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Effects of Age and Ambient Temperature on the Response of Infant Mice to Heat-Stable Enterotoxin of *Escherichia coli*: Assay Modifications

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The response of infant mice to heat-stable enterotoxin from *Escherichia coli* was affected by the age of the mice (2, 4, 6, and 8 days) and by the ambient temperature (25, 30, and 37°C) after exposure to the enterotoxin. The younger mice and/or mice held at lower temperatures tended to accumulate intestinal fluid (high gut weight/body weight ratios), but older mice and/or mice held at higher temperatures tended to respond with diarrhea and had low gut weight/body weight ratios. The standard infant mouse assay for heat-stable *E. coli* enterotoxin can be simplified, without loss of sensitivity or reliability, by holding the mice at 37°C after exposure and using diarrhea as the index of response. Diarrhea can be detected easily by incorporating dye in the inocula and (at the end of the assay) checking for dye mixed with feces on the rear quarters of the mice or on a sheet of white paper placed under them during incubation.

In the standard infant mouse assay for heat-stable *Escherichia coli* enterotoxin (ST), mice 1 to 4 days old are given ST intragastrically, held at an ambient temperature of 28°C for 4 h, killed, and necropsied (1). The ratio of the weight of the intestinal tract and its contents to the weight of the remaining body is determined; accumulations of intestinal fluid resulting in ratios of 0.07 to 0.09 are considered questionably positive and those over 0.09, strongly positive.

When mice were held at 37°C after ST exposure, assay results were either negative or the ratio was markedly reduced (2). In our laboratory, the standard assay was also usually negative if the mice were more than 4 days old when exposed to ST. However, mice held at 37°C after exposure to ST, or held at 30°C but exposed when more than 4 days old, developed diarrhea (3). The objectives of the study reported here were: (i) to compare intestinal accumulation of fluid and diarrhea, as indexes of the response of infant mice to ST; (ii) to determine the effects of age (from 2 to 8 days) and ambient temperature (from 25 to 37°C) on these indexes; and (iii) to see whether the infant mouse assay for ST can be made simpler, more sensitive, and more reliable than the standard assay by using older mice, higher temperatures, and diarrhea as the index of response.

MATERIALS AND METHODS

Mice. Pregnant CF1 mice were obtained from ARS Sprague Dawley, Madison, Wis. Newborn mice obtained from them were exposed to ST or control material when 2, 4, 6, or 8 days old. Each mouse was given 0.1 ml of ST or control material via stomach tube (5). These inocula contained Evans blue dye (0.01%). After exposure, mice were weighed and placed in egg incubators (Favorite Incubators, model 624-E; Leahy Mfg. Co., Higginsville, Mo.) at 25°C and 78% relative humidity, 30°C and 69% relative humidity, or 37°C and 66% relative humidity. The incubators contained wooden boxes divided into compartments (7 cm square and 5 cm deep). The floor of each compartment was covered with white filter paper. Incubator fans ran constantly, and mice were held (four to a compartment) in the incubator until they were killed, weighed, and necropsied and the ratios of gut weight to body weight were determined. Unless stated otherwise, all mice were incubated for 4 h after exposure. At the end of the incubation period, the paper at the bottom of compartments containing mice with diarrhea was stained with a mixture of dye and feces. The amount of staining was assessed visually to give a diarrhea score. Diarrhea scores varied from 0 to 4, with 0 meaning no staining of the paper (no diarrhea), 1 meaning only a single small stained spot (minimal diarrhea probably affecting one mouse), and four meaning extensive staining over most of the paper (profuse diarrhea, probably all four mice).

Enterotoxins and negative control materials. The enterotoxins and negative control materials were cell-free supernatant fluids from Trypticase soy broth cultures produced and stored as described previously (3), except that sodium azide was not added. Most experiments were conducted with material produced by *E. coli* 2176E8, which was originally isolated from a pig and which produces ST but no heat-labile enterotoxin. The negative control preparation for most of

the work was from the non-enterotoxigenic pig *E. coli* strain 123. Unless stated otherwise, these preparations were given as 1:4 dilutions in 0.01 M tris-(hydroxymethyl)aminomethane buffer, pH 8.0. This dose was chosen because the strain 2176E8 preparation gave strong positive, but not maximal, responses in the standard ST assay at this dilution. Other strains tested as cell-free supernatant culture fluids are described in Table 1.

RESULTS

Effects of mouse age. When 2-, 4-, 6-, and 8-day-old mice (12 mice per age group) were held at 30°C ambient temperature for 4 h postexposure to the negative control preparation, none developed diarrhea. Gut weight/body weight ratios were below 0.070, mean weight loss was less than 1% of total body weight, and there were no apparent differences in gut weight/body weight ratios or percent weight loss among the four age groups. The 2-day-old mice given ST accumulated intestinal fluid (gut weight/body weight ratios >0.09), but did not develop diarrhea, nor did they lose more weight than controls. In contrast, 8-day-old mice given ST developed diarrhea and lost more weight than controls, but did not accumulate intestinal fluid (gut weight/body weight ratios <0.070). The responses of 4- and 6-day-old mice were intermediate between those of 2- and 8-day-old mice.

In additional experiments when mice were held at 30°C after ST exposure, 8-day-old mice had diarrhea and weight loss (>control values) at 2 h (the earliest time tested). Diarrhea and weight loss also occurred in 2-day-old mice but not until 5 to 6 h after ST exposure.

Effects of temperature. None of the mice in any of the four age groups that were given the negative control preparation and then held at 25, 30, or 37°C developed diarrhea nor had positive gut weight/body weight ratios by 4 h postexposure. Furthermore, mice at different ages and ambient temperatures all had similar gut weight/body weight ratios (age and temperature group means ranged from 0.051 to 0.065, standard deviation 0.003) and lost a similar percentage of their body weight (age and temperature group means ranged from 0.43 to 1.11%, standard deviation of 0.02). More than 100 2-day-old mice were given this negative control preparation and held at 37°C for 4 h, and none excreted dye-stained feces. However, 1 of 24 8-day-old mice so treated did excrete dye-stained feces.

Mice from each of the four age groups also responded to ST when held at 25°C or at 37°C for 4 h postexposure. However, the nature of the response was affected by temperature (Fig. 1). In general, 25°C tended to enhance the accumulation of intestinal fluid while decreasing diarrhea score and weight loss. In contrast, 37°C tended to decrease fluid accumulation and to enhance diarrhea score and weight loss. At 37°C, the 2-day-old mice responded with diarrhea and weight loss but had negative gut weight/body weight ratios. At 25°C, the 6- and 8-day-old mice accumulated enough intestinal fluid to have (respectively) strongly positive and questionable gut weight/body weight ratios.

Sensitivity to ST. The sensitivity of mice to ST from strain 2176E8 was approximately equal at 2 and 8 days of age and at either 30 or 37°C. For example, when serial twofold dilutions of ST were tested, the highest dilution causing diar-

TABLE 1. *Sensitivity of 2-day-old mice held at 30 or 37°C for 4 h postexposure to culture fluids from enterotoxigenic and non-enterotoxigenic E. coli*

E. coli strain			30°C		37°C	
Source	Toxin type	Strain no.	Toxin titer[a]	No. died/no. tested[b]	Toxin titer[c]	No. died/no. tested
Pig	ST	431	1:16	2/4	1:32	2/4
Pig	ST	987	1:16	1/4	1:16	2/4
Calf	ST	B44	1:16	3/4	1:16	4/4
Human	ST & LT[d]	H10407	U[e]	0/4	1:4	0/4
Pig	ST & LT	72-2502	1:8	1/4	1:16	2/4
Chicken	ST & LT	F11 (P155)[f]	1:64	4/4	1:64	4/4
Chicken	None	F11	0	0/4	0	0/4
Pig	LT	263	0	0/12	0	0/12
Pig	None	123	0	0/12	0	0/12
Pig	None	124	0	0/4	0	0/4
Pig	None	252	0	0/4	0	0/4

[a] Highest dilution giving gut weight/body weight ratio ≥0.070.
[b] Mice given undiluted culture fluids.
[c] Highest dilution resulting in diarrhea score ≥1.0.
[d] LT, Heat-labile enterotoxin.
[e] Undiluted was positive, 1:4 was negative, 1:2 was not tested.
[f] Strain F11 which had received an LT and ST plasmid from pig strain P155.

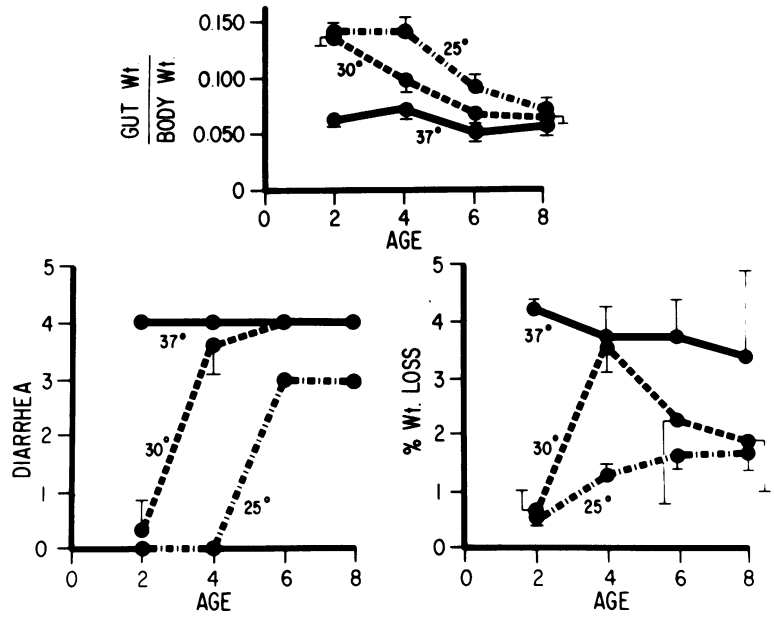

FIG. 1. *Responses of 2-, 4-, 6-, or 8-day-old mice held at ambient temperatures of 25, 30, or 37°C for 4 h after exposure to ST produced by E. coli. Diarrhea scores were based on the amount of feces accumulated on white papers placed under the mice after exposure. Mean of 12 mice per data point, ±1 standard deviation.*

rhea in 8-day-old mice was 1:8 at both temperatures and 1:16 in 2-day-old 37°C mice, and the highest dilution causing fluid accumulation in 2-day-old 30°C mice was 1:8. Two-day-old mice held at 37°C also responded to culture fluids from several other ST-producing *E. coli* strains with diarrhea rather than by accumulating intestinal fluids (Table 1). In each case they were at least as sensitive at 37°C as at 30°C. Holding at 37°C did not make the mice respond to heat-labile enterotoxin produced by strain 263, nor to fluids from cultures of non-enterotoxigenic strains (Table 1). The possibilities that minimal doses of ST might induce some fluid accumulation prior to the onset of diarrhea (or instead of diarrhea) in 8-day-old mice or in 2-day-old mice held at 37°C, and that this might be used to increase the sensitivity of the assay, were considered. However, at the highest dilutions of ST causing diarrhea (Table 1) and at all dilutions beyond, gut weight/body weight ratios were consistently <0.07.

Mice given undiluted preparations from ST-producing strains frequently died during the 4-h exposure period. This occurred both at 30°C and at 37°C (Table 1), and among both 8- and 2-day-old mice. Some mice that died had diarrhea, some had abdomens distended with accumulated intestinal fluid, and some had both, but an occasional dead mouse (<10%) had none of these conditions. In contrast, none of the mice given undiluted preparations from the non-enterotoxigenic strains or from the heat-labile enterotoxin-producing strain 263 died.

DISCUSSION

The transit of dye in the small intestine of normal mice (not exposed to ST) from 2 to 8 days old accelerated with age and ambient temperature (25 to 37°C) (3). This accelerated transit probably explains why younger mice and/or mice held at lower temperatures tended to respond to ST by accumulating intestinal fluids, whereas older mice and/or those held at higher temperatures tended to respond with diarrhea. Alteration of the response from fluid accumulation to diarrhea with increasing age and temperature is one reason why some mice more than 4 days old or held at 37°C give negative responses in the standard ST assay.

The standard infant mouse assay for ST can be simplified by holding the mice at 37°C, or by using 6- to 8-day-old mice, or both, if diarrhea score rather than gut weight/body weight is used as the index of response. This eliminates the necropsies, weighing, and calculations, and reduces the age restrictions required in the standard assay. Diarrhea scores of 1 or 2 in 2-day-old 37°C mice appear to be equivalent to questionable positives in the standard assay, whereas death with fluid accumulation, death with diarrhea scores ≥1, or diarrhea scores ≥3 without

death appear to be equivalent to strong positive responses in the standard assay. In routine assays we inoculate three mice (2-day-old, 37°C) per sample. Mice inoculated with negative and positive control preparations are included in each assay. Samples that cause death as well as either diarrhea or abdominal distention with accumulated intestinal fluid are recorded as positive. Samples that do not cause death but cause sufficient diarrhea to leave four or more spots of dye-stained feces on the filter paper are also recorded as positive. Samples that do not cause death but result in one to three spots of dye-stained feces on the paper are recorded as questionable. The percentage of body weight loss could also be used as a semiquantitative measure of response. In that case, diarrhea with ≥2% weight loss would be positive and diarrhea with <2% weight loss would be questionable.

We use 2-day-old mice because, under these conditions, 1 of 24 8-day-old mice did excrete dye-stained feces. Secondly, some of the data (Table 1, and titration of strain 2176E8 as above) were interpreted to indicate that mice are marginally more sensitive to ST at 2 days and 37°C than at other ages and/or temperatures. However, 8-day-old mice are easier to inoculate by stomach tube than 2-day-old mice. If the assay is confined to 8-day-old mice, the incubation period should be shortened to 2 h or less, and/or some index of diarrhea other than excretion of dye-stained feces should be used, because the feces of an occasional 8-day-old mouse will probably contain dye by 4 h postinoculation even without exposure to ST.

The simplified procedure suggested is at least as reliable as the standard infant mouse assay for ST, and may be more reliable, assuming that in the standard assay some 4-day-old mice develop diarrhea, and that mice more than 4 days old are occasionally included by oversight. The standard infant mouse assay for ST can be simplified, without loss of sensitivity or reliability, by holding the mice at 37°C after exposure and using diarrhea as the index of response.

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