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Antibodies to the Enterobacterial Common Antigen: Standardization of the Passive Hemagglutination Test and Levels in Normal Human Sera

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The passive hemagglutination test for antibodies against the enterobacterial common antigen (ECA) of Kunin was standardized for diagnostic purposes. Human erythrocytes were coated with a soluble ECA^+ preparation from *Salmonella typhimurium* or, as specificity controls, with a similar ECA^- preparation from congenic ECA-negative bacteria or with saline, and the hemagglutination assay was performed on microtiter plates. The specificity of the test was ascertained further by inhibition assays with purified ECA and with crude ECA^+ and ECA^- preparations. The reproducibility of the test was 96.4%; on this basis, a fourfold or larger difference in titers was regarded as significant. The anti-ECA titers in 649 serum samples from healthy persons ranged from <4 to 8,192. The titers increased with age, so that the geometric mean titers were 57 at 1.5 years of age and 201 at 45 years of age. After this, the titers decreased again, to a low of 52 in persons more than 70 years old. Women had higher titers than men up to the age of 40 years.

The enterobacterial common antigen (ECA) was first described by Kunin et al. in 1962 (4, 6, 7). In accordance with the original description, ECA is defined as a cross-reactive antigen that is detectable in all genera of *Enterobacteriaceae* by indirect hemagglutination and by other methods with antisera to *Escherichia coli* O14 (10).

At first, an antigen shared by several common human pathogens was expected to offer diagnostic and even prophylactic possibilities. However, the original hopes have been frustrated partially. Attempts to test the protective value of anti-ECA have been inconclusive (10, 14). Diagnostic applications have not become established despite several promising reports describing high titers or increases in titers in cases of enterobacterial peritonitis (17), bacteremia (15, 16), and chronic pyelonephritis (20; V. Thomas, B. Sanford, S. Carson, M. Forland, and A. Shelokov, Clin. Res. **25**:588A, 1977). The lag in finding diagnostic applications seems to be partly due to the lack of standardized methods; each group of workers has used different strains for antigen extraction and slightly different sensitization methods or agglutination methods or both. The specificity of the reaction measured has not always been evaluated.

As a basis for defining the diagnostic usefulness of anti-ECA, I examined the specificity and accuracy of the hemagglutination method and added an ECA-negative antigen as a specificity control. To find the normal range, I assayed a series of sera from healthy persons of varying ages. On this basis, a fourfold change in titer or a single high titer of ≥4,096 could be considered indicative of a recent enterobacterial infection. This method has been applied successfully to serological diagnosis of pelvic inflammatory disease (18) and of acute complications in gastrointestinal surgery (A. Palmu, V. V. Valtonen, M. Malkamäki, P. H. Mäkelä, and D. Kasper, manuscript in preparation).

MATERIALS AND METHODS

Sera from apparently healthy persons. Single serum specimens were obtained from 649 healthy children and adults as controls before vaccination (3), for screening of Rh sensitization during the first trimester of pregnancy (19), for a community health study (unpublished data), or from blood donors. The two sexes were distributed equally in each age group (Fig. 1). Paired serum samples were obtained from 50 army recruits before and 4 weeks after a series of vaccinations (killed poliomyelitis vaccine, killed mumps virus vaccine, diphtheria and tetanus toxoid vaccines, and group A meningococcal polysaccharide vaccine) (8).

Anti-ECA-positive sera. The single or paired sera from six patients with enterobacterial infections treated at the University Central Hospital, Helsinki, Finland, have been described by Palmu et al. (manuscript in preparation). The high titer serum K713 was from a rabbit hyperimmunized with *E. coli* O14 (11).

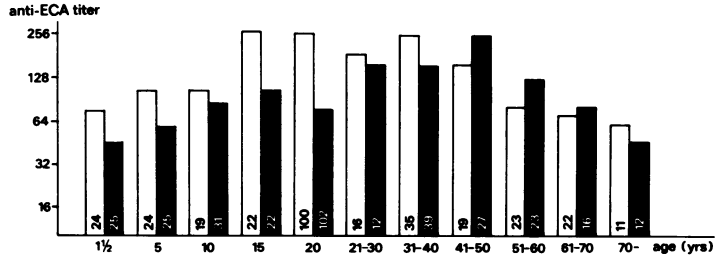

FIG. 1. *Anti-ECA titers in sera of apparently healthy males and females of varying ages. Open columns, Females; solid columns, males. Numbers within columns indicate numbers of sera tested. Titers are expressed as reciprocals of serum dilutions and are given as geometric means of each group.*

Antigens. Antigens for coating erythrocytes were prepared from a congenic pair of ECA-positive and ECA-negative strains of *Salmonella typhimurium*. These strains were derived from a rough (*rfb-4020*) *S. typhimurium* LT2 strain which had received the *ilv*, *rfe*, and *metE* genes in conjugation from *Salmonella montevideo* (9). Of these strains, SH 4892 was *rfe*$^+$ and ECA positive, and SH 4893 was *rfe*$^-$ (*rfe-3853*) and ECA negative (9). Overnight growth of each of these bacteria on a nutrient agar plate was suspended in 5 ml of phosphate-buffered saline (PBS) and heated for 1 h at 100°C. After centrifugation, the supernatant fluids from strains SH 4892 and SH 4893 were used as the ECA$^+$ and ECA$^-$ preparations, respectively.

A purified (13) preparation of ECA from *S. montevideo* strain SH 94 was received from H. Mayer (Max Planck Institute for Immunobiology, Freiburg, West Germany) and was used as a control in some experiments.

Anti-ECA determination. The indirect hemagglutination method was used essentially as described previously (11), except that bovine serum albumin was added to the buffer and an ECA-negative control antigen was included. Human blood group O erythrocytes were washed three times with PBS and suspended in PBS to a concentration of 2.5%, and 0.1 volume of the ECA$^+$ preparation, the ECA$^-$ preparation, or saline was added. After 30 min at 37°C, the sensitized cells were washed three times with PBS and suspended to a concentration of 0.5% in PBS containing 0.25% bovine serum albumin.

Serial twofold dilutions of the sera were prepared with PBS in acrylic microtiter plates containing V-shaped cups (Cooke Microtiter System; Dynatech Laboratories, Inc., Alexandria, Va.). A 50-μl amount of the 0.5% suspension of sensitized cells 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 hemagglutination titer was expressed as the reciprocal of the highest serum dilution producing definite agglutination.

Hemagglutination inhibition experiments. 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 ECA$^+$-sensitized cells (concentration, 0.5%) was then added to each well, and the test was completed as described above.

Statistical methods. Geometric means, 95% confidence limits, *t* tests, and analyses of variance were computed by using an SR-52 model calculator and its Statistics Library (Texas Instruments, Inc., Dallas, Tex.).

RESULTS

Reproducibility. A 1:8 dilution of the anti-ECA rabbit serum K713 was used as a reference serum and was included in every plate. The reproducibility of the method was tested by comparing the titers of the reference serum on parallel plates assayed on one day or on consecutive days. The anti-ECA titer of the reference serum on 193 plates for a total of 44 assay days varied between 256 and 4,096. On any one day, the titers differed by more than one dilution step on 3 of 193 plates (1.6%). The reproducibility estimate, computed as proposed by Wood and Durham (23), was 0.964. The titer of 1,024 was closest to the geometric mean of the titers recorded and was chosen as the standard. The titers of the test sera on each plate were corrected by the factor by which the titer of the reference serum on that plate differed from 1,024. All plates on which the titer of the reference serum differed by more than one dilution step from the value 1,024 were discarded, and the test was repeated. During the test period of 44 days, this meant that 4 of 193 plates (2.1%) were discarded.

On the above-described basis, a fourfold difference in titer between two samples tested simultaneously was due to experimental error in less than 2% of the tests; therefore, this difference seemed to be an acceptable diagnostic criterion. Even if the use of the standard serum minimized differences between days, it seemed to be good practice to titrate both members of a serum pair on the same day. This criterion was tested further by comparing the titers in 50 serum pairs drawn 4 weeks apart from men who in the meantime had received a series of vaccinations unrelated to enteric bacteria. In 34 pairs the anti-ECA titer remained unchanged. In six cases the titer increased, and in seven cases it

decreased by one dilution step (values that could have been due to experimental variation). However, in three men the titer increased by factors of 16, 64, and 128. These changes were too large to be caused by experimental error, and I propose that although the medical histories of the men were not available, these changes occurred as responses to intervening infections.

Specificity. The specificity of the test was studied by hemagglutination inhibition. The anti-ECA rabbit serum K713 was titrated in the presence of PBS, the ECA^+ preparation, the ECA^- preparation, or purified ECA. Table 1 shows that the titer was reduced only by the two ECA-containing preparations. A concentration of 1 μg of purified ECA per ml reduced the titer by a factor of 32 and was used in inhibition tests with several sera from patients with enterobacterial bacteremia or peritonitis very probably caused by members of the family *Enterobacteriaceae* (Table 2). As analyzed in detail elsewhere (Palmu et al., manuscript in preparation), these sera had high anti-ECA titers or an increase in anti-ECA titers in paired sera. In each case, the titer was reduced very substantially after absorption with purified ECA.

Neither the rabbit sera nor the human sera agglutinated the control cells sensitized with the ECA^- preparation or with saline.

Anti-ECA antibodies in normal human sera. Sera from 649 healthy persons were assayed (Table 3 and Fig. 1 and 2). The specificity of the assay for anti-ECA was demonstrated by the absence in most sera of agglutination of the cells sensitized with the ECA^- preparation. Only four sera agglutinated these cells and in each case to a low titer only. None of the sera agglutinated the cells sensitized with saline. The anti-ECA titers ranged from <4 to 8,192, with an essentially normal distribution. Titers of ≥4,096 and ≥2,048 were found in 1.7 and 4.2% of the material, respectively.

The titers varied with sex ($t = 4.65$; $P < 0.001$) and age ($F = 5.68$; $P < 0.001$) (Fig. 1 and 2). The mean serum titer rose from 57 at age 1 year to 160 to 200 between ages 15 and 50 years, after which it started decreasing again. The serum titers of females were higher than the titers of males in all age groups up to 40 years, after which the titers were equal. The differences were largest and statistically significant at the ages of 15 years ($t = 2.62$; $P < 0.05$) and 20 years ($t = 6.06$; $P < 0.001$). Although the sera of 20-year-old males and females were drawn from different populations (army recruits versus pregnant women), all of the sera from 15-year-old males and females were from classmates in the same school.

TABLE 1. *Inhibition of anti-ECA of hyperimmune rabbit serum K713*

Material added to the test[a]	Titer
PBS	16,384
Purified ECA (0.1 μg/ml)	4,096
Purified ECA (1 μg/ml)	512
Purified ECA (10 μg/ml)	32
Purified ECA (100 μg/ml)	4
ECA^+ prepn[b]	512
ECA^- prepn[b]	16,384

[a] A 25-μl amount was added to 25 μl of each serum dilution 30 min before 50 μl of ECA^+-sensitized cells was added.

[b] Diluted 1:10.

DISCUSSION

The passive hemagglutination method of measuring anti-ECA antibodies used here was both specific and reproducible. Whereas the chemical identity of ECA has long been in doubt, recent studies by Männel et al. (12, 13) have established that ECA is an acidic glycolipid characterized by the relatively unusual component *N*-acetyl mannosaminuronic acid. Because the final purification of ECA is difficult (13), I used a crude extract of ECA-positive bacteria to sensitize erythrocytes. Under the conditions used, ECA appeared to be the main component of this extract which attached to the cells; the hemagglutination of both a hyperimmune rabbit serum and several patient sera could be inhibited by low concentrations of purified ECA.

As a specificity control in each test, I used a parallel titration with cells sensitized with a similarly prepared antigen extract from otherwise similar bacteria which lacked ECA because of an *rfe* mutation (9). In the series of normal sera, this control did not appear to be necessary, since only 4 of the 649 sera tested agglutinated these control cells and even then only to a low titer. Nevertheless, until more experience has been gathered from various patient groups, I recommend that such a control be included. In addition, nonsensitized cells were used to exclude the effect of unrelated antibodies (e.g., cold agglutinins).

Human blood group O erythrocytes were used because with sheep cells, low titers have also been obtained with ECA^--sensitized cells (M. Malkamäki, M.S. thesis, Helsinki University, Helsinki, Finland, 1979). Hemagglutination rather than the more sensitive hemolysis (10, 21) was used because hemagglutination is easier to perform and the guinea pig serum normally used as the source of complement often contains antibodies to ECA (Malkamäki, M.S. thesis). Besides, sensitivity did not appear to be particu-

TABLE 2. *Inhibition by purified ECA (1 μg/ml) of anti-ECA of patient sera*

Patient	Serum[a]	Infection	Anti-ECA titer with:		Fold reduction in titer
			PBS	ECA	
A	Con	*Proteus rettgeri*[b]	512	32	16
B	Con	*Klebsiella pneumoniae*[b]	512	32	16
C	Ac	*E. coli*[b]	2,048	16	128
C	Con		≥65,536	512	≥128
D	Ac	*Klebsiella pneumoniae*[b]	1,024	32	32
D	Con		1,024	32	32
E	Ac	Peritonitis[c]	16	<4	>4
E	Con		4,096	64	64
F	Ac	Peritonitis[d]	16	<4	>4
F	Con		1,024	32	32

[a] Ac, Acute-phase serum; Con, convalescent serum.
[b] Bacteremia.
[c] After perforated gangrenous acute appendicitis.
[d] After perforation of the ileum.

TABLE 3. *Distribution of the passive hemagglutination titers in 649 sera from apparently healthy persons*[a]

Titer	% of sera with the following prepn for sensitization of erythrocytes:	
	ECA$^+$	ECA$^-$
<4	0.2	99.4
4	0.6	0.15
8	2.9	0.15
16	9.2	0.15
32	14.1	0.15
64	16.5	0
128	18.8	0
256	16.2	0
512	9.7	0
1,024	7.6	0
2,048	2.5	0
4,096	1.1	0
8,192	0.6	0
>8,192	0	0

[a] For age distribution, see Fig. 1.

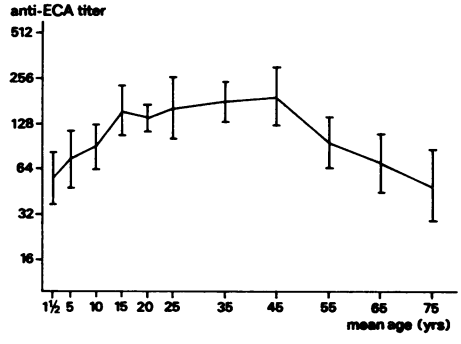

FIG. 2. *Distribution of anti-ECA titers in a normal population. Titers are expressed as reciprocals of serum dilutions and are given as geometric means for each age group. The vertical bars represent the 95% confidence limits of the geometric means.*

larly important since hemagglutinating anti-ECA was found in almost all human sera.

The endpoints of titers could be made clearer by two improvements over previous methods: the use of acrylic plates in which the surfaces of the cups are slightly rough and the addition of bovine serum albumin to the buffer. In this way variations in the titer of the reference serum on one day were minimized, so that a difference larger than one dilution step was observed in only 1.6% of the determinations. Somewhat more variation in the titer of the reference serum occurred from day to day, probably due to subtle variations in the quality of the sensitized erythrocytes; the effect of this was largely eliminated by correcting all titers observed to a normal value of the reference serum. Nevertheless, it is generally good practice to assay serial serum specimens of each patient on the same day, which in this method can mean side by side on the same microtiter plate. Then, a fourfold or larger difference in titer is only seldom caused by experimental error.

With this method, anti-ECA was found in most normal human sera. This is not surprising since members of the family *Enterobacteriaceae* are both major normal constituents of gut flora and common causes of infections. The titers recorded in this study were higher than the maximal values of 16 to 80 reported in many

previous reports (1, 2, 22); on the other hand, McCabe et al. (15) have reported equally high titers. These differences are probably due to slightly different assay methods but emphasize the need to use a reference set of normal values established with exactly the same method.

Conclusions based on the anti-ECA value of a single patient serum sample must be made with caution, taking into consideration the probability of finding such a value in comparable normal material. A titer of ≥4,096 was observed in 1.7% of the normal sera in this study and thus had a high predictive value of presumptive diagnosis of enterobacterial infection; lower values may also support such a diagnosis, but with progressively lower predictive values. A change in titer is always preferred as a diagnostic criterion.

As is the case with serum antibodies in general, the anti-ECA titers increased with increasing age in childhood, remained at a high level in adults, and decreased again in old age. I also tested some sera from newborn infants. Of the 21 sera tested, the anti-ECA titers in 20 were <4, whereas 1 had a titer of 256. Since the mean anti-ECA titer of young women was 256, this indicates that in most cases the anti-ECA antibodies of the mother did not pass the placental barrier (probably because they were immunoglobulin M). In the one case in which antibodies were found in the infant, they appeared to be of the immunoglobulin G class, as judged by being removed by staphylococcal protein A but not by concanavalin A (data not shown).

There were also marked differences between the sexes in the occurrence of anti-ECA; the mean titers of females reached the maximum value at a younger age than the mean titers of males and were higher until middle age. Later on, the titers were equal. Early pregnancy did not seem to affect the anti-ECA titers. All of the sera of 20-year-old women (but none in the other age groups) were taken during the first trimester of pregnancy. Although the differences in anti-ECA titers between females and males in this age group were large, the titers of 20-year-old women did not differ from the titers of the neighboring age groups. It seems possible that these differences reflect sex differences in the frequency of enterobacterial infections; up to middle age, females have a much higher frequency of urinary tract infections (5).

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