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Unexpected β Adrenergic Effects of the Antitumor Agent, Cyclocytidine, on Rat Salivary Glands[1] (38718)

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Arabinosyl cytosine (1-β-D-arabinofuranosyl cytosine hydrochloride) has been used in man in the treatment of leukemia (1). However, to maintain a constant and effective blood concentration of arabinosyl cytosine (Ara-C), daily intravenous infusions for long periods of time are necessary. Recently, it was reported that 2,2′-*O*-cyclocytidine (2,2′-anhydro-1-β-D-arabinofuranosyl cytosine hydrochloride, cyclocytidine, cyclo-C, NSC-145668) is also an effective inhibitor of various tumors (2), and chiefly because it is converted to arabinosyl C (3). The conversion of cyclocytidine to the tumoractive Ara-C proceeds slowly (3, 4); consequently, a single daily dose (oral or ip) of cyclocytidine can be employed to attain effective blood levels of Ara-C (4). Thus, use of cyclocytidine eliminates the inconvenience associated with direct administration of Ara-C itself. However, cyclocytidine produces certain undesirable side effects that are not seen when Ara-C is used. These include sialorrhea (5), parotid pain (6), and postural hypotension (6). While these effects do not preclude use of the drug, they do limit its usefulness; therefore, the present investigation was undertaken to determine the mechanism of some of these side effects, particularly the sialorrhea and parotid pain.

Materials and Methods. Female Long–Evans rats, 4 mo of age and weighing approximately 200 g, were used in these experiments. They were maintained on lab chow and water except for the 18 hr preceding collection of saliva when food, but not water, was removed from the animals. For saliva collection, animals were anesthetized with 1% Nembutal and the salivary ducts were cannulated or surgically freed. Cyclocytidine in 0.9% saline or distilled water, was administered ip as a single dose of 600 mg/kg. In some animals, autonomic antagonists were administered ip 20–25 min prior to the injection of cyclocytidine. The antagonists were atropine sulfate, 0.5 mg per rat; dibenzyline, 2 mg per rat; and propranolol, as single doses of 1 mg, 4 mg, or in doses of 0.5 mg, administered every 30 min for 3 hr. Salivary secretion was stimulated by the cyclocytidine, and the fluids were collected by micropipet. Flow rate was measured by determining the interval required to collect a specific quantity of saliva, and relating this to the weight of the gland. Gland weight was determined after the experiment by rapid weighing on a torsion balance. [Na] and [K] were determined by IL flame photometer. Amylase of saliva was determined using the method of Myers, Free, and Rosinski (7), and expressed as milligrams reducing substance formed per milligram of saliva. After glands were weighed, they were preserved in Bouin's for subsequent histological examination, or at −15° for subsequent amylase assay. Amylase assay was made on buffered saline homogenates of gland aliquots. In other experiments cyclocytidine was administered daily for 3 days. Twenty-four hours after the last injection of cyclocytidine, animals were anesthetized with Nembutal and salivary glands and heart ventricles were removed. Organs were preserved for histological examination or for DNA and RNA content. For determination of nucleic acids, organs were homogenized at 0–4° with 0.4 *N* $HClO_4$, and then centrifuged. The supernatant fluid was discarded. The precipitate was then dispersed, washed three times with cold $HClO_4$, and then hydrolyzed at 90° for 15 min in 0.4 *N* $HClO_4$. Total nucleic acids were then determined by measurement of optical density at 260 nm (8). Total DNA

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was determined using the diphenylamine reaction (9), and estimates of total RNA were obtained by subtracting DNA from total nucleic acids. In the histological examination cell size was estimated by counting of nuclei per area consisting only of acinar cells; for each determination, 10 areas per slide were counted.

Results. Intraperitoneal administration of cyclocytidine, in doses ranging from 300–600 mg/kg, caused secretion from submaxillary and parotid glands of rat. Flow was initiated within 3 min after injection of the agent and persisted at a very slow but constant rate (usually between 0.003–0.008 mg/min × mg of gland) for at least 3 and usually 7–8 hr. Flow of submaxillary saliva was somewhat greater than parotid flow rate (0.008 as compared with 0.006 mg/min × mg) (Table I). Mean value (and SE) for K concentration of submaxillary saliva was 121 ± 6 (eight rats), and that of parotid was 42 ± 3 (five rats); mean Na^+ was, respectively, 35 ± 4 and 110 ± 4 meq/liter (Table I), and concentrations of these ions did not change significantly throughout a 7-hr period of collection. Amylase activity of parotid saliva was about 800 mg/mg of saliva, and was not appreciably decreased during the first hour of secretion, but within the next hour, fell to levels of 400 mg/mg. This decrease in saliva levels was a reflection of the concurrent depletion of gland stores of amylase caused by the cyclocytidine stimulation. After 1 hr of stimulation, there was a 65% reduction in gland level of amylase; by 3 hr, stores were decreased by 95% (Fig. 1).

TABLE I. COMPOSITION OF SALIVARY SECRETIONS EVOKED BY CYCLOCYTIDINE STIMULATION.[a]

	Submaxillary (8)[b]	Parotid (5)[b]
Na (meq/liter)	35 ± 3.8	110 ± 4.4
K (meq/liter)	121 ± 5.6	42 ± 2.9
Flow rate (mg/min × mg)	.008 ± .002	.006 ± .001
Amylase activity (mg/mg)[c]		805 ± 32.7

[a] Values are means ± SE; secretions collected during first hour of stimulation.

[b] Number in parentheses indicates number of rats. In three additional rats, a superior cervical ganglion was unilaterally removed just prior to ip injection of cyclocytidine; the Na, K concentrations of the submaxillary saliva were 32 ± 3 and 120 ± 3 meq/liter, respectively; the amylase concentrations of the parotid saliva from the innervated glands was 1183 ± 454 and that from the denervated mates was 1180 ± 458.

[c] Amylase activity is expressed as mg reducing substance per mg saliva formed in 15 min at 37°.

The high K in the gland secretions, reaching 120 meq/liter in the case of submaxillary saliva, and the high amylase in parotid saliva were reminiscent of the action of the β adrenergic agent, isoproterenol (10). Isoproterenol has a further distinctive effect related to its β adrenergic action, i.e., its ability to induce enlargement of both salivary glands and heart when administered chronically (10–12). Accordingly, as a further test of the β adrenergic action of cyclocytidine, the cyclocytidine was administered chronically. After 3 days of daily administration of cyclocytidine, parotid and submaxillary glands and heart were appreciably enlarged. Submaxillary gland showed a 28% increase in weight, while parotid weight was increased by 100%. Total RNA of the parotid was also doubled, but total DNA was increased by only 12% (Table II). Weight of the heart ventricles was 28% greater, and total RNA 45% greater than ventricular weight and RNA of untreated rats but total DNA was not significantly increased (Table II). An increase in size of cells of salivary glands was also evident from histological examination. The increase in size of acinar cells of parotid was especially marked, and total number of nuclei/area was reduced from 17 ± 0.5 in parotid of untreated rats to 10 ± 0.4 in cyclocytidine-injected rats. The enlargement of heart and salivary gland induced by the cyclocytidine was similar in magnitude to that caused by isoproterenol (10). Thus, these effects as well as the secretory effects implicate β adrenergic receptors as the site of cyclocytidine action. To demonstrate that the action of cyclocytidine does involve β receptors, the β blocking agent propranolol was administered prior to injection of the cyclocytidine. Under these conditions, there was no secretion from either submaxillary

TABLE II. EFFECTS OF CHRONIC ADMINISTRATION OF CYCLOCYTIDINE ON WEIGHT, TOTAL DNA AND RNA OF RAT PAROTID GLAND AND HEART.[a]

No. of days on Cyclo-C	No. nuclei per area[b]	Parotid Wt (mg)	Parotid DNA (μg/gland)	Parotid RNA (μg/gland)	Heart (ventricles) Wt (mg)	Heart DNA (μg/gland)	Heart RNA (μg/gland)	No. rats
0	17±.5	162± 7	743±24	1980±104	518±11	700±24	877±30	7
3Δ[c]	10±.4	343±17	828±15	3980±169	700±28	755±31*	1270±57	7

[a] Values are means ± SE; for each parameter, cyclo-C values are significantly different from controls ($P < 0.01$) except where indicated with asterisk.

[b] One slide per rat was used to count No. nuclei/area of acinar cells, and for each slide, 10 areas were counted: values, thus, represent means for all rats.

[c] Δ100 mg cyclo-C ip daily for 3 days.

or parotid for at least 90 min after administration of the cyclocytidine, and amylase activity of parotid gland was very little reduced (Fig. 1). However, a flow of saliva was again evident after 90 min, and within 2 hr after cyclocytidine, amylase activity of the propranolol-treated parotid was reduced by 15%; by 3 hr the reduction amounted to 40%. Thus, effective inhibition by propranolol of cyclocytidine-induced secretory activity persisted for only the first 90 min. It was, therefore, necessary to determine why the β blockade did not persist beyond this time.

Since the propranolol was administered as a single ip injection, there was the possibility that after 90 min, β receptor blockade was no longer complete or at least was inadequate. Therefore, the amount of propranolol administered was increased, first by increasing the amount of the single dose (to 1 and 4 mg) and second, by continuing to administer the smaller (0.5 mg) doses throughout, at 30-min intervals. Neither of these expedients changed the course of events and within 90 min after cyclocytidine administration, secretion was again evident (Table III).

This suggested that not only β adrenergic receptors but others in addition were stimulated by the action of cyclocytidine. However, the characteristics of the secretory response strongly argue against any prominent role for other receptor sites during the first 90 min of cyclocytidine-induced secretion (13, 14). Furthermore, cyclocytidine-induced secretion cannot be abolished by

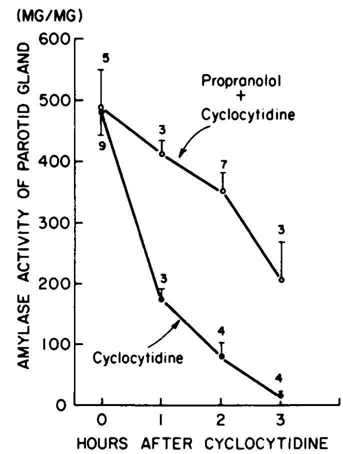

FIG. 1. Amylase concentration of rat parotid gland after administration of propranolol and cyclocytidine or of cyclocytidine alone. Propranolol was administered ip, 5 mg/kg, 25 min prior to injection of cyclocytidine, 600 mg/kg; amylase activity of parotid gland expressed as concentration of reducing substance formed per mg of wet weight of tissue in 15 min at 37°. Values are means ± SE and propranolol–cyclocytidine values differ significantly from cyclocytidine values ($P < 0.01$).

prior administration of either atropine or dibenamine, according to Hirayama *et al.* (5) and present data. It thus became clear that effects of cyclocytidine involving other receptor sites were initially absent or masked by the more prominent effect of cyclocytidine involving β receptor sites. Accordingly, atropine and propranolol were administered in close succession prior to injection of cyclocytidine; secretion was, as with propranolol alone, blocked for 90 min; again,

TABLE III. Duration of Cyclocytidine-Induced Salivary Secretion and Modification by Autonomic Antagonists.

Agents injected	Amount of antagonist per rat	Presence of salivary secretion at intervals after administration of cyclocytidine (Cyclo-C) (min)[a] 30	60	90	120	240
Cyclo-C	0	+	+	+	+	+
ATR + Cyclo-C	0.4 mg	+	+	+	+	+
DI + Cyclo-C	2 mg	+	+	+	+	+
IN + Cyclo-C	1 mg	–	–	–	+	+
	4 mg	–	–	–	+	+
	0.5 mg, each 30 min*	–	–	–	+	+
ATR + IN + Cyclo-C	0.4, 1 mg resp.	–	–	–	+	+
DI + IN + Cyclo-C	2, 1 mg resp.	–	–	–	–	–

[a] Presence of salivary flow indicated by +; absence of flow by –. Except where indicated with asterisk, cyclocytidine and antagonists were administered in a single dose; in each case, a dose of 120 mg of Cyclo-C was injected ip 20–25 min after ip administration of the blocking agents: IN = propranolol, DI = dibenzyline, and ATR = atropine.

as with propranolol alone, it resumed after 90 min. When dibenzyline and propranolol were administered in close succession prior to cyclocytidine injection, the secretion was also blocked for the first 90 min. However, in this case, it was also blocked thereafter, suggesting that some α adrenergic receptors were also stimulated by the cyclocytidine action, but that, at least for the first 90 min, this effect was minor. The effects of the antagonists on the course of secretion induced by cyclocytidine are summarized in Table III.

Finally, the β adrenergic effects of cyclocytidine on salivary glands are probably not mediated through indirect central or ganglionic actions. Thus, unilateral removal of the superior cervical ganglion immediately prior to injection of cyclocytidine did not in any discernible way prevent or even modify the secretory responses induced by the cyclocytidine. The data in Table I show that flow rate, Na, K, and amylase concentration of cyclocytidine-evoked saliva from innervated glands were generally indistinguishable from the values for saliva elicited from the acutely sympathectomized glands of the same animal. The cyclocytidine effects were thus not mediated through a central or ganglionic action (13). However, cyclocytidine could exert an indirect sialagogic effect by causing release of norepinephrine from the still intact nerve endings of the acutely sympathectomized gland (13). Accordingly, cyclocytidine was also administered 2 wk after removal of the superior cervical ganglion when the adrenergic nerves degenerated (14). Cyclocytidine still elicited secretion from the denervated as well as the innervated gland, indicating that the effects of cyclocytidine are, at least in part, not mediated indirectly through an action on the postganglionic nerve endings.

Discussion. A sialogogue action of cyclocytidine, first reported by Hirayama *et al.* (5), has been confirmed. The present work shows that this action of cyclocytidine depends on stimulation of β adrenergic receptors of the salivary glands. This conclusion is based on the characteristics of the cyclocytidine-evoked saliva and on the fact that the β blocking agent, propranolol, can prevent the sialagogic effect of cyclocytidine. The marked enlargement of the glands and heart ventricles is also consistent with a β adrenergic action (15).

The characteristics of the saliva and the effects on gland levels of amylase have previously been shown to be useful indicators of the kind of autonomic receptors stimulated (14). Thus, stimulation of cholinergic receptors results in elaboration of a watery saliva of high flow rate, and with levels of K and amylase distinctly less than those evoked with adrenergic stimulation. In fact, the viscous saliva of low flow rate evoked when β adrenoceptors are stimulated is much higher in amylase and K concentrations than that evoked by stimulation of α adrenoceptors (14). Emptying of gland amylase in response to autonomic stimulation shows the same pattern: very marked depletion with β stimulation, less marked with α, and least marked with stimulation of cholinergic receptors (15). The cyclocytidine-evoked saliva has the same high concentrations of amylase (800 mg/mg) and K (120–150 meq/liter) exhibited by isoproterenol-evoked secretion, is similarly viscous, and of similarly low flow rate; in addition, cyclocytidine, like isoproterenol, causes a virtual depletion of gland amylase within 2–3 hr (14, 15).

While these data demonstrate the similarities between β adrenergic effects and those caused by cyclocytidine, the experiments using autonomic antagonists firmly establish that cyclocytidine produces its effects principally by an action involving β adrenergic receptors. Thus, the secretory effects (salivary flow and emptying of gland amylase) were completely prevented for at least the first 90 min after cyclocytidine administration if β receptors were blocked by propranolol, but during this same time interval, neither α-blockade nor cholinergic-blockade inhibited the secretory effects. Blockade of the secretion after 90 min was obtained only when both α and β adrenergic antagonists were present. Thus, while cholinergic receptors are not involved at all in cyclocytidine-induced effects, the fact that cyclocytidine effects can be permanently blocked when α and β antagonists are present raises the question of the role of α receptors, especially in the later phases of the secretory response. Thus far, however, all of the evidence points to a predominant effect at β adrenergic receptors and only a minor effect at the α receptors. Stimulation at α adrenergic receptors does not appear to be involved in the organ enlargement caused by cyclocytidine since chronic stimulation of α receptors usually results in a decrease in gland size (16). On the other hand, the enlargement of salivary glands and heart caused by cyclocytidine closely mimics the organ hypertrophy caused by the β agonist isoproterenol; since the isoproterenol-induced hypertrophy can be prevented if β receptors are blocked by propranolol, the inference is that cyclocytidine causes organ enlargement by acting at β adrenergic receptors (14). However, this remains to be established. Whatever the mechanism, the data show that, in rat, cardiac and salivary gland enlargement are produced with chronic administration of doses of cyclocytidine directly comparable to those used therapeutically in man for treatment of leukemia. The usefulness of this agent in tumor chemotherapy would be increased if these undesirable side effects as well as others (sialorrhea (5), parotid pain, and postural hypotension (6) were eliminated. Since the evidence presented indicates that the sialorrhea (and parotid pain stemming from it) results principally from an action of cyclocytidine involving β adrenergic receptors, and that a similar action probably accounts for the organ enlargement, it appears probable that judicious use of the β adrenergic antagonist in combination with cyclocytidine would eliminate (or at least alleviate) the undesirable side actions attributable to the β adrenergic effects. This is entirely feasible since the β adrenergic properties of cyclocytidine appear to be distinct from its chemotherapeutic properties. Ara-C to which cyclocytidine is converted and from which the chemotherapeutic potency is principally derived (3, 4) has no sialagogic effects. This is important since cyclocytidine is potentially a more useful drug than Ara-C for treatment of leukemia. It, unlike Ara-C, is long lasting, and can be administered orally (4), instead of by continuous iv infusion.

Summary. In addition to its potent antileukemic properties, cyclocytidine has a sialogogue action that depends on stimulation of β adrenergic ereceptors of salivary glands. Furthermore, when chronically administered (for 3 days), cyclocytidine caused enlargement of parotid and submaxillary glands and heart that resembled the hypertrophy caused by chronic isoproterenol administration. The salivas evoked by cyclocytidine also closely resembled those evoked by isoproterenol, and were extremely viscous, and high in K^+, (121 ± 5.6, for submaxillary, and 42 ± 2.9, for parotid), low in flow rate (0.007 mg/min × mg) and parotid saliva

contained high concentrations of amylase (805 ± 33 mg/mg gland). Cyclocytidine also caused marked emptying of parotid gland amylase. The cyclocytidine-induced salivary flow and gland emptying of amylase were prevented for 90 min when propranolol (but not dibenzyline or atropine) was administered prior to injection of the cyclocytidine. In addition, when the superior cervical ganglion was acutely removed, administration of cyclocytidine elicited salivary flow from the denervated as well as the innervated glands. These findings suggest that cyclocytidine does not affect salivary glands through indirect central or ganglionic actions. Cyclocytidine action does not exclusively involve β receptors, since even in the presence of propranolol, secretory flow was evident after 90 min but when dibenzyline was given with the propranolol, complete blockade of cyclocytidine-stimulated saliva was effected. The dominant effect is, however, a β adrenergic one. The undesirable side effects of cyclocytidine (parotid pain, postural hypotension, and cardiac hypertrophy) probably stem chiefly from its β adrenergic properties and might be eliminated (or at least modified) by administration of propranolol with the cyclocytidine.

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