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Antibody to the Enterobacterial Common Antigen in Normal Human Sera and in Sera of Acute Pelvic Inflammatory Patients

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SUMMARY **Antibody titres to Enterobacterial common antigen (ECA) were assayed in 482 serum specimens from healthy adults and in 40 serum specimens from patients with acute pelvic inflammatory disease by indirect haemagglutination. Normal levels of anti-ECA ranged from 1:4 to 1:256. The mean serum titre was 1:64. The titres were found to be maximal in subjects between ages 31 and 40. The serum titres of females were slightly higher than the titres of males. Significant increases in the titres of anti-ECA were found in 11 of the 17 anti-ECA positive patients who had no concomitant gonococcal infection. ECA antibodies in this study were mostly IgM. The IgG class of antibody was found in 1.1 percent.**

The enterobacterial common antigen (ECA) represents a bacterial surface antigen shared by almost all Enterobacteriaceae.[1] The antigen can be present in two distinct forms: the free non-immunogenic form which is produced by both smooth and rough strains of Enterobacteriaceae and the lipopolysaccharide-linked, immunogenic form which is restricted to a few rough mutants of *Escherichia coli* or *Shigella* with complete R cores of the R_1 or R_4 types.[2,3] The free form of ECA was isolated by two independent methods, and its major sugar components were determined to be N-acetylglucosamine and N-acetylmannosaminuronic acid.[4,5] ECA is composed of a hydrophilic amino sugar chain and a hydrophobic L-glycerophosphatide part. The amino sugar chain carries the serological specificity of ECA whereas the physicochemical properties of the molecule depend on the lipid part.

The progressively increasing importance of gram-negative bacilli as a cause of many complications including nosocomial infections has prompted a series of investigations on the protective effect and other features of antibodies to O-specific and cross-reactive antigens in man and experimental animals.[6-8] Despite several promising reports describing high titres or increased titres in several enterobacterial complications, diagnostic and prophylactic applications of ECA antibody have thus far been inconclusive. The lag in finding diagnostic applications seems to be partly due to the lack of standard method: each group of investigators has used different strains for antigen extraction and slightly different assay methods.

As a basis for defining the diagnostic and prophylactic applications of ECA antibody, information concerning its level in normal human sera and in patient sera, particularly when measured by a standard method, may be useful. This study reports the distribution of antibody to ECA in sera of normal adult populations and changes in anti-ECA levels in acute pelvic inflammatory patients who had no concomitant gonococcal infection by using the standardised indirect haemagglutination method.[9]

MATERIALS AND METHODS

Bacteria

The organisms used were *Salmonella typhimurium* SH 4892 which was *rfe*$^+$ and ECA-positive, *S. typhimurium* SH 4893 which was *rfe*$^-$ (*rfe*-3853) and ECA-negative, and the ECA-immunogenic strain *E. coli* 014:K17 (F2387).[10] These organisms were kindly supplied by the Central Public Health Laboratory, Helsinki, Finland. The two *Salmonella*

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strains were used for the production of ECA-positive and ECA-negative preparations, respectively. They were derived from a rough (*rfb*-4020) *S. typhimurium* LT2 strain which had received the *ilv, rfe,* and *MetE* genes in conjugation from *S. montevideo.*[10] The ECA-immunogenic strain, *E. coli* 014, was used for the production of anti-ECA positive serum.

Antigens

Antigens were prepared from the bacteria according to a procedure described previously.[10] Briefly, the 18-h culture of each of the two *Salmonella* strains grown on a nutrient agar plate was suspended in 5 ml of phosphate-buffered saline (PBS) and heated at 100°C for 1 h. The clear supernatant fluids from strains SH 4892 and 4893 were used as the ECA-positive and ECA-negative preparations, respectively, and they were kept at -20°C before being used.

Anti-ECA positive serum

High-titre serum was obtained by immunisation of rabbits with the antigen prepared from *E. coli* 014:K17. The antiserum was produced as described previously.[11] Briefly, rabbits were intravenously immunised three times at 4-day intervals with increasing amounts (0.25, 0.5 and 1.0 ml) of bacterial suspensions (10^{10} cells/ml) which had previously been heated to 100°C for 1 h and washed with PBS. Rabbits were bled 6 days after the last injection. The sera were decomplemented by heating 30 min at 56°C.

Serum specimens

Single normal serum specimens were obtained from 482 blood donors at Ramathibodi Hospital in Bangkok, Thailand. In all sera, complement was inactivated by heating at 56°C for 30 min.

Both acute-phase and convalescent-phase sera (interval 1 to 3 weeks) were collected from 40 consecutive women with acute pelvic inflammatory disease not associated with instrumentation or surgery. These patients were visiting the Lumpini Public Health Service Centre of Bangkok Metropolis during the period of November, 1984 to October, 1985. The diagnosis of the disease was based on common criteria including fever, lower-abdominal pain, vaginal discharge, and adnexal tenderness, combined with a lower genital-tract-culture for *Neisseria gonorrhoeae.* The sera were stored at -20°C until both sera for each pair could be assayed together on the same day.

Anti-ECA determination

The indirect haemagglutination method adapted to a microtitre system as standardised by Malkamäki[9] was used. Briefly, human group O erythrocytes were washed thrice in PBS and suspended in PBS to a concentration of 2.5%, and 0.1 volume of ECA-positive preparation, ECA-negative preparation, or saline was added. After incubation in a water bath at 37°C for 30 min, the sensitised cells were washed three times with PBS and suspended to a concentration of 0.5% in PBS containing 0.25% bovine serum albumin.

Duplicate two-fold serial dilutions of the sera were made with PBS in V-shaped microtitre plates (Dynatech Laboratories, Inc., Alexandria, Va.). A 50-μl amount of the 0.5% suspension of sensitised erythrocytes was added to 50 μl of each serum dilution. The plates were covered and incubated at 37°C for 1 h, and then kept overnight at 4°C. The haemagglutination titre was expressed as the highest serum dilution producing definite agglutination.

The specificity of the method was studied by haemagglutination inhibition tests. A 25-μl amount of an inhibitor was added to 25 μl of serially diluted serum. The plates were incubated at room temperature for 30 min, 50 μl of 0.5% ECA-positive-sensitised cells was then added to each well, and the test was completed as described above.

Concomitant titrations of anti-ECA antibody before and after 2-

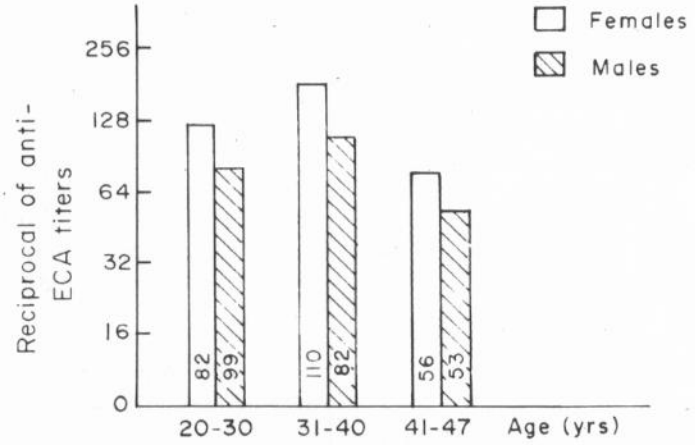

Fig. 1 Anti-ECA titres in the sera of healthy males and females. Titres are expressed as reciprocals of serum dilutions and are given as geometric means of each group. Numbers within columns indicate numbers of sera tested.

mercaptoethanol treatment of serum were also carried out. Serial dilutions of serum were treated with 0.05 ml of 1 M 2-mercaptoethanol for 1 h at 37°C. Sensitised erythrocytes were then added, and the mixture was incubated at 37°C for an additional hour, refrigerated overnight, and read.

RESULTS

Sera from 482 healthy persons at 20 to 47 years of age were assayed. A 1:8 dilution of the anti-ECA rabbit serum was included in every assay plate. The anti-ECA titres of this reference serum on 176 plates for a total of 20 assay days were equal, i.e., the titre was 1:8,192 on every assay day. The specificity of the method was demonstrated by the absence in most human sera and in rabbit sera of agglutination of the cells sensitised with the ECA-negative preparation. Only 5 sera agglutinated these cells and in each case to a low titre (<1:4) only. None of the sera agglutinated the unsensitised cells.

Table 1 Distribution of the anti-ECA antibody in 482 sera from healthy adults.

Passive haemagglutination titre	Positive sera	
	Number	Percent
4	6	1.2
8	34	7.1
16	93	19.4
32	138	28.7
64	127	26.3
128	53	10.9
256	31	6.4

Table 2 Anti-ECA responses of patients with acute pelvic inflammatory disease.

Lower-genital-tract cultures	Anti-ECA titres (reciprocal)		Number
	Acute sera	Convalescent sera	
N. gonorrhoeae positive	8	16	1
	16	16	6
	32	32	7
N. gonorrhoeae negative	16	16	3
	16	256	4
	16	512	5
	32	32	3
	32	512	1
	32	1,024	1

Table 3 Correlation of anti-ECA responses with *N. gonorrhoeae* cultures from the lower genital tract of 40 patients with acute pelvic inflammatory disease.

N. gonorrhoeae cultures	Number of patients		
	Total	With positive anti-ECA	With significant titre changes
+	20	14 (70%)	-
−	20	17 (85%)	11 (64.7%)

Titres of anti-ECA in normal serum specimens in this study ranged from 1:4 to 1:256 (Table 1), with a mean titre of 1:64. The titres were found to be maximal between ages 31 and 40, and decreased between ages 41 and 47 (Figure 1). The serum titres of females were slightly higher than the titres of males.

The anti-ECA antibody in this study appeared to be predominantly of the IgM mercaptoethanol sensitive variety. Treatment with 2-mercaptoethanol almost completely destroyed antibody activity against ECA-coated erythrocytes. Only 5 serum specimens were found to be mercaptoethanol resistant, with titres of 1:16 in 4 cases and 1:128 in 1 case.

Paired serum specimens from 40 patients with acute pelvic inflammatory disease at 23 to 47 years of age, 20 of which were with concomitant gonococcal infection, were tested for antibody levels against ECA. Thirty-one of the 40 patients gave positive tests for anti-ECA (Table 2). It is noted that a four-fold or greater increase in the titres of ECA antibodies occurred in 11 of the 17 patients (64.7%) who had no concomitant gonococcal infection (Tables 2 and 3). Fourteen of the 20 patients (70%) with gonococcal infection had positive anti-ECA but without a significant titre change. All of the ECA antibodies found in the patients in this study were of the IgM mercaptoethanol sensitive variety.

DISCUSSION

Haemagglutination rather than the more sensitive haemolysis[1] was used for measuring antibody to ECA since haemagglutination is easier to carry out and the guinea pig serum normally used as the source of complement often contains ECA antibody.[9] A crude extract of ECA-positive bacteria was used to sensitise erythrocytes since the final purification of ECA is difficult.[5] However, ECA under the conditions used appeared to be the main component of this extract which attached to the cells.[9]

The method used for measuring ECA antibody in this study was reproducible. The endpoints were clear. A difference in the titre of the reference serum of only one dilution step was observed in only 2.4 percent of the determinations on any one day. The method was also specific, since only 5 of the 482 sera tested agglutinated the control cells sensitised with a similarly prepared antigen extracted from bacteria which lacked ECA but to a low titre only. In addition, neither the reference serum nor the human sera agglutinated unsensitised cells used in each test to exclude the effect of unrelated antibodies (e.g., cold agglutinins).

The finding of antibody to ECA in most normal human sera in this study is not surprising since bacteria in the family Enterobacteriaceae are major components of normal flora and are common causes of infections of the gastrointestinal tract. The occurrence of higher anti-ECA titres in females than in males may reflect sex differences in the frequency of enterobacterial infections. For example, females probably have a higher frequency of urinary tract infection.[12] Further, the titres obtained herein were lower than those recorded in previous reports using the same standard of measurement.[9] In contrast, the titres in this study were relatively similar to the values reported in several previous studies using slightly different assay methods. The difference in titres in different places, therefore, may not due only to differences in assay techniques but also to some other factors such as the frequency of infection or the infecting bacterial strain. The presence of low titres of antibody to ECA in sera of the healthy subjects in this study is also probably due to the rarity of *E. coli* 014, the highly immunogenic strain, or of the emergence of R mutants, in causing human infections.

The anti-ECA antibodies have been found to be incapable of passing the placental barrier,[9] probably because they are of the IgM class, but rare cases of IgG antibody have also been reported.[9] In this study, most of the anti-ECA antibody in human sera were found to be IgM, while the IgG class of antibody was observed in 1.1 percent of the material.

High titres or increased titres of ECA antibodies have been found in cases of bacteraemia,[13] pyogenic peritonitis,[14] pyelonephritis,[15] and acute pelvic inflammatory disease.[16] As a basis for diagnostic application, a change in titre or a four-fold or larger increase in titre from normal levels of ECA antibody is regarded as significant.[9]

In addition to *N. gonorrhoeae* and some other kinds of bacteria, enteric bacteria have been found to play an important role in acute pelvic inflammatory disease.[17] Investigation of the microbial aetiology of the disease has been made from cultures of either endocervical (lower-genital-tract) swabs or pus from the pelvic abscesses. Only *N. gonorrhoeae* and *Chlamydia trachomatis* can be considered of aetiological significance in the endocervical cultures. On the other hand, mixed cultures of bacteria have very often been seen in cultures from pelvic abscesses and their presence is difficult to interpret because of possible contamination by normal vaginal flora. In the belief that a serum-antibody response would be a more clear-cut indicator of the involvement of enteric bacteria in pelvic infection, sera from pelvic inflammatory patients were tested for changes in ECA antibody titres. Significant increased ECA antibody titres were found in a number of patients whether or not the lower-genital-tract culture yielded gonococci.[16] Increased levels of anti-ECA were also observed in acute pelvic inflammatory patients without concomitant gonococcal infection in this study. The findings reported previously[16] and herein support a polymicrobial aetiology of acute pelvic inflammatory disease and provide a serodiagnosis of the disease when enteric bacteria are suspected to be the cause in patients without concomitant gonococcal infection.

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