Vascular Permeability Activity of *Escherichia coli* Heat-Stable Enterotoxin

JOHN P. CRAIG,^{1*} KOICHIRO YAMAMOTO,² TAE TAKEDA,² YOSHIFUMI TAKEDA,² AND TOSHIO MIWATANI²

Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203,¹ and Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka 565, Japan²

Received 26 January 1981/Accepted 24 April 1981

Purified heat-stable enterotoxin (ST) prepared from supernatants of *Escherichia coli* strain 53402 A-1, isolated from a patient with diarrhea, caused an increase in the permeability of the small blood vessels of the skin of adult rabbits after intracutaneous injection. Increased permeability was manifested by localized accumulation of intravenously injected blue dye at the injection sites. Permeability factor (PF) activity reached a peak 1 h after injection, and recovery of normal permeability was nearly complete in 3 h. Residual PF activity had disappeared by 24 h. Dose-response curves demonstrated a straight-line relationship between the logarithm of the dose and mean blueing diameter over a range of 3 to 12 mm. The PF assay was less sensitive than the suckling mouse assay, and adult rabbits varied in their sensitivity to the PF effect. Five to 100 mouse units of ST were required to consistently evoke strong and unequivocal blue lesions of 7 mm or more in diameter. PF activity was reduced 50 to 70% by heating at 70°C for 30 min, 60 to 80% by boiling for 30 min, and 95 to 98% by autoclaving for 15 min, but it was unaffected by treatment with cholera antitoxin. These findings are consistent with the notion that PF activity is a property of the ST molecule itself. This PF assay cannot be used for the detection of ST in crude culture supernatant fluid because of low sensitivity and the presence of nonspecific PF effects of culture media and other *E. coli* products.

Heat-stable enterotoxin of enterotoxinogenic *Escherichia coli* (ST) has been extensively purified by several workers (1, 14, 15), and the physicochemical properties of the purified ST have been well characterized. In contrast to cholera enterotoxin and *E. coli* heat-labile enterotoxin (LT), which stimulate adenylate cyclase (7, 11), ST does not affect the activity of adenylate cyclase (9). Thus, assay methods used for cholera enterotoxin and *E. coli* LT, such as the Chinese hamster ovary cell (11) and Y-1 adrenal cell assays (6, 13), which are based principally on the intracellular accumulation of cyclic adenosine-3',5'-monophosphate, are not applicable to the assay of ST. Therefore, biological assay of ST has depended chiefly on the suckling mouse assay described by Dean et al. (5). However, since the suckling mouse assay is tedious and time-consuming, a search has been made to develop better methods for the accurate and reproducible measurement of ST.

Takeda et al. (15) have shown that one preparation of purified ST caused increased vascular permeability in guinea pig skin. Since the permeability factor (PF) test in rabbit skin has long

been used as a reliable method for the assay of cholera enterotoxin and *E. coli* LT (2, 3, 8), we have attempted to examine whether the rabbit PF test can be used for assaying ST activity.

MATERIALS AND METHODS

Preparation of purified ST. ST was purified essentially as described previously (15). *E. coli* 53402 A-1, a strain producing only ST, was isolated from a patient with diarrhea in Bangladesh. It was cultured in CAYE medium (12) without glucose at 37°C for 24 h with vigorous shaking. CAYE medium consisted of 2% Casamino Acids (Difco Laboratories, Detroit, Mich.)–0.6% yeast extract (Difco)–0.25% NaCl–0.871% K₂HPO₄–0.1% (vol/vol) trace salts solution (5% MgSO₄, 0.5% MnCl₂, 0.5% FeCl₃, and 0.001 N H₂SO₄). The pH of the medium was 8.6. Culture supernatants were collected by centrifugation, and protamine sulfate (50 mg/100 ml) was added in the cold. The resulting precipitate was discarded after centrifugation. The supernatant was filtered through an Amicon PM-10 membrane, the filtrate was concentrated in a rotary evaporator, and the concentrated filtrate was dialyzed against 0.01 M phosphate buffer (pH 7.0), using Spectra/por 6 membrane tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.), which retains molecules with molecular weights of >1,000. The dialyzed ma-

material was chromatographed successively on a diethylaminoethyl cellulose column, a hydroxyapatite column, and a Bio-Gel P-10 column as described previously (15). The material was then treated with 90% ethanol, as described previously (15), and is referred to as preparation I. Preparation I was further purified with preparative polyacrylamide gel disc electrophoresis, as described previously (15), and is referred to as preparation II.

Measurement of PF activity. The method for measurement of PF activity was essentially that described by Craig (2, 3) with minor modifications. Materials to be tested were diluted in borate-buffered saline of the following composition: H_3BO_3 , 0.05 M; NaCl, 0.12 M; gelatin, 0.1%; pH 7.5. Volumes, 0.1 ml, of serial threefold dilutions were injected intracutaneously on the clipped skin of the backs of adult albino rabbits, usually at randomized sites, except for the injections made for photography. Intravenous blue dye (Diphenyl Brilliant Blue FF Supra [Ciba-Geigy Corp., Greensboro, N.C.]), 1.2 ml of a 5% solution in 0.45% NaCl per kg, was given at various intervals after intracutaneous injection. For time-course studies, ST was injected at different times before the dye so that the skin was bearing lesions of different ages at the time that permeability to dye was examined. Readings of blue lesions were made 1 h after dye injection both by directly measuring the diameter and intensity of the blue areas and by removing the skin, spreading on glass, and observing the transilluminated lesions. The latter method proved to be more satisfactory and less influenced by variations in the depth of intracutaneous injection. Lesion diameters were plotted against the logarithm of the dilution of the test material, and toxic potency of each preparation was expressed as the number of 4-mm blueing doses (BD_4) per ml or per 1,000 mouse units. One BD_4 was defined as that amount of toxin which produced a mean lesion diameter of 4 mm under the conditions described above.

Assay of ST activity in suckling mice. ST activity in suckling mice was assayed as described previously (15). One mouse unit was defined as that amount of ST yielding a fluid accumulation ratio of 0.09 in suckling mice. Preparations I and II contained 147 and 400 mouse units per μg , respectively.

Cholera antitoxin. Provisional U.S. standard cholera antitoxin, NIH lot 001 (goat; 4,400 arbitrary units per ml) was kindly supplied by E. Seligmann, Bureau of Biologics, Food and Drug Administration, Rockville, Md. (4).

RESULTS AND DISCUSSION

Time-course studies examined PF activity from 30 min to 24 h after intracutaneous injection of ST, and the results are shown in Fig. 1. Measurable increases in permeability were observed between 0.5 and 3 h, with a sharp peak of activity at 1 h. Permeability was expressed in BD_4 per 1,000 mouse units. Figure 1 shows PF activity calculated in two ways: (i) that based on measurement of lesion diameter only; and (ii) that based on a blueing score, which takes into account the intensity of the blue color. Blueing score is an arbitrary unit of measurement which

is obtained by multiplying blueing diameter by the intensity of color, using an arbitrary scale of 1 to 8. One BD on the scale is the amount of PF yielding a score of 5. The area of increased vascular permeability peaked at 1 h, and the lesions were also relatively more intensely blue at that time. Recovery of normal permeability began between 1 and 2 h and was nearly complete at 3 h. Vascular integrity returned essentially to normal at 24 h, although some erythema persisted.

To determine dose response of ST permeability activity, graded doses were injected in triplicate on either side of a rabbit. Intravenous dye was given 1 h after the intracutaneous injections. One hour later the rabbit was killed by intravenous injection of sodium pentobarbital. The skin was removed and transilluminated on a light box. Figure 2 shows the appearance of the transilluminated skin.

The lesions were measured, and the dose response was plotted as shown in Fig. 3. The response was plotted in three ways: (i) blueing diameter, (ii) blueing intensity, and (iii) blueing score. The dose-response curve obtained by using diameter only was less steep than the curves based on intensity. Thus, lower doses of up to 5 mouse units (12.5 ng) produced pale lesions of increasing size, whereas 10 to 100 mouse units (25 to 250 ng) produced lesions of increasing intensity. In this experiment, 1 BD_4 was equal to about 0.6 mouse unit. This value varied from rabbit to rabbit from 0.5 to 10 mouse units. Although the BD_4 represented a very small and pale lesion, it was used as an endpoint because it is near the null point on the dose-response scale and because we have used it for a long time as a unit of measure of the PF activity of cholera enterotoxin and *E. coli* LT. In using this assay, it is clear that at least 10 BD_4 s (5 to 100 mouse units) must be injected to assure the observer that a specific PF effect has occurred. Responses evoked by lower doses could be confused with nonspecific traumatic lesions. Thus, it is clear that the PF assay for ST is considerably less sensitive and more variable than the suckling mouse assay.

The effect of heat on the PF of ST was studied by determining dose-response curves to graded doses of ST after heating at various temperatures. Dilutions were prepared after heating. Table 1 summarizes two such studies. In these experiments, sensitivity of the rabbits was somewhat less than in the time-course and dose-response studies shown in Fig. 1, 2, and 3, but the percentage of activity remaining could be compared with the internal untreated controls. About 65 to 75% of activity was destroyed by boiling for 30 min. These values were consistent

FIG. 1. Time course of increased cutaneous vascular permeability in rabbits produced as a result of injection of purified *E. coli* ST (preparation II). One BD_4 (○) is that amount of PF yielding a blueing diameter of 4 mm; one BD (×) is that amount of PF yielding a blueing score of 5.

FIG. 2. Appearance of lesions of increased cutaneous vascular permeability in transilluminated skin of a rabbit in response to intracutaneous injection of graded doses of purified *E. coli* ST (preparation II). Left to right: 100, 33, 11, 3.7, 1.2, and 0.4 mouse units; diluent control.

with the heat inactivation studies on the enterotoxigenic activity of ST in suckling mice (15). Somewhat greater inactivation occurred at 70°C when heating was carried out at the lower concentration of 2.5 µg/ml, as was reported previously (Table 1; 15).

The PF of ST was not affected by preincubation with an excessive amount of cholera anti-

toxin. Compared with the response to cholera enterotoxin, the sensitivity of rabbit skin to the PF of ST was very low. One BD_4 of purified cholera enterotoxin is about 10 to 30 pg (J. P. Craig, K. Yamamoto, Y. Takeda, and T. Miwatani, submitted for publication), whereas 1 BD_4 of purified ST was 2 to 25 ng. On a molar basis, this difference is even more striking.

FIG. 3. Dose response to intracutaneous injection of graded doses of purified *E. coli* ST (preparation II). Plot of lesions shown in Fig. 2.

TABLE 1. Effect of heat on 1-h PF activity of purified *E. coli* ST

Temp (°C) and duration (min) of heating	Prepn I ^a		Prepn II ^b	
	BD ₄ /1,000 MU ^c	% of activity	BD ₄ /1,000 MU	% of activity
Untreated	107	100	161	100
70, 30	50	47	47	29
100, 30	36	34	42	26
121, 15	3	3	8	5

^a Concentration during heat treatment: 12.5 µg/ml.

^b Concentration during heat treatment: 2.5 µg/ml.

^c MU, Mouse units.

Heat-stable enterotoxins active in the suckling mouse intestine have been purified and characterized by other investigators (1, 14). Their results indicate that several *E. coli* ST preparations, all peptides of various molecular weights, may possess biological activity. We have tested only one kind of preparation and have shown it to possess PF activity. It may be useful to find out whether other preparations, including both those which are active and those which are inactive in the suckling mouse assay, possess PF activity. Such studies might indicate which regions of the molecule(s) are associated with each biological activity.

Variability of sensitivity in rabbit skin made it necessary to use a rather wide dose range to assure satisfactory endpoints. In view of this low sensitivity, the early and transient response, and the early nonspecific PF effects of culture media and other *E. coli* products in undiluted filtrates, this assay cannot be used as a screening test for detection of ST in crude culture supernatants. However, it may be useful for monitoring purification and for immunological and pharmacological studies of the mode of action of *E. coli* ST. The chief advantage would be that in a

given experiment 50 to 100 quanta of data can be obtained from a single animal.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan, a grant from the Yamada Science Foundation, and Public Health Service research contract 1A19602 from the National Institute of Allergy and Infectious Diseases.

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