

# Enterotoxin antibodies in relation to diarrhoea in Swedish soldiers in Cyprus

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*The development of antibodies to Escherichia coli heat-labile enterotoxin (LT) and its relationship to diarrhoea was studied in two Swedish battalions (one in the winter, WB, and one in the summer, SB) of the United Nations Peace-keeping Force in Cyprus. Whereas only 6-15% of the soldiers had detectable levels of anti-LT antibodies in their sera before leaving Sweden, as many as 69% of the SB and 43% of the WB soldiers who developed diarrhoea in Cyprus acquired significant titres of enterotoxin antibodies in their sera (acute phase serum). During the summer, many soldiers without diarrhoea also obtained anti-LT antibodies, whereas no such response was observed in healthy soldiers during the winter. Civilian Turkish Cypriots had positive sera in high frequency (60-66%) during both study periods. Parallel titration of positive sera against E. coli LT and cholera toxin indicated that the enterotoxin-neutralizing antibodies registered were induced by bacteria producing enterotoxin identical or closely related to LT.*

Recent studies have demonstrated that enterotoxinogenic *Escherichia coli* (ETEC), rather than the classical enteropathogens such as *Vibrio cholerae*, *Salmonella*, or *Shigella*, are responsible for acute diarrhoeal symptoms in North Americans visiting Mexico or Kenya (1). Whether this is true also for the diarrhoeas frequently occurring in North Europeans travelling to southern Europe has not been studied. It has been noticed, however, that most of the ETEC-induced diarrhoeas diagnosed in Sweden have been acquired during or immediately after a visit to southern countries (2).

To examine the exposure to enterotoxinogenic bacteria and its relationship to diarrhoea in travellers to the Mediterranean area, we conducted a prospective serological study in Swedish soldiers serving for a limited period in the United Nations Peace-keeping Force in Cyprus. The specific aims were to establish the frequency of sera with enterotoxin-neutralizing antibodies from civilian Cypriots, as an indicator of the occurrence of ETEC in the

native population, and to determine the development of such antibodies in the Swedish soldiers in the presence and absence of diarrhoeal symptoms.

## MATERIALS AND METHODS

### Outline of study

Two Swedish battalions of the United Nations Peace-keeping Force in Cyprus were studied. The soldiers were recruited from all parts of Sweden for a period of service of 6 months. The first battalion of 560 men (referred to as Winter Battalion, WB) was posted in October 1975 and was studied for the first 3 months in Cyprus. The second battalion of 450 men (Summer Battalion, SB) was studied during the fourth month of duty, in August 1976.

The main camp of the battalion was situated outside Famagusta on the east coast of the island, and the soldiers were on duty in the Famagusta district along the border between the Greek and Turkish sectors. Most of the food was imported and distributed by the British base in Cyprus. It was stored in freezers or refrigerators in the camp, prepared in a central kitchen, and either served in the messes or delivered to the observation posts. Fruit and vegetables were obtained from the local market and were disinfected in iodine before being

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served. The water used was chlorinated and was regularly tested for bacteria. When off duty, the soldiers usually ate in local restaurants or simple kebab houses.

Sera were collected from most individuals before leaving Sweden (pre-Cyprus sera), as well as on their return from Cyprus (post-Cyprus sera). All soldiers were informed about the investigation and instructed to report, as early as possible, any symptoms of diarrhoea. Diarrhoea was defined as two or more stools in one day, subjectively considered abnormally loose. A blood sample was taken from all soldiers with diarrhoea, usually within 24 h and always less than 2 days after the first loose stool (acute phase serum). Whenever possible serum was also collected from a "matched control", i.e., a soldier occupying an adjacent bed but without diarrhoea. A convalescent serum sample was taken 2 weeks later from the individuals in the diarrhoea group.

During each of the study periods, serum samples were also collected from civilian Turkish Cypriots representing different socioeconomic groups and geographical areas of the Famagusta district (personnel at the main battalion camp, outpatients at the surgical clinic of the Famagusta hospital, and 15-18-year-old pupils at the secondary school in Famagusta).

In close connexion with the serum sampling, stool specimens for microbiological examination were collected. The results of this study will be reported elsewhere (Bäck *et al.*, unpublished data).

### Serological tests

The sera were analysed for neutralizing antibodies against *E. coli* heat-labile enterotoxin (LT) by means of the YI adrenal cell system (3). The LT preparation used was kindly supplied by Dr R. Möllby, Stockholm, and was obtained by fermentor cultivation of *E. coli* 411-5 (originally supplied by Dr R. B. Sack) in a defined medium under controlled conditions (Landwall & Möllby, unpublished data). The culture supernatant was dialysed to remove medium components and concentrated 50 times by lyophilization and dialysis against polyethylene glycol. The LT preparation was used at a final dilution 1:160; this toxin concentration, as well as further dilutions varying from 1:2 to 1:8 on the various test occasions, produced typical rounding of  $\geq 75\%$  of the adrenal cells. The heat-inactivated sera were tested on two occasions at final dilutions of 1:2 and 1:4. When the results were discrepant a third test was

performed. Neutralization was defined as an inhibition of toxin activity resulting in less than 25% rounded cells. Sera neutralizing the toxin at a titre of 4 were further tested at least twice at a final dilution of 1:30.

Selected samples were titrated twice in parallel, by two-fold serial dilution against the *E. coli* LT preparation and against a crude enterotoxin preparation of *V. cholerae* (Cholera Toxin Lot 4493G, obtained from the National Institutes of Health, Bethesda, MD, USA; at least two such titrations were performed with each sample. Rabbit antisera against these toxins, obtained by repeated subcutaneous immunizations, were run for comparison.

### RESULTS

In the WB, 50 individuals (8.9%) developed diarrhoea during the study period of three months. In the SB, 29 cases of diarrhoea (6.4%) were detected during August, i.e., the incidence per month was about twice that in the WB. The diarrhoeal episodes were generally brief (<3 days) and mild, and in no case was parenteral fluid therapy required.

Of the WB soldiers with diarrhoea, 43% had detectable neutralizing antibodies against *E. coli* LT in the acute phase serum (Table 1). This frequency was significantly higher than that for the matched controls ( $P < 0.01$ ,  $\chi^2 = 8.61$ ). In the corresponding Cypriot control group as many as 66% of the sera were positive, i.e., significantly more than in both the Swedish matched controls ( $P < 0.001$ ) and in the group with diarrhoea ( $P < 0.02$ ). The proportion of sera with titres  $\geq 30$  was also higher for the Cypriots (17%) than for the Swedish soldiers (2%) (see Table 1).

In the SB, 69% of the soldiers with diarrhoea had antibodies neutralizing *E. coli* LT in the acute phase serum. This frequency is higher than was observed in the WB ( $P < 0.05$ ). In the matched control group, there was also a significantly higher proportion of positive sera (50%) in the SB than in the WB ( $P < 0.01$ ). Such seasonal variations were not found in civilian Cypriots, among whom the number of positive sera and their titre distribution during the summer did not differ from the winter study period (Table 1). The difference in frequency of positive sera between the diarrhoea and matched control groups in the SB was not significant, nor were the differences between any of these groups and the Cypriot civilians.

Table 1. Neutralizing antibodies against *E. coli* LT in Swedish soldiers (diarrhoea cases and matched controls) and Cypriot civilians

Battalion	Serum sample	No. of samples	No. of sera with anti-LT activity				
			Titre			Total	
			2	4-29	≥ 30	No.	%
Winter	Diarrhoea cases	47	9	10	1	20	43
	Matched controls	40	1	4	1	6	15
	Cypriot controls	103	33	17	18	68	66
Summer	Diarrhoea cases	29	8	10	2	20	69
	Matched controls	24	6	2	4	12	50
	Cypriot controls	25	5	4	6	15	60

Table 2. Development of anti-LT antibodies in relation to diarrhoea cases in Cyprus

Battalion	Serum sample	No. of samples	No. of sera with anti-LT activity				
			Titre			Total	
			2	4-29	≥ 30	No.	%
Winter	Pre-Cyprus	36	2	—	—	2	6
	Acute phase (0-2 days)	47	9	10	1	20	43
	Convalescence (12-14 days)	41	1	2	3	6	15
	Post-Cyprus	25	5	1	—	6	24
Summer	Pre-Cyprus	20	3	—	—	3	15
	Acute phase (0-2 days)	29	8	10	2	20	69
	Convalescence (12-14 days)	16	4	8	1	13	81
	Post-Cyprus	21	1	5	5	11	52

The occurrence of *E. coli* LT neutralizing antibodies in serum of the soldiers seems to reflect enterotoxin exposure in Cyprus. Thus, only a few of the soldiers who later experienced diarrhoea during the study period had anti-LT antibodies in their pre-Cyprus serum sample, and then in low titres (Table 2); the difference between pre-Cyprus and acute phase sera was highly significant ( $P < 0.001$ ) for both battalions. There was a striking difference between the WB and SB with regard to positive convalescence sera. In comparison with the acute phase samples, the frequency decreased in the WB

whereas it increased in the SB (Table 2). Nonetheless, in three instances in the WB, antibodies were demonstrated only in the convalescent samples. Such seroconversion was found in 2 SB cases, and in 1 other with a low titre in acute phase serum there was a more than 15-fold titre increase.

Post-Cyprus serum samples of the soldiers of the WB diarrhoea group were positive with about the same frequency as were the convalescent sera. In the SB, the proportion of positive post-Cyprus sera from the diarrhoea group had decreased slightly as compared with the convalescent samples (Table 2). On

their return to Sweden, more than twice as many of the SB soldiers had LT antibodies in serum as the WB soldiers.

To verify that the neutralizing antibodies were directed against *E. coli* LT and not against immunologically cross-reactive enterotoxin of *V. cholerae* (4, 5), selected sera were titrated in parallel against these toxins (Table 3). In similarity with rabbit antiserum against the *E. coli* LT used in this study, the sera of the soldiers as well as those of the Cypriot controls had, with one exception, a higher neutralizing capacity against LT than against cholera toxin. Rabbit antiserum to cholera toxin, on the other hand, had considerably higher titres against the homologous toxin than against LT (Table 3). These findings indicate that the enterotoxin-neutralizing antibodies registered in the human sera were induced by toxin identical or related to *E. coli* LT rather than by a more cholera-like enterotoxin.

#### DISCUSSION

This study shows that a high proportion of the Swedish soldiers who developed diarrhoea in Cyprus had significant levels of serum antibodies capable of neutralizing *E. coli* heat-labile enterotoxin (LT). During the summer period, many of their healthy comrades also produced detectable anti-LT antibodies, whereas there was no increase in antibodies in the absence of diarrhoea during the winter. For the WB, the correlation between the incidence of diarrhoea and the appearance of enterotoxin antibodies in serum is highly significant. For the SB, no such relationship was noted, due to the high frequency of positive sera in the matched control group.

In contrast to the soldiers, the Cypriot controls showed no seasonal variation in enterotoxin antibodies, with 60–65% of the population having positive sera during both study periods. This is considerably more than in healthy Swedes, since only 6–15% of the serum samples taken from the soldiers before leaving Sweden contained anti-LT antibodies, and then only in very low titres. This difference, together with the finding in a parallel study (Bäck *et al.*, unpublished data) that LT-producing bacteria could be isolated in 7% of faecal specimens taken from healthy Cypriots, but not from any of the more than 400 stools taken from the soldiers before leaving Sweden, indicates a higher risk of being exposed to ETEC in Cyprus than in Sweden. This is in agreement with a recent report that ETEC is isolated from less than 5% of individuals with acute diarrhoea in

Table 3. Comparison of neutralizing capacity against *E. coli* LT and cholera toxin (CT)

Sera	Titre against		Neutralizing capacity LT/CT <sup>c</sup>
	LT <sup>a</sup>	CT <sup>b</sup>	
Rabbit anti-LT	410 <sup>d</sup>	50	16.4
Rabbit-anti-CT	37	2050	0.04
Human diarrhoea cases			
Acute phase WB	10	11	2.0
Convalescence WB	24	12	4.0
Acute phase SB	20	16	2.8
Convalescence SB	30	16	3.8
Cypriot controls			
Winter I	20	8	5.0
II	38	19	4.0
Summer I	12	40	0.6
II	16	8	4.0

<sup>a</sup> The concentration of LT used was twice that minimally required for  $\geq 75\%$  rounded cells. For the Cypriot controls, sera I and II correspond to the acute phase and convalescence sera.

<sup>b</sup> The CT was used in the minimal effective concentration for  $\geq 75\%$  rounded cells.

<sup>c</sup> Titre per toxin unit ratio. Titres against LT have been multiplied by 2, since neutralization was performed with 2 toxin units.

<sup>d</sup> Titres were determined by interpolation from the results with serial 2-fold dilutions, as the serum dilution resulting in 25% rounded cells; the mean values of 2–3 experiments are given.

Sweden, and then usually from cases who have acquired the illness during travel in southern Europe or North Africa (2).

Few studies of the antibody response in man to *E. coli* LT have been undertaken. In individuals living in areas where exposure to ETEC is frequent, such as Bengal and Bangladesh (6, 7), high levels of neutralizing antibodies have been observed after ETEC-induced diarrhoea, usually with fourfold or greater titre increases between acute phase and convalescence. On the other hand, North Americans acquiring enterotoxin diarrhoea during travel in Mexico or Kenya have only rarely shown such titre increases (1). In the present study, although development of antitoxin antibodies was frequent in relation to diarrhoea, a titre rise between acute phase and convalescence was registered in only a few instances. An increase (although not significant) in frequency of positive sera from acute phase to convalescence,

from 69 to 81%, was noted for the SB, while for the WB there was a significant decrease. A possible explanation for this difference is that the WB soldiers were studied in relation to their first exposure to immunogenic amounts of enterotoxin, whereas during the summer and at a later stage of service many of the soldiers had probably been immunologically primed prior to their diarrhoeal episode. The higher frequency of positive sera among the healthy controls in the SB, as compared to the WB, is consistent with this hypothesis.

Although the development of enterotoxin-neutralizing antibodies could be related to diarrhoea in the WB soldiers, the isolation of enterotoxin-producing bacteria was far from regular (Bäck *et al.*, unpublished data). This complicates judgment of the

bacterial 'species' responsible for induction of the anti-enterotoxin antibody response, since *E. coli* LT cross-reacts immunologically with the enterotoxin produced by *V. cholerae*, and possibly also with enterotoxins from other bacteria (4, 5). The considerably higher titres found against the homologous than against the heterologous toxin for hyperimmune sera to *E. coli* LT and to cholera toxin, indicate the presence of strong type-specific determinants on the two toxins in addition to their shared determinants. In view of the higher neutralization titres against *E. coli* LT than against cholera toxin in the soldiers' sera, it appears that in Cyprus the exposure was to bacteria producing enterotoxin identical or related to LT rather than to organisms producing more cholera-like toxin.

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### RÉSUMÉ

#### ANTICORPS CONTRE L'ENTÉROTOXINE ET DIARRHÉE CHEZ LES SOLDATS SUÉDOIS A CHYPRE

La relation entre l'apparition dans le sérum d'anticorps contre l'entérottoxine thermolabile (LT) d'*Escherichia coli* et les manifestations diarrhéiques a été étudiée sur environ 1000 soldats composant deux bataillons suédois de la Force de Maintien de la Paix des Nations Unies à Chypre au cours de leur séjour de 6 mois dans l'île.

Un bataillon d'hiver (WB) a été contrôlé pendant ses trois premiers mois dans l'île et un bataillon d'été (SB) pendant son quatrième mois de service, en août. Un groupe de 128 civils cypristes turcs a été soumis à la même étude aux fins de comparaison.

La présence d'anticorps neutralisants contre LT a été déterminée au moyen d'un test sur des cellules de la glande surrénale. Un niveau significatif de ces anticorps a été observé dans le sérum de 6% seulement des soldats du WB et de 15% de ceux du SB à leur départ de Suède. Dans le WB, l'incidence de la diarrhée a été de 3% par mois. Parmi les soldats atteints, la proportion de ceux dont le sérum, prélevé en phase aiguë, contenait des anticorps contre LT, était passée à 43% — proportion beaucoup plus élevée ( $P < 0,01$ ,  $\chi^2 = 8,61$ ) que celle de 15% établie pour les soldats exempts de diarrhée dont le sérum a été analysé au même moment. L'incidence de la

diarrhée dans le SB a été à peu près le double de celle constatée dans le WB. Des anticorps sériques contre LT ont été trouvés en quantité importante chez 69% des soldats du SB présentant ce symptôme. Chez les soldats « appairés » non atteints de diarrhée, le taux de séropositivité (50%) ne présentait cependant pas de différence significative. Les analyses de sérum prélevé en phase de convalescence ont également montré un écart frappant entre le WB et le SB: Par rapport au sérum prélevé en phase aiguë, la fréquence de séropositivité est passée chez les convalescents du SB à 81%, alors qu'elle a décliné à 15% dans le WB. Au retour en Suède, le nombre de soldats du SB dont le sérum contenait des anticorps contre LT était de 52%, soit plus du double de celui des soldats du WB à sérum positif (24%).

Les variations saisonnières du taux d'anticorps contre l'entérottoxine observées chez les soldats suédois n'ont pas été constatées chez les civils cypristes, chez qui ce taux est demeuré d'environ 65% durant les deux périodes d'étude.

Le titrage parallèle sur des sérums choisis des anticorps contre LT d'*E. coli* et contre l'entérottoxine brute de *Vibrio cholerae* a permis de conclure que les anticorps

présents avaient été induits par une toxine identique à LT d'*E. coli* ou apparentée plutôt que par une toxine plus voisine de celle liée au choléra.

Les résultats obtenus indiquent que l'exposition à *E. coli* entérotoxigène est, à Chypre, aussi commune

chez les visiteurs étrangers que chez les civils de l'île et qu'elle suscite souvent un taux élevé d'anticorps spécifiques. Ils suggèrent également que cette exposition est une cause fréquente de diarrhée du voyageur, bien qu'il existe de nombreux cas asymptomatiques.

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