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Best, John Adam

Quinton, P M

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Salivary secretion assay for drug efficacy for cystic fibrosis in mice

John Adam Best[1] and P. M. Quinton[1,2]

[1]*Department of Pediatrics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0831, USA*
[2]*Division of Biomedical Sciences, University of California Riverside, Riverside, CA 92521-0121*

Computerized assays on cultured cells *ex vivo* have been used to screen thousands of compounds for their effectiveness in correcting the basic physiological defect in cystic fibrosis (CF). While a number of these compounds appear promising, their effectiveness will almost certainly need to be demonstrated in animals before therapeutic tests in humans will be possible. We show herein that the function of salivary secretion in the mouse model for CF could be used as a simple, easy and rapid *in vivo* assay for drug effects. We demonstrate that salivary secretory capacity stimulated with a β-adrenergic agonist closely reflects the genotype of origin. Specifically, the mean maximal secretory rate of saliva in normal wild type (+/+) mice was about 1.5 times higher than that of the mean rate in heterozygote (+/−) mice and more than 50 times greater than in CF (−/−) mice. Total saliva secreted per stimulated period obeyed a similar phenotype–genotype segregation. The data indicate that salivary secretory rates in CF mice could be used to assay potential drugs for their effectiveness in correcting the secretory defect in cystic fibrosis.

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Corresponding author P. M. Quinton: University of California, San Diego, Department of Pediatrics, 9500 Gilman Drive, La Jolla, CA 92093-0831, USA. Email: pquinton@ucsd.edu

With the recent development of computer-automated technology, it is now possible to test thousands of different compounds for physiological effects on cultured cell functions in relatively short periods of time (Verkman, 2004). Unfortunately, the effect in culture is often at variance with, or limited by, its effects *in vivo* in animals. Thus, before most drugs can be tested in humans, safety and efficacy must be established in animals first. Moreover, given the number of compounds flagged in mass screening, a simple, reliable assay for efficacy and safety in an animal model should be of significant benefit in furthering drug development. A number of compounds have already been identified (Singh *et al.* 2002; Caci *et al.* 2003; Xiao *et al.* 2003) that appear to affect the function of cystic fibrosis transmembrane conductance regulator (CFTR) in cultured cell systems, indicating that an efficient assay in animals would be of significant use at present.

We based this model assay on the fact that in humans with cystic fibrosis (CF), the response of most, if not all, exocrine gland secretion is refractory to β-adrenergic stimulation (Sato & Sato, 1984; Quinton, 1990; Johnson *et al.* 1991). In general, mutations of CFTR that cause disease, when expressed in cells *in vivo* or *in vitro,* do not respond normally to cAMP-mediated activation. More specifically, human eccrine sweat glands normally respond to both β-adrenergic and cholinergic stimulation (Sato, 1977), but these glands in CF patients fail to respond to stimulation with isoprenaline, a β-adrenergic agonist (Sato & Sato, 1984). Submucosal glands of human airways show the same pattern of CF and normal responses (Joo *et al.* 2002). Mice, however, do not have thermoregulatory sweat glands like humans. Instead, they lick saliva onto their fur to provide evaporative cooling (Quinton, 1979). Herein we show that the salivary gland responses of the CF and wild type (WT) mouse respond in exactly the same way as human sweat glands, so that the restoration of this β-adrenergic-dependent function could be used as an assay of the ability of potential drugs to restore CFTR function *in vivo*. That is, a drug that increases the salivary secretory response to β-adrenergic stimulation in mutant mouse models of CF should be more likely to qualify as therapeutic.

Methods

Animals

A colony of knockout *cftr*m1UNC mice was established by mating animals heterozygous for the CFTR gene

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disruption (+/−) (The Jackson Laboratory, Bar Harbour, ME, USA) to produce WT (+/+) and CF knockout (−/−) mice. All animals were housed and handled as approved by the UCSD Animal Care Program Internal Review Board. Because intestinal blockage is the most common cause of death in CF knockout mice, all mice were maintained on an osmotic laxative, an electrolyte solution containing polyethylene glycol 3350 (GoLYTELY; Braintree Laboratories, Inc., Braintree, MA, USA) administered *ad libitum* in the drinking water (Clarke *et al.* 1996). Mice were studied between the ages of 10 and 12 weeks. Six male WT (+/+) mice with an average weight of 24.9 ± 2.4 g (mean ± S.D.) and five female WT (+/+) mice with an average weight of 22.5 ± 5.2 g were used as controls. Seven male heterozygote (+/−) mice with an average weight of 25.2 ± 2.0 g and seven female heterozygote (+/−) mice with an average weight of 22.4 ± 1.3 g were used as carriers. Eight homozygous knockout (−/−) male mice with an average weight of 21.6 ± 1.4 g and four female heterozygote (+/−) mice with an average weight of 17.6 ± 2.4 g were used as animal models for CF. The genotypes of mice were determined by polymerase chain reaction (PCR) for the CF gene from tail clippings of pups at weaning. Clippings were digested with lysis buffer and proteinase K (Qiagen DNeasy Kit; Qiagen Inc., Valencia, CA, USA) at 55°C overnight. DNA was extracted using the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen Inc.). The WT genotype was reflected by a single band of 526 bp from CFTR, heterozygous by two bands of 526 and 357 bp from the neomycin insert, and the homozygous knockout by a single band at 357 bp as visualized under ultraviolet light when run on a 1.5% agarose gel for 45 min at 150 V.

Mice were anaesthetized with a combination of ketamine and diazepam. A stock solution of ketamine and diazepam was prepared to contain 0.5 ml of 5 mg ml^{-1} diazepam and 0.5 ml of 100 mg ml^{-1} ketamine in 4.0 ml sterile saline (Abbott Laboratories, Chicago, IL; Fort Dodge Animal Health, Fort Dodge, IA). Anaesthesia was induced by intraperitoneal injection of the stock solution (100 μl per 10 g body weight) through a 0.2 μm filter. Usually mice became quiescent within 5 min of injection. After anaesthesia, each animal was maintained on a flat surface in a supine orientation with adhesive tape. The mouth was held open by cephalic retraction of the dorsal front teeth with a small rubber band. An indirect heat lamp warmed the animals during the saliva collection period. Animals tolerated the protocol well and were returned to the vivarium after the procedure.

Solutions

Solutions of atropine (10^{-3} M) with and without isoprenaline (10^{-4} M) and with and without acetylcholine (ACh; 10^{-4} M) were prepared in standard Ringer solution immediately preceding each protocol. Appropriate doses were determined from preliminary trials and previous data from rats (Martinez & Camden, 1983).

Salivary stimulation

Salivary secretion can normally be stimulated by cholinergic or adrenergic mechanisms. Stimulating with a β-adrenergic agonist alone can, however, cross-stimulate cholinergic receptors (Sato & Sato, 1984). We avoided this complication by pretreating mice with a subcutaneous injection of the cholinergic antagonist atropine (50 μl at 10^{-3} M) into the left cheek. A 2 × 25 mm piece of Whatman ashless filter paper was then inserted inside the previously injected cheek and left in place for approximately 4 min in order to absorb any saliva secreted after the injection of atropine. This first piece of filter paper was removed and replaced with a second. If, under visual inspection, the second piece of paper appeared dry, 100 μl of a combination of isoprenaline (10^{-4} M) and atropine (10^{-3} M) was injected subcutaneously into the left cheek of each mouse at the site of the prior injection of atropine. Isoprenaline was used as a selective, potent β-adrenergic agonist. The time of the isoprenaline injection was taken as time zero, and the time in minutes from this point was recorded as each sample was collected.

Measurements

Prior to each experiment, a piece of filter paper 2 × 25 mm was marked at 7 mm from one end and placed in a small, hermetically capped plastic vial. The total weight of the vial with paper was measured to the nearest 0.00001 g using a Metzler AE 250 balance. Following the subcutaneous injection of isoprenaline, a series of filter papers were inserted sequentially into the cavity of the left cheek of each mouse. Each filter paper was inserted toward the dorsal left corner of the cheek until the 7 mm mark was level with the two ventral incisors. The distance of 7 mm was determined by postmortem dissection of test mice to be the approximate length of the inner cheek for maximum insertion of the filter paper. Each piece of filter paper was left in the mouth for 3 min or until it became visibly wet up to the 7 mm mark. When each piece of filter paper was removed, the time of removal was recorded, and it was immediately placed and sealed in its preweighed vial. Another fresh piece of filter paper was immediately inserted into the mouth at the same location. Each collection period was recorded to the nearest tenth of a minute. This process was repeated for approximately 30 min or, rarely, until the mouse started to awaken, whichever occurred first.

After all samples had been collected, each vial was re-measured and the weights of all samples were recorded. The difference in total weight of vial plus paper measured before and after collecting saliva was taken as the net

Table 1. Mean maximum secretory rates and mean total weights of stimulated saliva from two genotypes of mice

Drug	Maximum rate (μg g^{-1} min^{-1})	Total secretion (μg g^{-1})
Acetylcholine		
Wild type ($n = 3$)	50.0 ± 22.1	0.36 ± 0.15
Knockout ($n = 4$)	69.8 ± 31.5	0.45 ± 0.13
Atropine + ACh		
Wild type ($n = 3$)	1.94 ± 1.09	0.022 ± 0.02

Wild type (+/+) and CFTR knockout (−/−) mice stimulated by subcutaneous injection of acetylcholine (ACh) alone did not respond significantly differently to cholinergic stimulation ($P > 0.37$). Atropine almost completely blocked the ACh stimulation.

weight of saliva secreted during the collection period. The secretory rate was calculated as the weight of saliva divided by the number of minutes required for each collection and then normalized by dividing the result by the weight in grams of the mouse. The total amount of saliva secreted by each mouse was calculated as the sum of the weights of all saliva samples collected during the sequential collection periods.

Statistics

Student's paired *t* test was used to determine statistical significance. A probability value of $P < 0.05$ was taken as significantly different.

Results

We first tested the secretory capacity of salivary secretion in (−/−) mice by stimulating the salivary glands of (−/−), (−/+) and (+/+) mice with acetylcholine alone. The mean maximum rate and mean total secretion from four −/− mice were 137 and 122%, respectively, of wild type ($n = 3$; Table 1). There was no significant difference in the secretory rates or in total saliva secreted between CF and WT mice. Then, to be sure of blocking any cross-stimulation of cholinergic secretion with isoprenaline, we tested the effectiveness of atropine to block secretion stimulated by ACh in WT mice. After pretreatment with atropine and in the presence of atropine, ACh-stimulated salivary secretory rates fell almost to zero (Table 1). However, when stimulated with isoprenaline in the presence of the atropine block, the mean peak saliva secretion rates (Fig. 1) in male heterozygote (+/−; $n = 7$) and knockout (−/−; $n = 8$) mice were 70.0 and 1.5% of WT values (i.e. 148.7 μg (g body weight)$^{-1}$ min^{-1}; $n = 6$). Correspondingly, the average weight of total saliva secreted per injection (Fig. 2) was 56.9 and 1.8%, respectively, of WT (1.69 mg (g body weight)$^{-1}$). In female heterozygote (+/−; $n = 7$) and knockout (−/−; $n = 4$) mice, the average maximum salivary rates (Fig. 1) were 78.9 and 4.5% of WT (86.6 μg (g body weight)$^{-1}$ min^{-1}), respectively. The average weights of total secretions from female(+/−) and (−/−) mice were 49.3 and 5.3% of WT (1.69 mg g^{-1}), respectively (Fig. 2). The average peak secretion rates for both sexes combined were 110.5 ± 51.0, 82.4 ± 32.8 and 2.67 ± 0.98 μg g^{-1} min^{-1} for WT, heterozygote and knockout mice, respectively. Most significantly, there was no overlap of any values from CF mice with those of either WT or heterozygote genotypes of either sex.

Discussion

One of the most characteristic defects in cystic fibrosis is a failure of many exocrine tissues to respond to β-adrenergic stimulation. The failure was first reported in sweat glands (Sato & Sato, 1984, 1988; Johnson *et al.* 1991) but has been observed since in all tissues affected in CF (Quinton, 1990; Steagall *et al.* 1998; McPherson *et al.* 2001). The defect occurs because β-adrenergically stimulated secretion in these cases is dependent upon the anion channel, CFTR, which is absent or dysfunctional in the disease. The most common genetic mutations in the CFTR gene result in a protein product that fails to be processed normally to its proper place in the cell membrane or which, if processed, fails to conduct anions normally (Quinton, 1999). A major objective in developing a therapy for cystic fibrosis is to restore pharmacologically at least some capacity to process CFTR and/or increase its function in cell membranes. In the past few years, both academic and industrial efforts have screened thousands of potential drugs for their effects on the function or processing of CFTR. These screening procedures generally assay drug effects on special cultured cells *ex vivo*. Positive effects,

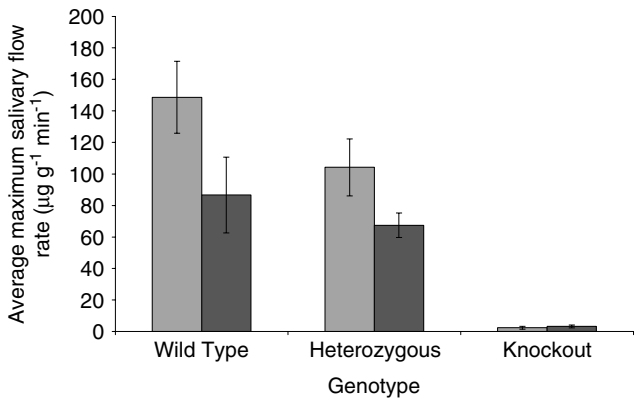

Figure 1. Mean secretory rates of male (left columns) and female mice (right columns) stimulated with isoprenaline
Male (+/+) and (+/−) mice secreted faster in general than their female cohorts, but there was no significant difference in maximum secretory rates between sex-matched (+/+) and (+/−) genotypes ($P > 0.0035$ males, $P > 0.15$ females). The CF (−/−) knockout mice of either sex had very low response ($P < 0.000002$) compared to either sex of either genotype. Error bars represent S.D.

or 'hits', indicate that a compound may have promise as a therapeutic compound. Unfortunately, these tests are still far removed from possible effects, both beneficial and deleterious, in humans. Needless to say, any 'hits' that might be applied to humans generally must undergo extensive animal testing first. To date rapid, convenient and accurate animal assays are largely lacking. To that end, we undertook the task of determining whether a simple, benign test of salivary secretion might be used to assay corrective effects of potential hit compounds. We show here that the physiological response to β-adrenergic stimulation of salivary glands in mice should be an excellent assay for *in vivo* drug efficacy. The idea is based on much earlier, preliminary data suggesting that the response of salivary secretion in CF mice to isoprenaline is poor or missing (Bergler *et al.* 1994).

Presently, the only animal model available for drug testing in cystic fibrosis is the mouse. Current assays of efficacy are time consuming and/or complicated. Some investigators have used changes in survival, life-span, weight gain (Egan *et al.* 2004), fibrosis in the lung (after 2–3 months only in the BL/6 strain of mice), and/or correction of faecal bile salt loss and goblet cell hyperplasia in ileal crypts as assays of efficacy. Another approach has been to determine the ability of treated mice to survive on a solid diet without developing distal intestine obstruction syndrome (DIOS). These approaches (H. R. de Jonge, personal communication) suffer from the requirments for relatively long periods for breeding and survival, numerous animals and kill of the animal. A more rapid, but also more complicated assay entails measurement of the nasal potential difference (Grubb, 2002). These approaches may be useful, but they may not yield information sufficiently quantitative to indicate the degree of efficacy; i.e. the extent to which the drug restores normal phenotype.

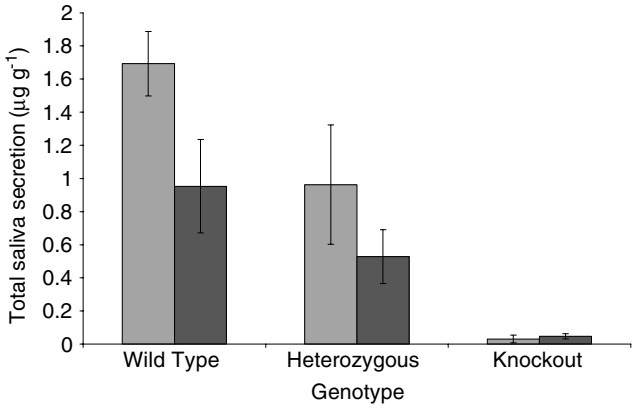

Figure 2. Mean total saliva secreted by male (left columns) and female mice (right columns) stimulated with isoprenaline
Male (+/+) mice secreted significantly larger total amounts of saliva during the collection period than did their male (+/−) cohorts ($P < 0.001$). Likewise, female (+/+) mice secreted significantly more than their female (+/−) cohorts ($P < 0.026$). There was no significant difference between female (+/+) and male (+/−) mice ($P > 0.95$). The CF (−/−) knockout mouse had virtually no response in either sex and was significantly lower than either sex of the other genotypes, with no overlapping values ($P < 0.000001$). Error bars represent S.D.

There are two possible pitfalls in the assays presented herein. First, the lack of β-adrenergic response might be due to the lack of a developed, functional salivary secretory apparatus in the CF mouse. However, the ACh-stimulated mean salivary secretion rate in CF (−/−) mice was actually higher, but not significantly higher, than the mean rate in WT mice (Table 1). These data show that the cholinergically activated apparatus for salivary secretion is not only present, but also apparently functions normally in (−/−) mice. Thus, differences in responses to β-adrenergically stimulated secretion cannot be due to a non-specific failure of the CF salivary gland to secrete.

Second, since salivary and other exocrine glands generally respond to both β-adrenergically and cholinergically mediated stimulation, and since the cholinergic component is competent in CF mice, potential cross-talk and inadvertent stimulation of cholinergic receptors must be avoided. In preliminary data from a similar study (Grubb & Boucher, 1999), salivary secretory rates for WT and knockout mice were compared after a single intraperitoneal injection of isoprenaline. In contrast to the results reported here, those results indicated that even though the salivary rates after β-adrenergic stimulation in WT mice were about twice those of knockout mice, there was significant overlap. Superficially, these previous results suggest that salivary rates exhibit too much overlap to be used as an end-point in a rigorous assay for corrector or potentiator drug efficacy. No doubt, the elevated secretion in knockout mice and consequent overlap with WT rates was due to the fact that cross-stimulation of cholinergic innervation was not blocked during the β-adrenergic challenge. Table 1 shows that pretreatment and concomitant stimulation in the presence of atropine inhibited 99% of the cholinergic stimulation and therefore any possible cross-talk, avoiding this pitfall (Martinez & Cassity, 1982; Bergler *et al.* 1994).

The assay as presented herein has several immediately apparent benefits and advantages, as follows. (1) The assay is non-invasive. No surgical intervention is required. (2) The measurement is quantitative. The degree of correction should be related to the magnitude of the secretory response and therefore quantifiable. Note that heterozygote values are less than WT. (3) The animal survives. The assay does not require killing, so it can be used for multiple assays or to provide an intra-animal control. (4) The assay is simple. No instrumentation other than a precision gravimetric balance is required. (5) The assay is rapid. In general, pertinent data can be obtained in less than 30 min (6) Multiple simultaneous assays should be

practical. Multiple animals could be tested in the same time frame.

In conclusion, it seems likely that the salivary secretory response of any (−/−) mouse (presumably, any mutation of CFTR incorporated into the animal could be tested) to β-adrenergic stimulation could be used as an *in vivo* assay for potential therapeutic effects of corrector or potentiator drugs administered systemically. To wit, (+/+) mice should secrete more than uncorrected (+/−) mice, and both (+/+) and (+/−) mice should secrete significantly more than uncorrected (−/−) mice. The degree of correction effected by a given drug should be correlated with the degree to which it restores the salivary secretion function of CF genotype to normal when subjected to selective β-adrenergic stimulation. The assay has numerous advantages over present approaches, including simplicity, rapidity and repeatability in the same animal as well as among cohorts.

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