Effect of heat-labile enterotoxin(LT) produced by *Escherichia coli* on in vitro cell proliferation[1]

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Summary. Filtrates from *E. coli* H10407 cultures, giving a positive response for heat-labile enterotoxin(LT) in the Y-1 cell test, show an inhibitory activity both on ^{3}H-thymidine uptake by Ehrlich ascites cells and on granulocytic-macrophagic precursors (CFU-C) in murine bone marrow.

It is well known that enterotoxigenic strains of *E. coli* cause diarrheal disease in man and several animal species[3–5]. *E. coli* strains can produce 2 enterotoxins; a low-molecular weight heat-stable toxin (ST) and a heat-labile toxin (LT). Diarrheal disease results from production of heat-labile enterotoxin which acts through stimulation of membrane-bound adenylate cyclase in epithelial cells of the small intestine[6]. LT may be produced 'in vitro' by using different media to grow the bacteria but the results indicate a significant chemical and biological heterogeneity of most LT preparations[7,8].

In this paper we describe studies of 'in vitro' production in different media (Penassay Broth; RPMI-1640; Ham's F-10; M-199; and Leibovitz's L-15) and compare the effect exerted by some of these preparations on: a) ^{3}H-thymidine-uptake by Ehrlich ascites carcinoma (EAC) cells, b) the proliferation of granulocytic-macrophagic precursors (CFU-c) in murine bone marrow.

Materials and methods. Animals. BDF-1 female mice, 10–12 weeks old from Charles River, Italy (Calco-Como) were used as source of normal bone marrow cells for the CFU-c assay.

Bacterial strains. Heat-labile toxin (LT) was prepared from *E. coli* strain H-10407 (serotype O78:H11) kindly provided by Dr Dolores G. Evans (University of Texas Medical School, Houston, Texas, USA). This strain was isolated from a patient with acute diarrhea in Bangladesh and produces LT and heat-stable toxin (ST)[9]. The non-toxigenic strain *E. coli* K 12, from our laboratory, was used as a negative control.

Media. Bacteria were grown in the following media: Penassay Broth from Difco-USA, RPMI-1640, Ham's F-10, M-199 and Leibovitz's L-15 from Eurobio, France. The media containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) were prepared as suggested by Gilligan and Robertson[7] by adding glucose (to final concentration of 0.3%) L-methionine, L-lysine and glutamic acid (200 µg/ml for each) and $FeCl_3$ (0.005%). Penassay Broth alone did not receive any additional component.

Growth conditions and preparation of filtrates. Cultures were grown aerobically with shaking (120 rpm) in 500 ml Erlenmeyer flasks containing 80 ml of Penassay Broth or synthetic defined media. The inoculum was performed with 1 ml suspension of bacteria grown overnight in the corresponding media. After 8 h of incubation at 37 °C the pH was adjusted to 7.7–8.0 by adding 1 N NaOH. After 30 min the bacteria were removed by centrifugation in sterile conditions at 17,000 × g for 30 min (Servall refrigerated centrifuge) and the supernatant was passed through a 0.45 µm pore-size membrane filter (Millipore, USA). The filtrates were introduced into dialysis tubes and concentrated 5-fold by water extraction with polyethylene glycol (PEG 6000), dialyzed 24 h against 100 vol. of phosphate buffered saline, (PBS), filtered on 0.22 µm pore size and finally stored at −20 °C until assay.

Heat-labile enterotoxin assay. The Y-1 adrenal tumor cell assay suggested by Donta et al.[10] was used to determine the toxic activity of filtrates containing LT. The adrenal cells were maintained in culture with Ham's F-10 medium supplemented with 15% horse serum (Gibco, USA) 10% foetal calf serum (Gibco, USA) and 50 µg/ml gentamicin. The assay was performed in triplicate in 24-well plastic dishes (Falcon 3008, USA). Each well contained 1 ml of medium and was seeded with 10^5 cells. After 2 days of growth at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, 0.05 ml of filtrate was added to each well. Control wells received complete F-10 medium or filtrate treated for 15 min at 98 °C.

Toxin activity was determined by estimation of the amount of rounding of cells at 24 h by using the following scoring system: 0 ≤ 5% rounded cells; 1 = between 5 and 25%; 2 = between 25 and 50%; 3 = between 50 and 75%; 4 = between 75 and 95%; 5 : ≥ 95%.

CFU-c assay. Anaesthetized mice were killed by cervical dislocation and the bone marrow cells, collected by flushing both femura using a syringe filled with Hank's balanced salt solution (HBSS), were washed 3 times with HBSS. The ability of bone marrow to form granulocytic-macrophagic colonies (CFU-c) was assayed in a soft-agar layer system as suggested by Bradley and Metcalf[11]. The standard colony stimulating factor (CSF) used to stimulate CFU-c was obtained from L-cell cultures maintained in our laboratory. Briefly, 10^5 nucleated viable cells from bone marrow were cultured in triplicate on 35 mm Petri dishes containing 1 ml of McCoy's-agar (0.3%) mixture supplemented with foetal calf serum 20%, colony stimulating factor 10% and 10% of filtrates from bacterial cultures. Control dishes did not receive filtrates (positive control) or CSF (negative control). After 7 days at 37 °C in a water-saturated atmosphere of 5% CO_2 in air, the cultures were scored for the count of colonies (more than 50 cells) by an inverted microscope at 25-fold magnification.

Table 1. Response of Y-1 cell line to filtrates from *Escherichia coli* cultured in different nutritional media

Culture medium	*E. coli* strain	Y-1 response*	Mean of response ± SEM
Penassay broth	None	000120010000 0	0.3
	K 12	011100010101 0	0.5
	H 10407	444334444434 4	3.7 ± 0.1
RPMI-1640	None	001000	0.2
	K 12	111000	0.5
	H 10407	231212	1.8 ± 0.3
Ham's F-10	None	000000	0.0
	K 12	000000	0.0
	H 10407	333334	3.2 ± 0.1
M-199	None	110000	0.3
	K 12	110000	0.3
	H 10407	321112	1.7 ± 0.3
Leibovitz's L-15	None	000000000	0.0
	K 12	110000000	0.2
	H 10407	441222232	2.5 ± 0.3

* Data are reported as estimation of the amount of rounded cells using the following scoring system: 0: ≤ 5% rounded cells; 1 = between 5 and 25%; 2 = between 25 and 50%; 3 = between 50 and 75%; 4 = between 75 and 95%; 5: ≥ 95%. See materials and methods.

^{3}H-thymidine-uptake. A half-diploid and half-tetraploid strain of Ehrlich ascites carcinoma[12] was used as source of cells for ^{3}H-thymidine-uptake. 10^5 viable cells were cultured in triplicate in microtiter wells with minimum essential medium (MEM) + 10% foetal calf serum containing 10% of filtrates from cultured bacteria and ^{3}H-thymidine (0.5 µCi/well; Sp.act. = 5 Ci/mmole, Amersham, England). Controls did not receive filtrates. After 24 h the cells were harvested by collection on glass-wool dishes and ^{3}H-thymidine-uptake was determined in a Packard scintillation counter as cpm.

Statistical analysis. The statistical significance of the differences between the experimental and control groups was determined by Student's t-test. p-Values exceeding 0.05 were considered not to be significant.

Results. As shown on table 1, filtrates of *E. coli* H 10407 cultures induce a cytotoxic response in Y-1 cell assay. However, a different degree of this toxic effect was expressed by various filtrates obtained by growing the same bacterial strain in different synthetic media.

The maximum effect was obtained with filtrates from Ham's F-10 and Leibovitz's L-15 culture media; smaller effects were obtained by using RPMI-1640 and M-199 as culture media for *E. coli* H 10407. After treatment of filtrates by heating at 98 °C for 15 min the toxic effect disappeared (data are not reported in the table). A low level of aspecific toxic activity was detected with filtrates from *E. coli* K 12 cultures.

Table 2 shows the effect of filtrates from cultures of *E. coli* H 10407 strain on ^{3}H-thymidine-uptake by EAC cells compared with the effect exerted by filtrates from *E. coli* K 12 cultures.

The data indicate that filtrates from *E. coli* H 10407 cultures produce a significant ($p \leq 0.01$) reduction in ^{3}H-thymidine-uptake. The most significant ($p \leq 0.005$) inhibition is obtained with filtrates from bacteria cultured in Leibovitz's L-15 medium.

Table 3 reports the effect of filtrates from *E. coli* H 10407 cultures on CFU-c proliferation in normal murine bone marrow. The data show a significant ($p \leq 0.02$) reduction in CFU-c proliferation more drastic than the effect exerted by the same filtrates on ^{3}H-thymidine uptake by EAC cells.

No significant difference was observed among the filtrates obtained by growing bacteria in the different nutritional media.

Discussion. The data reported in this paper suggest that Penassy Broth represents the best growing medium for the production of LT by *E. coli* H 10407. The preparation of a synthetic medium involves a series of difficulties related to the fact that the optimal conditions for bacterial growth do not coincide with the optimal production of LT. In fact, critical conditions for LT production are the pH-value (must be alkaline) and the presence of glutamic acid, L-methionine, L-lysine, glucose and $FeCl_3$ as suggested by various authors[7,13].

In appropriate conditions we obtained positive responses in the Y-1 assay with all the synthetic media, and among them the best result was obtained with the filtrate from *E. coli* H 10407 grown in F-10 medium, followed by the filtrate obtained from cultures in L-15 medium (table 1).

For these reasons, we performed further evaluations of LT activities by testing these 2 filtrates (from F-10 and L-15 cultured bacteria) compared with filtrates obtained by growing the bacteria in Penassay broth. In effect, as reported in tables 2 and 3, the 3 filtrates do not differ in significant way in inhibiting activity, either of ^{3}H-thymidine uptake by EAC cells or of CFU-c proliferation in murine bone marrow.

The filtrates added to the bone marrow cells in the absence of standard CSF do not stimulate the proliferation of CFU-c (data are not reported because they were constantly 'zero'). In the presence of CSF, the filtrates caused a severe inhibition of CFU-c proliferation. This phenomenon may be regarded as a result of an aspecific cytotoxic effect exerted by filtrates on the granulocytic-macrophagic committed stem cells or as a result of competition between LT and CSF. In effect, the membrane receptors for LT are gangliosides of the GM_1 type, very similar or identical to the receptors for many glycoproteic hormones and for CSF[14,15].

Further studies to verify this hypothesis and to investigate the mechanisms related to these phenomena are in progress in our laboratories.

Table 2. Effect of filtrates from *E. coli* H 10407 on ^{3}H-thymidine uptake by Ehrlich ascites carcinoma cells*

Bacterial strain	Media of bacterial cultures		
	Penassay	F-10	L-15
E. coli K 12	100.0 ± 4.2	100.0 ± 4.2	100.0 ± 1.7
E. coli H 10407	77.5 ± 4.6	84.0 ± 4.8	75.0 ± 5.7

* Data are expressed as response percent referred to the number of cpm obtained by testing filtrates from *E. coli* K 12 cultured in the same nutritional media (mean of absolute number of cpm = 51059). Filtrates were tested at 10% final concentration. Each value represents the mean ± SEM of 4 experiments performed in triplicate.

Table 3. Effect of filtrates from *E. coli* H 10407 on CFU-C proliferation of murine bone marrow*

Bacterial strain	Media of bacterial cultures		
	Penassay	F-10	L-15
E. coli K 12	100.0 ± 8.07	100.0 ± 9.0	100.0 ± 9.4
E. coli H 10407	50.0 ± 5.8	44.0 ± 20.3	53.8 ± 4.08

* Data are expressed as response percent referred to the number of CFU-C obtained by testing filtrates from *E. coli* K 12 cultured in the same nutritional media (mean of absolute number of CFU-C = 19.4). Filtrates were tested at 10% final concentration in presence of colony stimulating factor. Each value represents the mean ± SEM of 4 experiments performed in triplicate.

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