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BRIEF COMMUNICATION

A Potential Animal Model for Studying CF Heterozygote Advantage: Genetic Variation in Theophylline-Inducible Colonic Chloride Currents among Inbred Strains of Mice

We have used Ussing chambers to measure chloride secretion by colonic segments (mucosa, muscularis, and serosa) from various inbred strains of mice. We found lower theophylline-induced Cl^- secretion in the DBA/2J than in the C57BL/6J strain. Their F_1 showed significantly higher levels of Cl^- secretion than did the C57BL/6J parental strain while colonic segments from five recombinant inbred B × D lines ranged between the C57BL/6J and F_1 values. No major component of the variation appeared to be associated with alleles of the *met* oncogene region of chromosome 6 or the *H-2* region of chromsome 17.

Cystic fibrosis is an autosomally inherited recessive disease impairing the secretory ability of the epithelia of the pancreas, the bronchi, and the sweat gland secretory coil, as well as reabsorption into the sweat ducts. It is known to affect the regulation of apical chloride channels (1–3); the defective gene has been mapped to the 7q31 region of chromosome 7 (4) and has recently been cloned (5). Research into this illness has been slowed by the absence of animal models.

The physiology of the mouse colonic epithelium can be studied by measuring the short circuit current (Isc) in an Ussing chamber using a voltage clamp. Putative chloride secretion can be identified by two methods: by blockade (6) using bumetanide [3-(aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid] or induction by maneuvers which are expected to elevate cellular cAMP (2). The loop diuretic, bumetanide, blocks the basolateral Na^+, K^+, 2 Cl^- cotransporter which is responsible for Cl^- entry into the cells. Elevating cytosolic cAMP is thought to activate apical Cl^- channels and promote Cl^- exit from the cell to the lumenal side. In this study, both methods were used to identify chloride secretory components of Isc. The defect associated with cystic fibrosis affects chloride channel gating in response to elevations of cAMP (1–3).

The use of recombinant inbred (RI) mouse strains for genetic studies has been well established (7). Once a theophylline [3,7-dihydro-1,3-dimethyl-1*H*-purine-2,6-dione]-inducible chloride secretion variation had been identified for two such strains, the genetics of the variation in RI strains was then examined to determine if there are genetic correlations with chloride secretion. The *met* oncogene is

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known to map near the cystic fibrosis region in man (8), and has a homologous region on mouse chromosome 6 (9). Using the gene as a marker, we determined if a major genetic component of chloride secretion is linked to this region. In addition, we searched for any possible *H-2* component of the theophylline-induced Cl^- secretion since *H-2* influences several components of the cAMP response (10,11).

MATERIALS AND METHODS

Mice used in this study were obtained from the Jackson Laboratories (Bar Harbor, ME) or bred from such stocks. The mice were maintained in a light-controlled room until sacrifice by cervical dislocation.

The excised colon was placed at 0°C in mouse Ringer's solution (120 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM KH_2PO_4, 0.6 mM KH_2PO_4, 2 mM KCl, 1.2 mM $CaCl_2$, and 10 mM glucose), where it was flushed of its contents and the attached mesentery removed. The fragility of the epithelium did not permit the removal of the muscle. Colon segments were mounted in a 0.5-cm^2 gas lift Ussing chamber attached to an Iowa Bioelectronics voltage clamp. The circulating Ringer's solution was buffered with 95% O_2 and 5% CO_2 and maintained by a water jacket at 37°C.

For the bumetanide treatments, each segment was treated with 100 mM amiloride on the mucosal side 9 min after mounting to block electrogenic Na^+ absorption, followed by 100 mM bumetanide on the serosal side 15 min after mounting. The response to bumetanide was measured as a percentage decrease in the short-circuit current.

In the theophylline treatments, 10 mM theophylline was added to both the serosal and mucosal sides 9 min after mounting, sometimes followed by 100 mM bumetanide on the serosal side 15 min after mounting (to assess viability). The response to theophylline was recorded as the absolute increase in short-circuit current.

To determine if a significant genetic component of chloride secretion variation was linked to *met,* Southern analyses were performed (12): DNA from five DBA/2J × C57BL/6J recombinant inbred lines (lines 2, 12, 18, 23, and 25) were digested with *Eco*RI, as an RFLP between DBA/2J and C57BL/6J had previously been identified for this enzyme (13). The *met* oncogene probe, met G, was obtained from Michael Dean (14) and the insert was labeled with Amersham's multiprime oligolabeled kit (Amersham, Arlington Heights, IL). Blots were hybridized overnight in Collaborative Research hybridization solution (10% dextran sulfate, 30% formamide, 1 M NaCl, 0.5 M Tris, 25 mM EDTA, 2.5 mg/ml sonicated salmon sperm DNA) at 50°C. Final washes were at 65°C in 2 × SSC, 0.1% SDS.

RESULTS

We initially searched for quantitative variations in bumetanide inhibitable spontaneous Isc. To establish optimum experimental conditions and eliminate possible variables, a number of parameters were examined. Proximal, middle, and distal segments from the same animal were mounted from 1-year-old females to determine which segment gave the best response. Middle and distal segments were mounted 40, 70, and 100 min after sacrifice to determine if a decrease in response

TABLE 1
Theophylline-Induced Isc in Inbred Mouse Strains

Strain	Sex & age	*N*	Isc μA/0.5 cm^2[a]	*met* typing[b]	*H-2* typing[c]
DBA/2J	Males + females, 2–4 months	14	17.20 ± 3.72[d]	DBA	d
C57BL/6J	Males + females, 2–4 months	18	32.84 ± 3.60	BL/6	b
F_1 (B×D)	Males, 2 months	10	58.30 ± 9.42	Heterozygous	b/d
B×D2	Females, 2 months	4	43.00 ± 4.46	BL/6	b
B×D12	Females, 2 months	4	39.25 ± 7.41	DBA	d
B×D18	Females, 2 months	5	40.60 ± 5.53	DBA	d
B×D23	Females, 2 months	4	32.50 ± 4.29	DBA	b
B×D25	Females, 2 months	4	38.00 ± 10.01	BL/6	d

[a] Means ± standard error of the theophylline-induced transport expressed as the absolute increase in Isc for 0.5 cm^2 of mouse colon.

[b] DBA = Those like the DBA/2J strain for the *met* oncogene; BL/6 = those like the C57BL/6J strain for the *met* oncogene.

[c] *H-2*^{b} or *H-2*^{d} from Ref. (26).

[d] Includes four readings of zero on segments shown to be viable (see text).

could be detected. The results from these preliminary studies indicated that middle and distal colon segments should be mounted no later than 45 min after sacrifice (data not shown). Groups of mice representing five different inbred strains were also tested with some age and sex contrasts. No significant age difference was found in C57BL/6J females and no sex differences were found in the C57BL/6J and DBA/2J strains (date not shown). There was a suggestion of differences between some strains with C57BL/6J showing higher, and DBA/2J showing lower, bumetanide inhibitable currents. Since temporal drift in the conditions affecting the apparatus were a probable cause of slight apparent differences, mice from the different strains were alternated in the apparatus within a short time period and no significant differences were found with C57BL/6J alternating with DBA/2J and alternating separately with BALB/6J—all with 6-week-old males (data not shown).

We then studied theophylline-induced Isc among inbred strains of mice. The absolute increase in Isc induced by 10 mM theophylline in colons from DBA/2J varied significantly ($P < 0.005$) from the response in C57BL/6J (Table 1). Colon segments from four of the DBA/2J mice had zero theophylline response but responded strongly to bumetanide demonstrating tissue viability (not shown). Segments from two DBA/2J colons that had not responded to 10 mM theophylline responded to 40 mM theophylline with a mean of 21.5 μA. To ensure that chloride secretion was being measured, mouse Ringer's was replaced with a Ringer's low in chloride and the colons from a DBA/2J animal and one from a C57BL/6J were treated with theophylline as described previously. No induction of Isc was observed with the low chloride Ringer's.

In order to further define the genetics of the chloride secretion variable, F_1 animals from a DBA/2 and C57BL/6J cross were also studied. The theophylline-

induced current for these animals was significantly larger than either of the parental strains (Table 1). The *P* values equaled 0.0002 for the comparison to the DBA/2J strain and 0.0049 from the comparison to the C57BL/6J strain. To determine if a significant genetic component of the chloride transport variation segregated and/or was linked to the *met* oncogene on chromosome 6, the chloride transport of recombinant inbred lines created from the matings of DBA/2J with C57BL/6J was examined. First each RI line was typed as either DBA/2J-like or C57BL/6J-like with respect to the *met* oncogene (Table 1). Next the theophylline-induced Isc for the C57BL/6J-like RI line (for *met*) was compared to that for the DBA/2J-like RI lines. The RI lines showed theophylline-induced Isc ranging between the C57BL/6J and F_1 levels, and no correlation between typing for *met* and chloride secretion was observed. Finally, the values of theophylline-induced Isc for the *H-2*^{b} RI lines were contrasted with those for the *H-2*^{d} RI lines (Table 1). The *H-2*^{b} lines had a somewhat lower level of inducible Cl^- transport (37.75 ± 3.48 compared to 39.38 ± 4.00 for the *H-2*^{d} lines). Although the probability of this result by chance is less than 0.05 (t = 2.52), we do not consider this a significant difference given the numbers of statistical tests performed (corrected required significance = 0.05/10 tests).

DISCUSSION

Currently, the basic defect in CF is believed to affect regulation of apical chloride channels. The sweat gland coil has been shown to have a diminished secretory response to β-adrenergic agonists (15) and defective β-adrenergic regulation of chloride ion permeability has been shown in cultured cells from sweat glands (16). The defect in β-adrenergic responsiveness has been documented in other tissues such as submandibular glands (17) and the chloride transport defect has even been demonstrated in cultured skin fibroblasts (18). The predicted protein for the cloned gene has many properties expected for an ion channel (19). It is now generally believed that homozygotes for the CF gene are defective in regulation of Cl^- transport at a site distal to cAMP-dependent protein kinase (reviewed in Ref. (20)).

The CF defect in Cl^- transport has been documented in the gut: ileum (21), jejunum (22), colon (21), and rectum (23) where normal cAMP-content and cAMP-dependent kinase (among other cAMP-binding proteins) were found. It has been hypothesized that such a defect might be advantageous in the presence of diarrhea-producing bacterial toxins (24) thus explaining the high frequency of the CF gene. The genetic variation in theophylline-inducible CF transport which we have found among inbred strains may be of the order to be expected between ΔF508 heterozygotes (ΔF508 is the most common mutant and is correlated with variability in sweat electrolyte levels (25)) and normal homozygotes. As such, it may be useful for studies on heterozygote selection.

The significantly higher theophylline-induced Cl^- secretion found in the C57BL/6J × DBA/2J F_1 colons is possibly an example of overdominance ("hybrid vigor"). However, inasmuch as the five RI lines studied do not show segregation of theophylline-induced chloride secretion to parental strain values but have values which range from those of the high strain to the higher value of the F_1 mice,

multiple loci must affect colonic chloride secretion. No major gene effect on the quantitative variation in theophylline-induced colonic chloride secretion which we studied appears to segregate with the *met* locus on chromosome 6 or the *H-2* locus on chromosome 17.

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