (*7*). As reported elsewhere (*8, 9*), the growth of WT cells is blocked in the G_1 phase of the cell cycle by DB cyclic AMP, isoproterenol, or cholera toxin. In contrast (Fig. 1a), I^R.1 cells grow normally in the presence of isoproterenol or cholera toxin, although they are normally sensitive to DB cyclic AMP. Similarly, isoproterenol and DB cyclic AMP induce synthesis of cyclic AMP phosphodiesterase in WT cells (*8, 10*). DB cyclic AMP effectively induces phosphodiesterase in I^R.1 cells, but isoproterenol does not (Fig. 1b). Basal cyclic AMP content (3 to 6 pmole per milligram of protein, in the absence of drugs) is the same in WT and I^R.1 cells (*11*). However, cyclic AMP rises in WT, but not I^R.1 cells treated with isoproterenol, cholera toxin, or prostaglandin (PG) E_1 (Fig. 1c). Even in the presence of 30 μM RO 20-1724, which potentiated the isoproterenol-induced cyclic AMP response of WT cells fourfold, I^R.1 cells failed to accumulate cyclic AMP (not shown). The I^R.1 phenotype has remained stable for 6 months in continuous culture in medium without drugs.

The I^R.1 phenotype also contrasted with that of a previously derived class of mutant S49 cells (exemplified by clone cA^R.1) (Fig. 1), which are completely resistant not only to agents that stimulate accumulation of endogenous cyclic AMP but also to its exogenous DB derivative. The cA^R mutants lack the cytoplasmic receptor for cyclic AMP (*3, 5, 8–10*).

Measurements of adenylate cyclase activity in particulate cell fractions (Fig. 2) revealed the biochemical lesion of I^R.1 cells. Adenylate cyclase activity could not be detected in extracts of I^R.1 cells, even in the presence of isoproterenol, PGE_1, or NaF. Basal activity was easily measurable in WT and cA^R.1 extracts, and both responded to all three drugs (Fig. 2). These results suggest that the altered phenotype of I^R.1 cells is caused by a defective adenylate cyclase. Although enzyme activity was not detected in particulate fractions, the presence of cyclic AMP in intact I^R.1 cells makes it likely that they possess some adenylate cyclase (*12*). It was important to rule out the possibility that cyclic AMP was indeed synthesized by I^R.1 extracts but was degraded at an unusually fast rate, despite the presence of a phosphodiesterase inhibitor. This was not the case: Nonradioactive cyclic AMP in amounts (2.5 or 10 pmole) equivalent to that synthesized by WT extracts was quantitatively recovered from assay mixtures containing either WT or I^R.1 extracts incubated under standard conditions for the usual assay time (10 minutes). In addition, mixing experiments suggested that the defect of I^R.1 cells was not caused by an endogenous inhibitor or an altered modulator of adenylate cyclase. Extracts of I^R.1 cells did not inhibit WT enzyme activity, and WT extracts failed to restore I^R.1 activity (not shown) (*13*).

Current models of hormone-sensitive adenylate cyclase (*1, 2*) are based on the assumption that the catalytic activity of a single class of enzyme molecules in a cell can be activated by detergents, NaF, multiple hormone receptors, and the unique time-dependent action of cholera toxin (*14*). This assumption rests on two sets of observations. (i) The enzyme, from a variety of sources, exhibits similar kinetic properties and ion requirements, and can always be stimulated by NaF. (ii) Adenylate cyclase in membranes of homogeneous cell preparations, such as adipocytes, may be stimulated by more than one hormone, as well as by NaF; nonetheless, the effects of maximally active concentrations of different hormones are not additive (*1, 2*).

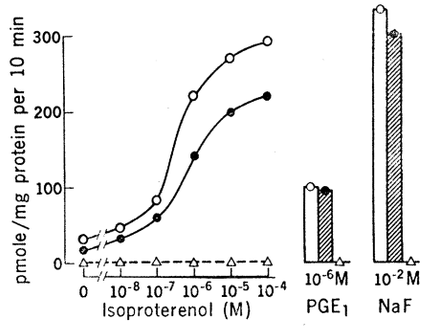

Fig. 2. Effects of isoproterenol, PGE_1 and NaF on adenylate cyclase activity of crude particulate fractions prepared from wild-type cells (WT) (open circles) and isoproterenol resistant (I^R.1) (triangles) or cyclic AMP resistant (cA^R.1) (closed circles) clones. Logarithmically growing cells were centrifuged, washed in ice-cold 0.32M sucrose, and resuspended in 0.05M sucrose containing 5 mM tris-Cl buffer (pH 7.0), 2 mM $MgCl_2$, and 2 mM dithiothreitol. The resuspended cells were immediately homogenized (12 strokes of a motor-driven Teflon-glass homogenizer), and the homogenate was centrifuged at 190g for 2 minutes. The pellet, containing nuclei and residual unbroken cells, was discarded. The supernatant was then centrifuged for 10 minutes at 10,000g, and the pellet was resuspended in the hypotonic sucrose buffer, washed, and assayed for adenylate cyclase. This fraction contained 70 to 80 percent of the total enzyme activity present in WT homogenates; the remaining activity was all in the low-speed fraction, and none could be detected in the 10,000g supernatant fraction. None of the fractions prepared from I^R.1 cells exhibited detectable adenylate cyclase. The enzyme was assayed (*12*) with adenylyl imidodiphosphate (AMP-PNP) as substrate, and the product, nonradioactive cyclic AMP, was measured by the competition binding method of Gilman (*11*). Assay incubations contained, in a total volume of 100 μl, 50 to 100 μg of enzyme protein, 20 mM tris-Cl, pH 8.5 (30°C), 2 mM $MgCl_2$, 0.3 mM 1-methyl-3-isobutylxanthine, and the indicated concentration of drug. The reaction was started by adding AMP-PNP, 0.5 mM. Enzyme activity was linear with increasing protein concentrations (5 to 150 μg) and time (0 to 20 minutes) at 30°C.

The phenotype of clone I^R.1, selected in a single step, is probably determined by mutation of a single gene and the resulting deficiency of a single gene product. If the affected gene product is adenylate cyclase itself, our experiments would suggest that S49 cells contain only one type of adenylate cyclase molecule, responsive to multiple stimuli. However, since basal cyclic AMP is present in I^R.1 cells, it is possible that the enzyme itself is not altered, but that the mutant cells lack an enzyme cofactor or membrane component required for stimulation by hormones, cholera toxin, and NaF.

Mutations affecting both the synthesis (*15*) and action (*16*) of cyclic AMP in *Escherichia coli* have proved essential for understanding the regulatory role of the cyclic nucleotide in prokaryotes. The genetic approach may prove equally useful in elucidating the cyclic AMP system of mammalian cells.

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11. Cellular cyclic AMP was assayed by the protein binding assay of A. Gilman [*Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970)]. Measurable cyclic AMP of both WT and $I^R.1$ cells was completely destroyed by beef heart cyclic AMP phosphodiesterase (obtained from Sigma).
12. The adenylate cyclase assay (M. E. Maguire and A. Gilman, *Biochim. Biophys. Acta*, in press) can reproducibly measure cyclic AMP production as low as 0.5 pmole per milligram of protein per minute. The apparent discrepancy between measurable cyclic AMP in intact $I^R.1$ cells and undetectable adenylate cyclase activity in $I^R.1$ particulate fractions is not surprising, however, since cyclic AMP synthesis has consistently been found much greater in intact mammalian cells than in broken cells (*1*), probably because the enzyme is damaged by homogenization.
13. Specifically, when crude particulate extracts (prepared as described in the legend to Fig. 2) of WT and $I^R.1$ cells were mixed, adenylate cyclase activity was identical to that measured in parallel tubes containing WT particulates alone. In addition, the supernatant fraction of a homogenate of WT cells centrifuged at 10,000*g* (see legend to Fig. 2), which contained negligible adenylate cyclase activity, failed to restore activity of $I^R.1$ particulate fractions, and similar supernatant fractions from $I^R.1$ cells did not affect WT enzyme activity.
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Early Signs of Language in Child and Chimpanzee

Abstract. *In a sequel to Project Washoe, chimpanzees are being taught American Sign Language from birth by humans who are fluent in the language, including persons who are themselves deaf or whose parents were deaf. The first two subjects began to use signs when they were 3 months old, and these early results indicate that the new conditions are significantly superior to the conditions of Project Washoe. More valid comparisons can now be made between the acquisition of language by children and by chimpanzees.*

The exposure of children to their native language begins at birth, and most theories of language acquisition assume that the exposure during the earliest years is particularly significant. What evidence there is on this point is, at best, indirect. Long-term negative effects of an impoverished linguistic environment can be demonstrated for children reared in orphanages during the first years of life (*1*). Favorable effects of early exposure to language—in this case, sign language—can also be demonstrated by comparing deaf children of deaf parents with deaf children of hearing parents on tests of the ability to speak, read, or write English (*2*). Moreover, with the recently developed techniques for recording behavior of neonates, it has been shown that the human infant is responsive to characteristics of adult speech, such as segmentation and the distinction between phonemes, within a month of birth (*3*). It seems likely that the beneficial effects of early exposure to language can also be demonstrated in attempts to teach language to animals.

Project Washoe was the first attempt to teach sign language to a chimpanzee. Washoe was about 11 months old when her training in American Sign Language (Ameslan) began. Within 51 months, she had acquired 132 signs of Ameslan, as determined by criteria for reliable usage developed during the research (*4*). As with humans using words, Washoe used her signs for classes of referents rather than for particular objects or events, and used signs in combinations (*5*). Brown (*6*), Klima and Bellugi (*7*), and other investigators of child language have commented on the many ways in which Washoe's acquisition of sign language parallels the acquisition of spoken language by children, as, for example, in Washoe's generalization of the meaning of signs, in the gradual increase in length of her sign combinations, and in the types of semantic relations expressed by early combinations. Thus, Project Washoe demonstrated that Ameslan is a suitable medium of communication for a chimpanzee, and that, given a suitable medium, a significant level of two-way communication could be achieved. Since then, several chimpanzees in several laboratories have acquired a vocabulary of signs (*8*), and it is appropriate to pose questions about individual differences, about limits, and about the effectiveness of different methods of teaching sign language. In our current project of teaching sign language to several chimpanzees, we are capitalizing upon our experience in the research with Washoe by improving key features of procedure, and we plan to maintain these more favorable conditions until the subjects reach intellectual maturity. In this way, we can come much closer to describing the highest level of two-way communication that can be achieved by chimpanzees taught a form of human language.

One of the significant improvements in procedure is that several fluent signers, including deaf persons and persons who have deaf parents, are research personnel in the current project. These "native speakers" of Ameslan provide far more adequate models of the language than those we provided for Washoe.

Another improvement is that the exposure of subjects to Ameslan begins 1 or 2 days after birth. Chimpanzee Moja was born at the Laboratory of Experimental Medicine and Surgery in Primates, Tuxedo Park, N.Y., on 18 November 1972 and arrived in our laboratory on the next day. Chimpanzee Pili was born at Yerkes Regional Primate Research Center, Atlanta, Ga., on 30 October 1973 and arrived in our laboratory on 1 November.

No special difficulties were encountered in maintaining the infants in good health. Their care is similar to that of the human infant; around-the-clock feedings, diapering, inoculations, sanitary precautions such as sterilization of bottles, and so on. In addition, we provided the infants with body contact whenever they were awake. Within a few weeks, the infants appeared re-