Measurement of Immunoglobulin M (IgM), IgG, and IgA Antibodies Against *Yersinia enterocolitica* by Enzyme-Linked Immunosorbent Assay: Persistence of Serum Antibodies During Disease

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An enzyme-linked immunosorbent assay for the detection and quantitation of human immunoglobulin M (IgM), IgG, and IgA antibodies against *Yersinia enterocolitica* is described. Formalinized or heat-treated bacteria were adsorbed onto specially designed microcuvettes, and antibodies were allowed to attach to the antigen-coated cuvettes. Rabbit anti-human μ , anti-human γ , and anti-human α antisera were allowed to react with human antibodies, and these class-specific anti-immunoglobulins were detected by alkaline phosphatase-labeled swine anti-rabbit IgG. A total of 423 sera were tested. The results obtained with the enzyme-linked immunosorbent assay were compared with the results of the conventional tube agglutination test. Persistence of different antibodies was studied in six patients. Antibodies of the IgM class persisted only for 1 to 3 months after onset of the disease; thus the occurrence of IgM-class *Yersinia* antibodies in a single sample indicates a recently acquired infection. The persistence of the IgG- and IgA-class antibodies was variable and not parallel with each other. Remarkably, all three patients in which the disease was complicated with arthritis had IgA-class *Yersinia* antibodies at the end of the follow-up period of 9 to 14 months, and in those without arthritis the IgA-class antibodies disappeared within 3 months after onset of the disease.

A radioimmunoassay for quantitation of human IgM and IgG-class antibodies against *Yersinia* has recently been developed in our laboratory (16). This sensitive method is suitable for analysis of a large number of serum specimens in a short period and offers a possibility of establishing the diagnosis of a recently acquired *Yersinia* infection on the basis of a single serum sample. However, the requirement for radioactive reagents and an expensive detection system makes the radioimmunoassay impractical for many laboratories. Several investigators have developed immunological assays in which enzymes instead of radioisotopes are conjugated to antigens or antibodies (3, 11, 13, 14, 29). These techniques have also been shown to be suitable for antibody detection in diagnostic microbiology (4-7, 10, 12, 17, 22, 26, 27, 30, 33-37).

There are two previous reports on the application of the enzyme-linked immunosorbent assay (ELISA) for determination of *Yersinia* antibodies. Carlsson et al. (4) have described quantitation of *Yersinia* and *Brucella* antibodies in rabbit sera using bacterial lipopolysaccharides

as antigens. Recently, Gripenberg et al. (M. Gripenberg, A. Nissinen, E. Väisänen, and E. Linder, submitted for publication) have described an ELISA for demonstration of IgG-class antibodies against *Yersinia enterocolitica* lipopolysaccharide in human sera. Both of these methods are direct assays using one antiserum, and they do not distinguish between antibodies of different immunoglobulin classes.

This paper describes a sandwich ELISA for determination of *Yersinia*-specific IgM-, IgG-, and IgA-class serum antibodies and its use for monitoring the antibody persistence during the disease. The method is based on the radioimmunoassay of class-specific antibodies developed in our laboratory (2, 16, 18-20, 23, 24, 28, 31, 32). Disposable microcuvettes and a nine-channel spectrophotometer were used (21); all the reagents used are commercially available.

(A preliminary report of this study was presented at the 8th Annual Meeting of the Scandinavian Society for Immunology, Turku, 25-27 May 1977 [K. Granfors, E.-L. Kiiskilä, M. Viljanen, K. Kalimo, P. Ahvonen, and P. Toivanen,

Scand. J. Immunol. 6:1186, 1977], and at the 4th European Immunology Meeting, Budapest, 12-15 April 1978.)

MATERIALS AND METHODS

Antigen preparation. The strains used as antigens included *Y. enterocolitica* serotype O:3 (M.Y. 0; S. Winblad, General Hospital, Malmö, Sweden), *Y. enterocolitica* serotype O:9 (M.Y. 79; B. Niléhn, General Hospital, Malmö), and *Y. pseudotuberculosis* IA (strain 2; W. Knapp, Institute of Hygiene and Medical Microbiology of the University of Erlangen-Nuernberg, Germany). All strains were cultured on tryptose agar (9) for 48 h at 20°C. The resulting growth was suspended in saline; formalinized OH-antigen of *Y. enterocolitica* O:3 and *Y. pseudotuberculosis* IA and heat treated O-antigen of *Y. enterocolitica* O:9 were prepared according to Winblad et al. (39). The bacteria were harvested by centrifugation and washed twice in saline. The stock suspensions, added with 0.5% Formalin, were stored at 4°C. The same preparations were used for the ELISA and agglutination.

Serum specimens. The serum material consisted of 423 specimens from our diagnostic laboratory. All samples had been tested by tube agglutination for antibodies against *Y. enterocolitica* serotypes O:3 and O:9 and *Y. pseudotuberculosis* IA. Sixty-one of these specimens were negative in the agglutination test, and 356 specimens gave an agglutination with *Y. enterocolitica* O:3 with titers from 80 to 20,480 (titers expressed as reciprocals). The specimens included serial serum samples from six patients. In addition, nine sera were tested for cross-reactions between different *Yersinia* strains. They were selected on the basis of high concentration of IgM-, IgG-, or IgA-class antibodies against *Y. enterocolitica* O:3 and O:9 and *Y. pseudotuberculosis* IA. All the specimens were stored at -20°C until used.

The procedure for ELISA. The tests were carried out in disposable polystyrene nine-microcuvette blocks (Finnpipette-Labsystems, Helsinki, Finland). These cuvettes were incubated with 200 µl of *Yersinia* antigen solution containing 0.3 mg (dry weight) of bacteria per ml of 0.1 M sodium phosphate buffer (pH 7.5) (PBS). The adsorption was allowed to take place overnight at 37°C. The cuvettes were washed once with 400 µl of PBS. Thereafter, 200 µl of 5% normal sheep serum (NSS) in PBS (NSS-PBS) was added to each cuvette. After an incubation of 2 h at 37°C the cuvettes were washed as before and stored at 4°C.

For the IgG test the serum specimens were diluted 1:500 and, for the IgM and IgA tests, 1:256 in NSS-PBS. Volumes of 150 µl of these dilutions were incubated for 2 h at 37°C. The cuvettes were washed three times with physiological saline containing 0.05% Tween 20 (PS-T), and 150 µl of rabbit anti-human γ , anti-human μ , or anti-human α antiserum (Behringwerke AG, Frankfurt am Main, Germany) was added. Anti-human γ was diluted 1:4,000 in NSS-PBS, and anti-human μ and anti-human α were diluted 1:1,000. After incubation overnight (18 h) at room temperature, the cuvettes were washed again three times with PS-T, and 150 µl of swine anti-rabbit IgG alkaline phosphatase conjugate (Orion Diagnostica, Helsinki,

Finland), diluted 1:200 in NSS-PBS, was added. The cuvettes were incubated for 5 h at 37°C, and the washing was carried out as described previously. A 150-µl volume of a fresh substrate solution was added to each cuvette and incubated for 30 min at 37°C. This solution was prepared by dissolving 200 mg of *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.) into 100 ml of glycine-MgCl₂ buffer (pH 10.0; Orion Diagnostica). After incubation, 250 µl of 1 N NaOH was added to stop the reaction.

The optical absorbance was measured vertically through the cuvettes using a nine-channel photometer (Finnpipette Analyzer System, Finnpipette-Labsystems) at a wavelength of 405 nm. The measurement was carried out against a sensitized cuvette treated with the substrate and 1 N NaOH as control.

The same procedure, with the only difference in the antigen, was applied for the antibodies against *Y. enterocolitica* serotypes O:3 and O:9 and *Y. pseudotuberculosis* IA.

Tube agglutination test. Serial twofold dilutions of serum starting at 1:20 were prepared in physiological saline. Tubes containing 0.25 ml of serum and 0.25 ml of the antigen suspension were incubated overnight at 50°C. The concentration of the antigen was same as in ELISA. The agglutination was read with the naked eye, and the titer was determined as the highest serum dilution giving visible agglutination.

RESULTS

Antigen adsorption onto polystyrene cuvettes. The optimal concentration of the antigen used for the adsorption on the polystyrene cuvettes was 0.3 mg (dry weight) per ml. Higher concentrations of the antigen resulted in a slight prozone effect and did not increase specificity or sensitivity of the assay, and the use of lower concentrations resulted in a decreased sensitivity. After antigen adsorption the cuvettes were treated with 5% NSS to decrease the binding of nonspecific immunoglobulins.

Incubation with serum dilutions. The effects of various serum dilutions, different blocking agents (NSS, bovine serum albumin) in the reaction mixture, different incubation times, and various washing schemes were studied. Optimal results were obtained when 150 µl of serum dilutions 1:500 (in the IgG test) and 1:256 (in the IgM and IgA tests) in PBS with 5% NSS were used. For the first step an incubation time of 2 h at 37°C and for the second step an incubation time of 18 h at room temperature were found to be the best. For the reaction of rabbit anti-human immunoglobulin antisera with the anti-rabbit IgG-enzyme conjugate, incubation for 5 h at 37°C was optimal. The washing of cuvettes three times with physiological saline containing 0.05% Tween 20 proved suitable in different steps.

Working dilutions of anti-human immunoglobulin antisera and the conjugate. To

FIG. 1. Standard curves for determination of IgM-, IgG-, and IgA-class antibodies against *Yersinia*.

determine the most suitable working dilution for the anti-human immunoglobulins, various dilutions of them and a constant dilution of the reference serum and the conjugate were used in the test. High concentrations of the anti-immunoglobulins resulted in a slight prozone effect, but beyond the critical dilution (for anti-human γ , 1:4,000; for anti-human μ and anti-human α , 1:1,000) a steep decrease was observed. The optimal dilution of the conjugate was found to be 1:200.

Assay results. The results were evaluated by means of a standard curve based on the use of six different dilutions (1:100, 1:500, 1:2,500, 1:12,500, 1:62,500 and 1:312,500 for IgG, and 1:16, 1:64, 1:256, 1:1,024, 1:4,096, and 1:16,384 for IgM and IgA) of serum with a high concentration of the corresponding *Yersinia* antibody (Fig. 1). The results are presented as percent values of the standard serum.

The results obtained with the tube agglutination test and with the ELISA of IgM-, IgG-, and IgA-class antibodies were compared (Table 1). All 61 sera that were negative by tube agglutination (titer < 80) were negative by ELISA in the IgM and IgG tests. Eight out of 61 sera (13%) negative by the tube agglutination were positive in the IgA ELISA. In all, 356 sera with an agglutination titer ≥ 80 were studied by the ELISA; from these 153 (43%) were positive for IgM antibodies, 171 (48%) were positive for IgG antibodies, and 246 (69%) were positive for IgA

antibodies. Seventy out of 356 (20%) sera with an agglutination titer ≥ 80 were negative in all three (IgM, IgG, and IgA) ELISAs (Table 1). Most of these ELISA-negative sera had an agglutination titer of 80 or 160.

Cross-reactions. Cross-reactions between sera originating from patients with different infections (*Y. enterocolitica* serotypes O:3 and O:9 and *Y. pseudotuberculosis* IA) were studied using nine different human sera (Table 2). If a net absorbance of 0.100 was taken as a limit for a positive reaction, *Y. enterocolitica* O:3-specific antiserum gave a slight cross-reaction with serotype O:9 antigen in the IgM and IgG tests. Antiserum originating from an infection with the serotype O:9 showed no cross-reaction with the other strains used, and furthermore, *Y. pseudotuberculosis* IA-specific antiserum gave a slight reaction with *Y. enterocolitica* O:9 in the IgA test. Altogether, the cross-reactions observed were weak and, in practice, insignificant.

Persistence of IgM-, IgG-, and IgA-class *Yersinia* antibodies. In six patients sera collected at different intervals were studied. In these patients the diagnosis of a *Y. enterocolitica* infection was established on the basis of serological and clinical findings, and the date for the onset of the symptoms was known. A total of 40 serum samples were available from these patients. Two patients (K.I. and M.P.) had infection with abdominal pains, diarrhea, and fever; one patient (I.T.) had in addition erythema nodosum; and three patients (I.K., N.K., and O.R.) had infection complicated with severe ar-

TABLE 1. Comparison of tube agglutination and ELISA of *Yersinia* antibodies

Agglutination titer (reciprocal)	ELISA* (% of sera)				Total no. of sera
	Positive in:			Negative in all tests	
	IgM test	IgG test	IgA test	All tests	
<80	—	—	13	—	87
80	—	15	21	—	64
160	12	16	42	—	49
320	4	15	50	—	30
640	14	34	66	7	20
1,280	50	48	78	17	4
2,560	82	82	97	56	2
$\geq 5,120$	97	90	95	84	—
$\geq 80^b$	43	48	69	28	20
					356

* The limit values of 21% for IgM, 7% for IgG, and 8% for IgA were used. To obtain these limit values 20 sera negative in the tube agglutination were tested by ELISA for antibodies of the IgM, IgG, and IgA class. The highest value for each immunoglobulin class was taken and multiplied by 3.

^b Total for sera with agglutination titer ≥ 80 .

TABLE 2. Cross-reactions between different *Yersinia* strains^a

ELISA test	Antigen	Net absorbance values for sera positive for:		
		<i>Y. enterocolitica</i> O:3	<i>Y. enterocolitica</i> O:9	<i>Y. pseudotuberculosis</i> IA
IgM	<i>Y. enterocolitica</i> O:3	1.153 (20.5)	0.001 (1.0)	0.003 (1.1)
	<i>Y. enterocolitica</i> O:9	0.114 (2.5)	1.188 (16.6)	0.011 (1.1)
	<i>Y. pseudotuberculosis</i> IA	0.000 (1.0)	-0.026 (0.6)	1.049 (17.1)
	IA			
IgG	<i>Y. enterocolitica</i> O:3	0.980 (12.5)	-0.039 (0.5)	-0.008 (0.9)
	<i>Y. enterocolitica</i> O:9	0.179 (2.0)	0.868 (5.7)	0.077 (1.4)
	<i>Y. pseudotuberculosis</i> IA	0.017 (1.3)	-0.024 (0.6)	0.322 (5.9)
	IA			
IgA	<i>Y. enterocolitica</i> O:3	0.949 (18.9)	0.011 (1.2)	0.018 (1.3)
	<i>Y. enterocolitica</i> O:9	0.086 (2.0)	1.308 (16.2)	0.204 (3.4)
	<i>Y. pseudotuberculosis</i> IA	0.018 (1.3)	0.018 (1.3)	0.939 (18.1)
	IA			

^a *Y. enterocolitica* O:3, *Y. enterocolitica* O:9, and *Y. pseudotuberculosis* IA positive sera were tested for IgM-, IgG-, and IgA-class antibodies. Nine different sera were studied, one specific for each bacterial strain and immunoglobulin class. Net absorbance values (absorbance in the sample minus absorbance in the negative control) are given. Figures in parentheses represent ratios of absorbance in the sample/absorbance in the negative control. Results of homologous reactions are printed in italics, and those showing cross-reactions (net absorbance >0.100) are given in boldface.

FIG. 2. Development of *Yersinia*-specific IgM, IgG, and IgA antibodies measured by ELISA in sera of six patients with a primary *Yersinia* infection. All patients represent *Y. enterocolitica* O:3 infection with abdominal pains, diarrhea, and fever at the onset of the disease. In addition, patient I.T. developed erythema nodosum, and the three patients presented at right (I.K., N.K., and O.R.) had arthritis. The bars at left indicate the scale for antibody concentrations from 0 to 300% of the standard. Open circles represent zero concentrations.

thrititis. The persistence of *Y. enterocolitica* O:3-specific IgM-, IgG-, and IgA-class serum antibodies in these patients is presented in Fig. 2.

The first samples were obtained 2 to 21 days after the onset of clinical symptoms. All the patients showed a roughly parallel development of the IgM-, IgG-, and IgA-class antibodies during the acute phase of the infection; during the first month after the onset of the illness antibodies of all three classes were demonstrable in the

sera of all six patients. IgM-*Yersinia* antibodies disappeared in all patients during the first 3 months of the disease. The IgG-class antibodies seemed to persist longer, at least for 5 months. In three of the six patients, the IgG-class antibodies were detectable at the end of the follow-up period of 9 to 14 months.

The persistence of the IgA-class antibodies was variable and did not parallel the occurrence of IgG-class antibodies. In three patients (two

with uncomplicated infection and one with erythema nodosum) the IgA-class antibodies disappeared within 1 to 2.5 months after onset of the disease. In contrast, in the three patients who developed arthritis these antibodies were still demonstrable at the end of the follow-up period of 9 to 14 months.

DISCUSSION

The ELISA described here utilizes two antisera of which one is enzyme labeled. The main advantage of a double-antibody system is that only one labeled antiserum, in this case anti-rabbit IgG, is required for detection of antibodies of different immunoglobulin classes. Another advantage over the direct system is that the class-specific anti-immunoglobulin sera used in the first step need not be purified free from other serum proteins; it is only necessary to make them specific without any cross-reacting antibodies. This also offers an advantage in the storage and stability of the antisera, since highly purified antibodies lose their activity considerably sooner than the unpurified ones. In the present work, no attempts have been made to modify this technique for microplates, due to the known heterogeneity of microplate surfaces (8) as well as to the availability of specifically designed cuvettes for the nine-channel spectrophotometer used.

When the results obtained with ELISA were compared with the agglutination titers, mostly agreement was observed. All of the 61 sera negative by the tube agglutination were also negative by the IgM and IgG ELISA. Eight (13%) of these sera showed IgA-class antibodies in the ELISA. On the other hand, 70 (20%) of 356 sera with an agglutination titer ≥ 80 were negative for IgM-, IgG-, and IgA-class antibodies in the ELISA (Table 1). I believe that these disagreements between the tube agglutination and the ELISA are due to false negative as well as to false positive results in the tube agglutination test. For instance, it is known that IgA- and IgG-class antibodies may lack agglutinating activity (38). I have also noticed that agglutination in sera containing only IgG- and IgA-class antibodies is very faint and therefore may remain undetected. Some of the results where agglutination was positive and nothing was detected by the ELISA might be explained by the fact that most of these sera (42/70) had an agglutination titer of 80 or 160. Such low titers are often considered unspecific or insignificant (1). Rheumatoid factor hardly can be a reason for the discrepancies, since all the sera negative by the agglutination were negative by the IgM ELISA. This is also the experience gained in an analo-

gous radioimmunoassay of rubella virus antibodies (24). However, interaction of rheumatoid factor with the IgM ELISA will be elucidated in more detail in our further studies.

In six patients, the appearance and persistence of *Y. enterocolitica* antibodies of the IgM, IgG, and IgA classes were studied. The IgM-class antibodies persisted only for 1 to 3 months after the onset of the disease, even in the patients who developed arthritis. Therefore, it seems that demonstration of IgM-class *Yersinia* antibodies can be considered to indicate a recently acquired infection. The IgG-class antibodies persisted at least for 5 months, and half of the patients studied (3/6) had them at the end of the follow-up period of 9 to 14 months.

I find the persistence of IgA-class *Yersinia* antibodies most interesting. In the three patients without arthritis, these antibodies disappeared within the first 3 months of the disease. In contrast, in the three patients with *Yersinia* arthritis they were demonstrable during the whole follow-up period up to 9 to 14 months (Fig. 2). Even though the number of patients monitored is so far limited, these observations are challenging, particularly since IgA-secreting plasma cells are known to exist in the synovium of patients with rheumatoid arthritis (15, 25).

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