present in the cultured infective forms, we measured the steady-state concentration of the terminal oxidases present. Culture medium (90 ml) from three inoculated Chinese hamster cell cultures was removed at a concentration of 2.5×10^6 trypanosomes per milliliter. The organisms were collected by centrifugation and the steady-state oxygen levels measured (Fig. 3). Salicylhydroxamic acid (SHAM) inhibited 90 percent of the cell respiration. Azide had little effect, inhibiting 7 percent of the cell respiration. Little significant azide- and SHAM-insensitive respiration was observed. These results are similar to our previous results for bloodstream trypomastigotes of *T. brucei* (*4*). They clearly indicate that the SHAM-sensitive α-glycerophosphate oxidase is the predominant terminal oxidase in cultured infective trypomastigotes of *T. rhodesiense*.

The procedures described herein provide a simplified technique for initiating and maintaining large quantities of bloodstream trypomastigotes of *T. rhodesiense* in culture on an established tissue culture cell line. The cultured infective trypomastigotes have also been stored as frozen stabilates at −90°C (that is, culture mixed 1 : 1 with 20 percent glycerol in the culture medium). The thawed stabilates remained infective for mice and rats and continued to develop in vitro on Chinese hamster lung cells.

At present, the *T. rhodesiense* trypomastigotes have retained infectivity after being maintained for 80 days in vitro at 37°C. The concentration of trypanosomes obtained in our experiments (3×10^6 to 5×10^6 cells per milliliter) should provide adequate numbers of cells to allow further biochemical and immunological studies, and this system may prove valuable in studies of the process of trypanosome antigenic variation.

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Cellular Interactions Uncouple β-Adrenergic Receptors from Adenylate Cyclase

Abstract. *C6 glioma cells and B104 neuroblastoma cells both possess adenylate cyclase activity, but only C6 cells have β-adrenergic receptors. However, when cocultured with B104 cells, C6 cells show a marked decrease in their ability to accumulate adenosine 3′,5′-monophosphate upon stimulation with β receptor agonists. Since both β receptors and cholera toxin–stimulated adenylate cyclase activities are present in C6/B104 cocultures, we conclude that the β receptor/adenylate cyclase transduction mechanism in cocultured C6 cells is uncoupled.*

The means by which cells communicate with each other include syncitial events [that is, the passage of ions or small molecules through intercellular junctions (*1*)], events involving soluble factors (for example, hormones, neurotransmitters), and those utilizing membrane-associated elements in intimate cellular contact. This last mode of communication is believed to involve cell surface molecules on an "instructor" cell interacting with its counterparts on a target cell to effect specific changes in the target cell's physiology (*2*). The membrane-associated molecules thought to mediate these interactions include gangliosides (*3*), glycosaminoglycans (*4*), and glycoproteins (*5*). Although the effects of neurohormonal communication have been well characterized in a variety of systems (*6*), similar biochemical effects of either syncytial- or surface molecular-interactions between cells of different types have been studied to a lesser extent (*7*).

In this report, we describe a technique for coculturing two cell types for studies of these intimate types of cellular interactions. This technique offers the advantages of enabling one to monitor the absolute number of each cell type at any time, as well as to coculture cells with very different growth rates without one cell type overgrowing the other. Using C6 glioma and B104 neuroblastoma cells—both cloned cell lines from chemically induced tumors of the rat central nervous system (*8, 9*)—we found that intimate cellular interactions with B104 cells cause the β-adrenergic receptors on C6 cells to become uncoupled from adenylate cyclase.

Trypsinized C6 (subclone 2B) cells, some of the cells previously labeled with [^{3}H]thymidine, were plated onto either confluent beds of B104 cells (C6/B104 cocultures) or onto tissue culture plastic surfaces (C6-only cultures) (*10*). The density of C6 cells in the inoculum was such that only two to three rounds of cell division could occur before their growth became contact-inhibited. (Confluent cultures of B104 cells not receiving C6 cells are termed "B104 only.") These cultures were then allowed to grow for various periods of time. Similar cultures were fixed and processed for autoradiography (*11*). To estimate the numbers of each cell type growing in coculture, we first examined autoradiographs of C6-only cultures and determined the proportion of C6 cells containing label. This ratio, divided into the number of labeled cells seen in autoradiographs of C6/B104 cocultures, yields the total number of C6 cells growing on the B104 cell bed. (At least three rounds of cell division can occur before the amount of label per daughter cell falls below that detectable by autoradiography.) The number of B104 cells growing in cocultures was obtained by subtracting the amount of C6 DNA (*12*) from total coculture DNA and then by dividing this remainder by the amount of DNA per B104 cell.

Figure 1 shows that from day 3 after the addition of C6 cells, there were marked differences among the cultures in their abilities to accumulate adenosine 3′, 5′-monophosphate (cyclic AMP) in response to incubation with the potent β-adrenergic agonist isoproterenol. The C6-only cultures showed a steady increase in this β responsiveness as a function of time. In contrast, C6 cells in the C6/B104 cocultures maintained a low level of β responsiveness, even after the C6 cells had reached their maximum cell density (inset, Fig. 1). The B104-only cultures showed a consistent, yet statistically insignificant (*13*) increase in cyclic AMP throughout the experimental period (for example, see Table 1). Similar

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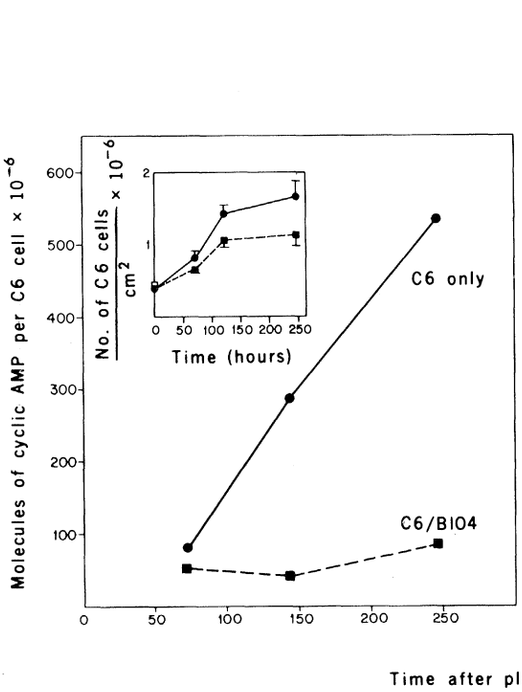
differences in β responsiveness were also observed when norepinephrine (3 μM) was substituted for isoproterenol (data not shown).

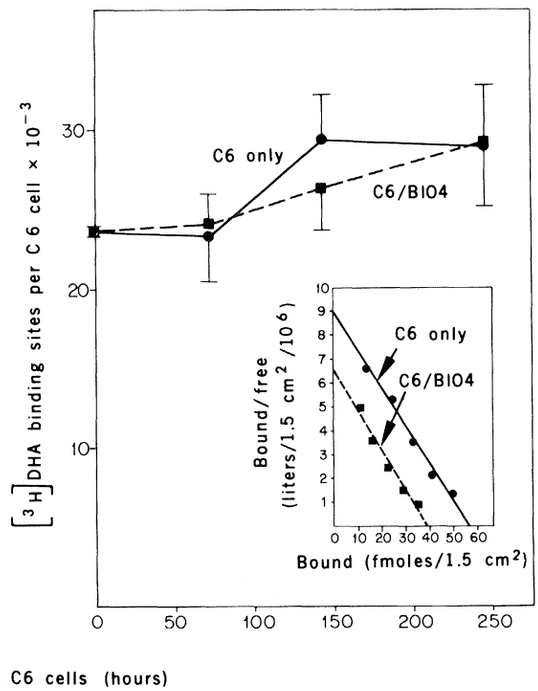
To determine whether the release of a soluble factor by the B104 cell bed was responsible for the decreased β responsiveness of the cocultured C6 cells, we added to newly plated C6-only cultures 50 percent fresh medium and 50 percent (by volume) conditioned medium from either C6 only, B104 only, or C6/B104 cocultures on every second day for 9 days. The cultures were then stimulated with isoproterenol as described above and assayed for cyclic AMP. There was no significant difference between C6 cells treated with any of these conditioned media (data not shown), suggesting that it is the presence of the B104 cell itself, rather than simply a B104-released factor, which is responsible for the diminished β responsiveness seen in the C6 cells.

We then determined the molecular site, within the cyclic AMP metabolic scheme, affected by the cellular interaction. Since the loss of β receptors accompanying desensitivity to β stimulation had been described in a variety of systems (*14*), a [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding assay (*15*) was used to quantify the number and binding characteristics of β receptors in the C6/B104 cocultures. Scatchard analyses of the binding data, however, demonstrate that the number of β receptors per C6 cell (*16*) did not significantly differ between C6-only and C6/B104 cocultures at any time during the 10-day experiment (Fig. 2). In contrast, B104-only cultures lacked detectable levels of β receptors at all times (data not shown). Receptor binding affinities for both agonist and antagonist ligands also did not vary significantly between C6-only and C6/B104 cocultures. Apparent dissociation constants (K_d) for the antagonist [^{3}H]DHA were 3.5 nM and 3.8 nM, respectively, whereas K_d values for the agonist isoproterenol were 0.33 μM and 0.50 μM, respectively (*17*). Taken together, this suggests that β receptors on cocultured C6 cells are qualitatively and quantitatively indistinguishable from those found on C6 cells grown by themselves—at least with respect to ligand binding.

A second site at which B104 cells could interfere with the β responsiveness of cocultured C6 cells involves phosphodiesterase (E.C. 3.1. 4.17), the enzyme responsible for the catabolism of cyclic nucleotides (*18*). Higher activities of this enzyme in the cocultured C6 cells, for instance, might act to mask the otherwise normal rates of cyclic AMP production in isoproterenol-stimulated, cocultured C6 cells. Therefore, to eliminate any possible contributions by phosphodiesterase to the decreased β responsiveness in C6/B104 cocultures, 10-day-old cultures were first incubated in Ham's F-10 medium with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Aldrich), a potent inhibitor of phosphodiesterase. This medium was then replaced with fresh medium containing IBMX in the absence or presence of isoproterenol, and, after another 15-minute incubation period, both the cells and the final incubation medium were assayed for cyclic AMP.

Table 1 shows that the increase in intracellular cyclic AMP per C6 cell was again significantly diminished in the C6/B104 condition when compared with the C6-only cultures (*13*). This suggests that the catabolism of cyclic AMP by





Time after plating C6 cells (hours)

Fig. 1 (left). The increase in β responsiveness in C6 cells as a function of time after plating. Cultures, grown on Falcon 3008 tissue culture plates, were washed once with warmed F-10 medium without serum and then incubated for 15 minutes at 37°C in F-10 with or without 1 μM isoproterenol (Sigma). The incubation was terminated by placing the plate on ice, removing the medium, washing the surface once with 0.9 percent (weight to volume) NaCl (0°C), and then extracting the cyclic AMP with 0.1N HCl (0°C). These extracts were stored at −20°C for 1 to 4 days. After the acid-extraction step, the remaining cell material was utilized for the DNA determination. To separate cyclic AMP from various interfering substances, the pH of the acid extract was first adjusted to 6.0 with tris buffer (0.5N, pH 9.0), the extract was passed over a 0.8 by 1.4 cm column of AG1-X2 (200 to 400 mesh, chloride form, Biorad), and the column was washed with 40 ml of deionized water. Cyclic AMP was eluted with 0.5N HCl, lyophilized, and a radioimmunoassay (Collaborative Research Co.) performed. Each point on the graph is the mean of four determinations. Symbols: ●, C6 cells in C6-only cultures; ■, C6 cells in C6/B104 cocultures. (Inset) Growth curve of C6 cells. Cultures were grown on Falcon 3001 plates and cell numbers were estimated by autoradiographic methods discussed in the text. Each point is the mean of three cultures ± standard deviation. Fig. 2 (right). The number of β receptors per C6 cell as a function of time after plating. Each point represents the number of [^{3}H]DHA binding sites (as calculated by Scatchard analyses) per square centimeter of tissue culture surface divided by the number of C6 cells (± standard deviation) per square centimeter of tissue culture surface. Symbols: ●, C6 cells in C6-only cultures; ■, C6 cells in C6/B104 cultures. (Inset) Scatchard plot of [^{3}H]DHA binding to membranes of 10-day-old cultures. The slopes and intercepts of the lines were calculated by linear regression analyses. Note that in the C6-only cultures, the number of C6 cells is 11.9 ± 1.6 $\times 10^5$/1.5 cm^2, whereas in the C6/B104 cocultures, the number of C6 cells is 8.0 ± 1.0 $\times$ 10^5/1.5 cm^2.

phosphodiesterase in these cultures is not a major factor in the decreased β responsiveness observed in cocultures. To preclude the possibility that cocultured C6 cells secrete more cyclic AMP than do C6 cells grown by themselves, the ratios of extracellular to intracellular concentrations of cyclic AMP were calculated from these same isoproterenol-stimulated cells. The C6-only cultures secreted 6.3 percent of their intracellular cyclic AMP content during the 15-minute incubation period with isoproterenol, whereas C6/B104 cocultures secreted 6.2 percent. Since these values are not significantly different from one another (*13*), the rate of cyclic AMP secretion by C6 cells in coculture can also be excluded as a contributing factor to their decreased β responsiveness.

Another site where B104 cells could influence the β responsiveness of cocultured C6 cells involves adenylate cyclase (E.C. 4.6.1.1). Therefore, stimulation of cyclic AMP concentrations by cholera toxin was used as an indirect measure of adenylate cyclase activity. This toxin is thought to irreversibly activate the adenylate cyclase molecule in virtually every vertebrate cell possessing this enzyme, by a process involving the toxin's uptake into the cell and its covalent modification of a component in the adenylate cyclase enzyme complex (*19*). In this experiment, 10-day-old cultures were incubated for 60 minutes at 37°C with cholera toxin (Sigma; 0.5 μg/ml) and IBMX (0.5 m*M*) after a 15-minute incubation period with IBMX at the same concentration. When cyclic AMP concentrations were determined at the end of the final incubation period, C6-only, C6/B104, and B104-only cultures had accumulated 530 ± 79, 886 ± 216, and 224 ± 42 pmole of cyclic AMP per 25-cm^2 growing area above control cultures, respectively (the numbers of C6 and B104 cells are the same as those reported in Table 1). Although it is impossible from these data to separate out the relative contributions of the two cell types toward the higher cholera toxin–stimulated cyclic AMP concentrations in the C6/B104 cocultures, these data are clearly inconsistent with the possibility that adenylate cyclase is grossly diminished in the cocultured C6 cells.

The site at which B104 cells exert their inhibitory influence on β responsiveness in cocultured C6 cells thus appears to involve the mechanism of β receptor/adenylate cyclase coupling, because cocultured C6 cells possess adequate levels of both β receptors and adenylate cyclase molecules, yet fail to show an accumulation of intracellular cyclic AMP in the presence of β agonists and a phosphodiesterase inhibitor.

Table 1. Hyporesponsiveness of β receptors occurs in the presence of a phosphodiesterase inhibitor. Ten-day-old cultures were incubated with 0.5 m*M* IBMX in Ham's F-10 medium for 15 minutes at 37°C. This medium was then replaced with fresh medium with or without 1.0 μM isoproterenol and incubated an additional 15 minutes at 37°C. Cyclic AMP concentrations were then determined in four cultures for each condition and are expressed here as the mean ± standard deviation (S.D.). Basal levels of cyclic AMP were subtracted. These levels were: C6-only cultures, 36.3 ± 7.7 pmole/25 cm^2; C6/B104, 18.3 ± 7.7 pmole/25 cm^2; B104-only cultures, 3.5 ± 1.0 pmole/25 cm^2. The numbers of each cell type in culture were calculated as described in the text and expressed as the mean ± standard error. In C6-only cultures, there were 10.39 ± 1.94 $\times 10^6$ cells per 25 cm^2. In the C6/B104 cocultures, there were 7.17 ± 0.11 $\times$ 10^6 C6 cells per 25 cm^2 and about 8.90 $\times$ 10^6 B104 cells per 25 cm^2. In the B104-only cultures, there were 11.39 ± 0.01 $\times$ 10^6 cells per 25 cm^2.

Condition	Isoproterenol-treated (pmoles of cultures of cyclic AMP per 25 cm^2)	Molecules of cyclic AMP accumulated per C6 cell
C6 only	9420 ± 1920	546 ± 151 $\times$ 10^6
C6/B104	111 ± 59.1	9.32 ± 4.96 $\times$ 10^6
B104 only	0.99 ± 1.05	

The degree of receptor-effector coupling can be quantified from the data in Fig. 2 and Table 1 by dividing the number of β receptors per cell into the number of cyclic AMP molecules accumulated per cell in the presence of isoproterenol and IBMX (cyclic AMP/β receptor). With this measure, C6-only cultures accumulate 18,900 cyclic AMP/β receptor during the 15-minute incubation period, whereas C6/B104 cocultures only accumulate 320 cyclic AMP/β receptor above basal levels. These numbers are similar to those recently reported by Haga *et al*. (*20*) who used several variants of the S49 lymphoma cell line in which β receptors are permanently uncoupled from adenylate cyclase. By reexpressing their data in terms of cyclic AMP/β receptor (per 15 minutes), one finds that the degree of receptor coupling in their membrane fractions falls from about 14,300 cyclic AMP/β receptor in the wild-type S49 to about 300 to 600 cyclic AMP/β receptor in the uncoupled variants. However, this is not to say that the molecular site or sites of the uncoupling in the S49 variants is necessarily the same as that in cocultured C6 cells. In fact, the recent reports of multiple intermediate components in the β receptor/adenylate cyclase system (*21*) suggest that uncoupling might occur at any one of several molecular loci.

Cultured by themselves, C6 cells have also been shown to express a diminished β-responsive adenylate cyclase activity when previously incubated for long periods with various β agonists or cyclic AMP analogs (*22, 23*). Although there is one report (*24*) of substantially decreased numbers of β receptors on refractory C6 cells (*24*), others (*22*) have found that the numbers of β receptors on the 2B subclone of C6 are, at most, only marginally diminished in the refractory state. This suggests that in refractory C6 cells, as in B104-cocultured C6 cells, β receptors have become uncoupled from adenylate cyclase. However, the means by which the B104 cells accomplish this is clearly not by the release of β agonist, because B104-conditioned medium does not decrease the β responsiveness of C6-only cultures. Moreover, B104 cells possess only low concentrations of tyrosine hydroxylase (*8*), the enzyme which catalyzes the rate-limiting step in catecholamine biosynthesis (*25*).

Our data indicate that intimate cellular interactions occur between C6 glioma and B104 neuroblastoma cells in culture. The biochemical effects on the C6 cells are specific in that only some aspects of this cell's physiology are affected while other aspects are left unchanged. Although the experiments reported here do not permit us to distinguish between the syncytial and surface molecular modes of intimate intercellular communication, the technique of coculture we describe here can be easily modified to accommodate these types of studies (*26*).

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11. Cells were fixed in 4 percent glutaraldehyde-phosphate buffered (*p*H 7.2, 0.01*M*) saline solution overnight at 4°C. After they were extensively washed in water and dried, the plates were processed for "high speed scintillation autoradiography" [B. G. M. Durie and S. E. Salmon, *Science* **190**, 1093 (1975)], Spectrafluor (Amersham/Searle) being used as the scintillator.
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16. Although the numbers of β receptors per C6 cell reported here are several times the number published by other groups *15*), several pieces of evidence confirm that these specific [^{3}H]DHA binding sites are indeed β-receptors: (i) the apparent dissociation constant [calculated as in (*17*)] for *l*-propranolol (Ayerst Labs) is $2.3 \times 10^{-8} M$, while that of *d*-propranolol (Ayerst Labs) is $27.1 \times 10^{-8} M$—suggesting stercospecificity of binding, and (ii) the specific binding of [^{3}H]DHA is linear with respect to the amount of protein added. Possible reasons for the discrepancy in receptor number include differences in the particular subclone of C6 used, the source of serum, or various modifications used in the preparation of membrane fractions.
17. The K_d values for [^{3}H]DHA were calculated directly from Scatchard plots of [^{3}H]DHA binding data. The K_d values for isoproterenol were calculated by first determining the concentration of isoproterenol required to inhibit 50 percent of specific [^{3}H]DHA binding to membranes. This concentration multiplied by the K_d of [^{3}H]DHA and divided by the concentration of [^{3}H]DHA used (20 n*M*) in the competition study, yields the K_d for isoproterenol.
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Neuronal Plasticity in Primate Telencephalon: Anomalous Projections Induced by Prenatal Removal of Frontal Cortex

Abstract. *When the dorsolateral prefrontal cortex in one hemisphere of a rhesus monkey is resected 6 weeks before birth and the fetus survives to postnatal ages, neurons of the corresponding cortex in the intact hemisphere issue a greatly expanded projection to the contralateral caudate nucleus in addition to a normal projection to the ipsilateral caudate. The enhancement of the crossed prefronto-caudate pathway after prenatal neurosurgery provides direct evidence for lesion-induced neuronal rearrangement in the primate telencephalon.*

Rearrangement of synaptic connections is potentially the most important biological mechanism underlying recovery of function after brain injury. Most of the evidence for such rearrangement in mammals has been obtained from studies of focal ablation of sensory, motor, and limbic structures in developing (*1*) and mature (*2*) rodents or carnivores. It is not known, however, whether and to what degree such neuronal plasticity can occur in the primate brain at maturity or at any stage of development. Primates, including humans, exhibit remarkable sparing of behavioral function after circumscribed brain injuries, particularly those occurring early in life (*3*, *4*). Knowledge of the capacity for axonal redistribution in the primate order is essential for understanding the mechanisms of both reversible and permanent consequences of brain damage. I now report that neocortical neurons in a nonhuman primate can alter their locus of termination in response to a focal brain lesion.

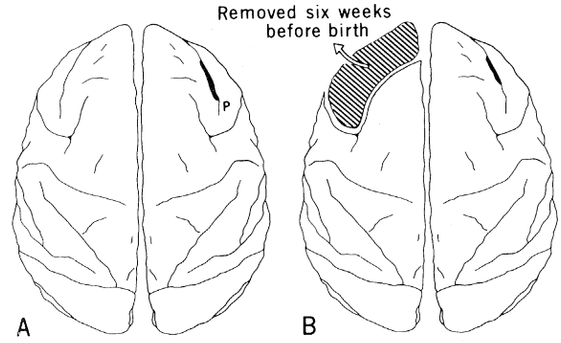

Fig. 1. Injections sites in (A) a control monkey and (B) the monkey whose prospective dorsolateral cortex in the left hemisphere was resected before birth and whose right hemisphere was injected after birth with [^{3}H]proline and [^{3}H]leucine. Injection sites were reconstructed from serial sections through the labeled area. They were defined by extremely dense labeling of the middle portion of the dorsal rim and about half the height of the dorsal bank of the principal sulcus (*P*) in the experimental animal and in both controls. The comparability of the cortical territory labeled in all cases was further corroborated by the similar distribution of labeled cortico-thalamic fibers in the appropriate sector (parvocellular division) of the dorsomedial nucleus which reciprocates projections to the principal sulcus (not shown) as well as by correspondence in the topographic location of ipsilateral cortico-caudate connections shown in Fig. 2. Although the injection site of the control animal was somewhat larger than that of the experimental animal, the autoradiograms (Fig. 2) did not display evidence of anomalous crossed projections.

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