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ORIGINAL PAPER

Serum Immunoglobulin IgG Subclass Distribution of Antibody Responses to Yop Proteins and Lipopolysaccharide of *Yersinia enterocolitica* in Patients with Yersiniosis

WALDEMAR RASTAWICKI* and ALEKSANDRA JAKUBCZAK

Department of Bacteriology, National Institute of Hygiene, Warsaw, Poland

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Abstract

To assess the humoral immunological responses at the IgG subclass level in yersiniosis specific antibody responses against lipopolysaccharide of *Yersinia enterocolitica* O3 (LPS) and *Yersinia* Yop proteins were analyzed by ELISA. Thirty five patients with arthritis and forty nine patients with uncomplicated yersiniosis were included in the study. Analysis of the IgG subclass responses to the LPS revealed that the subclass distribution for both groups of patients was IgG2>IgG1>IgG3. The concentration of IgG4 was below detection level. The predominant antibody responses to Yop proteins were IgG1>IgG3>IgG2>IgG4 but the frequency of detection of particular IgG subclass antibodies were dependent on the age of patients. Generally, the frequency of occurrence of IgG2 antibodies for Yop proteins of *Yersinia* together increased with age reaching its peak among individuals aged above 40 years. On the other hand, IgG1 for Yop proteins and IgG3 for *Y. enterocolitica* LPS were diagnosed more often in serum samples obtained from children than from adults. We also found significantly higher frequency of IgG4 to Yop proteins of *Y. enterocolitica* in men than in women.

Key words: *Yersinia enterocolitica*, IgG subclass antibodies, LPS, Yop proteins

Introduction

Infections produced by *Yersinia enterocolitica* are recognized as an important cause of enteric illness (Chandler and Parisi, 1994; Vantrappen *et al*., 1979). Sometimes a *Yersinia* infection is suspected on the basis of postinfectious complications such as reactive arthritis or erythema nodosum, after an infection which may have passed unnoticed (Gaston *et al*., 1999; Touraud *et al*., 2000). In the virulent *Y. enterocolitica* the major antigenic constituents are the invasion plasmid-encoded proteins antigens (Yop) and the lipopolysaccharide (LPS) (Straley *et al*., 1993; Holst, 2003). Infections with *Y. enterocolitica* result in the production of patients' serum antibodies which can be used for the serodiagnosis of acute infections in the absence of a culturable organism. Serum IgA, IgG and IgM antibodies to these bacteria can also be used to establish the role of *Yersinia* in post-infection sequelae, which may manifest several months after infection.

It has been recognized that human serum IgG is composed of four distinct subclasses. The IgG subclasses differ not only in their distribution in normal serum, but also in their biological properties and the nature of the antigens which elicit their production (Spiegelberg, 1974; Islam *et al*., 1995; Wilson and Hamilton, 1992). In general, bacterial protein antigens preferentially induce IgG1 antibodies in humans, with minor contributions of IgG3 and IgG4. In contrast, IgG response to polysaccharides is normally restricted to the IgG2 subclass, although some polysaccharides also induce substantial amounts of IgG1 (Hamilton, 1987).

There are many reports about the prevalence of IgA, IgG and IgM antibodies to Yop proteins and lipopolysaccharide of *Y. enterocolitica* both in healthy blood donors and patients suspected for yersiniosis (Granfors *et al*., 1981; Mäki-Ikola *et al*., 1997). However, there are no complex reports concerning IgG subclass responses to these antigens in yersiniosis so far. This information will aid the development of good assay system for diagnosis and monitoring the progression of the disease.

The present study was aimed at determining the IgG subclass distribution against a Yop proteins and lipopolysaccharide *Y. enterocolitica* serotype O3 in patients with yersiniosis.

* Corresponding author. W. Rastawicki, Department of Bacteriology, National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland; Phone: (48) 22 5421325; **e-mail: wrastawicki@pzh.gov.pl**

Experimental

Materials and Methods

Patients. The study population consisted of 84 patients with *Yersinia* infections. The diagnosis was established by bacterial isolation and/or by routine serological means – a serotype specific ELISA and ELISA with Yop proteins as antigen. Thirty-five patients (mean age 34 years; 19 men and 16 women) developed reactive arthritis (ReA) as a post-infections complication and the other 49 (mean age 11.1 years; 21 men and 28 women) had uncomplicated yersiniosis, with manifestation of diarrhoea, abdominal pain or mesenteric lymphadenitis mimicking appendicitis. Paired serum specimens were obtained from 34 patients with uncomplicated yersiniosis. The first set of serum samples was collected during 1–2 weeks after onset of illness, the second one, an average of a month later.

Additionally, 80 serum samples were obtained from clinically healthy persons – blood donors.

Preparation of LPS for ELISA. The LPS antigen was prepared from *Y. enterocolitica* serotype O3. For preparation of the antigen bacteria were cultured on nutrient agar plates, for 48 h at 20°C. The bacteria grown on the plates were suspended in physiological saline, harvested by centrifugation, and washed twice with saline. The LPS extraction was carried out by the Boivina method (Suzuki *et al*., 1964).

Preparation of Yop proteins. The strain of *Y. enterocolitica* serotype O:8 (WA-314) containing the 42 MDa plasmid pYV was used for production of Yop proteins. Briefly, bacteria were first grown in BHI broth at 37°C for 90 minutes, followed by incubation under calcium restriction by addition of 10 mM magnesium-EGTA (ethyleneglycol-bis (β-aminoethylether) tetra-acetate) for an additional 90 minutes. The bacteria were removed by centrifugation and the proteins precipitated from culture supernatant by ammonium sulphate. After overnight incubation at 4°C the precipitates were collected by centrifugation and resuspended in buffer containing 10 mM TRIS-HCl (pH 8) and 0.1 mM EDTA. The suspensions were recentrifuged, and the obtained pellets were dissolved in 1×Laemmli buffer and used for ELISA and SDS-PAGE (Heesemann *et al*., 1986).

Serology. In initial experiments, different ELISA conditions were tested to obtain maximum discrimination between positive and negative sera, while keeping the background at a low level. Polystyrene microtiter plates (Nunc, MaxiSorp) were separately coated with LPS *Y. enterocolitica* (25 μg/ml) and Yop proteins (10 μg/ml) in 0.05 M carbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were washed twice with phosphate-buffered saline (PBS; pH 7.4) with 0.05% (vol/vol Tween 20 (PBS-T), and then postcoated with 5% (wt/vol) solution of powder milk in PBS at room temperature for 2 h. The wells were washed twice, and 100 μl of the patient serum samples at a dilution of 1:25, 1:100, 1:400 and 1:1600 were added. The plates were incubated for 1.5 h at 37°C and washed four times with PBS-T, and then 100 μl of mouse-anti-human IgG, IgG1, IgG2, IgG3, or IgG4 monoclonal antibody conjugated with horseradish peroxidase (Invitrogen, clone no. HP6017, HP 6069, HP 6014, HP 6047, HP 6025, respectively) was added at a dilution of 1:500 to 1:2000. After 1 h incubation, the plates were washed as described above and the wells were filled with 100 μl of substrate solution (10 mg of 3,3',5,5'-tetramethylbenzidine dihydrochloride, 100 ml citrate buffer, pH 5.0, and 20 μl H_2O_2). The reaction was stopped after 10 min by the addition of 150 μl of 1N H_2SO_4 to each well. The A_{450} was read with a photometer (AsysTech). The blank was a well coated with antigen and incubated with the conjugates only. The results were evaluated by means of a curve constructed from measurements on four different dilutions of each diagnostic serum. Antibody concentration was expressed as the serum titre.

Statistic. Based on the results of determining the level of antibodies against LPS and Yop proteins in the sera of 80 clinically healthy persons, the cut-off limit of serum antibodies in IgG and in four IgG subclasses was set at $x + 3$ SD (x – arithmetic mean of antibody titre, SD – standard deviation). This included the results of determining more than 95% of the sera investigated in the particular tests.

The statistical significance of the differences was analyzed by Fisher's exact probability test with Yates' correction when at least one of the calculated figures was <5. In each case, $P<0.05$ was considered significantly different.

Results

The percentage distribution of specific anti-Yersinia IgG subclass response was calculated only on the basis of group of patients confirmed in the present study as positive for total IgG antibodies (61 patients to LPS antigen and 76 to Yop proteins). This allowed for more reliable evaluation of humoral response in each subclass.

The titres of antibodies detected by ELISA in serum samples obtained from patients with yersiniosis were found to vary from 0 to few thousands. The values for IgG were higher than those recorded for IgG1, IgG2, IgG3 and IgG4. As can be seen (Fig. 1, and Table I), the development of antibodies in particular IgG subclasses to Yop proteins showed a pattern different from that of LPS specific. Analysis of the IgG subclass responses to the LPS revealed that the

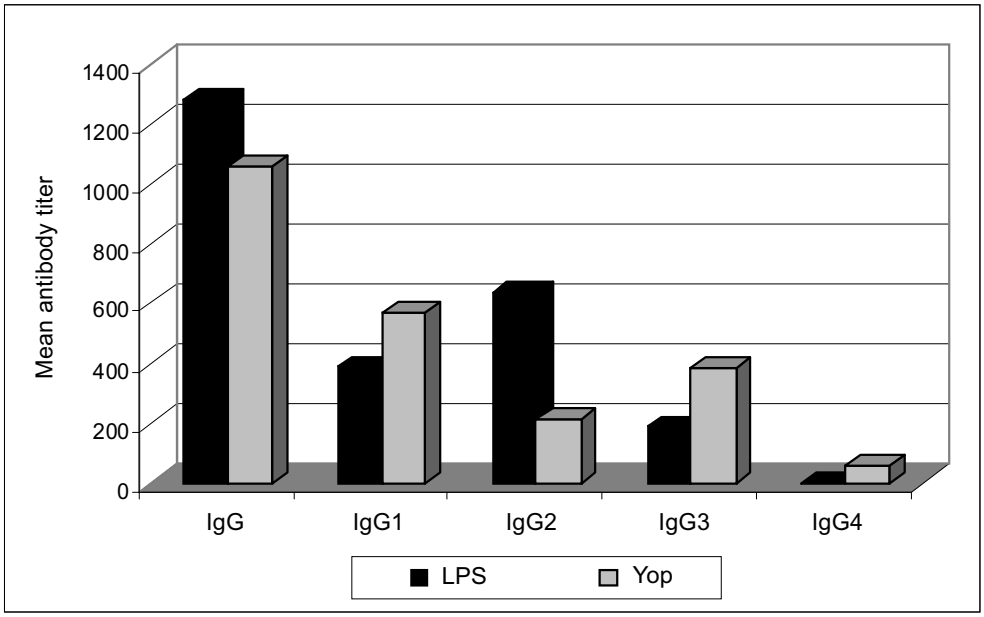

Fig.1. Mean arithmetic values of antibodies IgG and IgG1-IgG4 titers in serum samples of patients with yersiniosis.

subclass distribution was IgG2>IgG1>IgG3. The amount of IgG4 was below the detection level. In general, the predominant antibody responses to Yop proteins were IgG1>IgG3>IgG2>IgG4 but the frequency of detection of IgG2 antibodies at diagnostically significant titers was much higher in patients with arthritis (50.0%) than in patients with uncomplicated yersiniosis (15.2%) ($\lambda^2 = 9.05$; $P < 0.05$). There were no significant differences between mean levels of IgG1-IgG4 antibodies to LPS and Yop proteins in patients with arthritic and non-arthritic yersiniosis. It is notable that the concentrations of IgG subclass antibodies to these antigens varied greatly among the patients in both groups.

The percent distributions of IgG subclasses in patients with yersiniosis in relation to sex, age and phase of the disease are presented in Table II and Fig. 2–3. A comparison of the frequency of antibody detection for LPS and Yop proteins of *Y. enterocolitica* with reference to the sex of the individuals studied showed there were no significant differences in percentage of patients with IgG1, IgG2 and IgG3 (Table II).

Table I
Distribution of specific anti-*Yersinia* IgG subclass responses by antigen and disease status

Antigen	Clinical symptoms	Number of patients with IgG	Number and percentage of patients with:				Subclass distribution
			IgG1	IgG2	IgG3	IgG4	
LPS	ReA	27	16 (59.3)	21 (77.8)	5 (18.5)	0	2 > 1 > 3 > 4
	Uncomplicated yersiniosis	34	16 (47.1)	22 (64.7)	8 (23.5)	0	2 > 1 > 3 > 4
Yop	ReA	30	23 (76.7)	15 (50.0)	16 (53.3)	5 (16.7)	1 > 3 > 2 > 4
	Uncomplicated yersiniosis	46	34 (73.9)	7 (15.2)	30 (65.2)	7 (15.2)	1 > 3 > 2 = 4

Table II
Distribution of specific anti-*Yersinia* IgG subclass responses by antigen and gender

Antigen	Gender	Number of patients with IgG	Number and percentage of patients with:				Subclass distribution
			IgG1	IgG2	IgG3	IgG4	
LPS	Female	29	14 (48.3)	20 (69.0)	8 (27.6)	0	2 > 1 > 3 > 4
	Male	32	18 (56.3)	24 (75.0)	5 (15.6)	0	2 > 1 > 3 > 4
Yop	Female	34	27 (79.4)	13 (38.2)	20 (58.8)	2 (5.9)	1 > 3 > 2 > 4
	Male	42	30 (71.4)	10 (23.8)	27 (64.3)	10 (23.8)	1 > 3 > 2 = 4

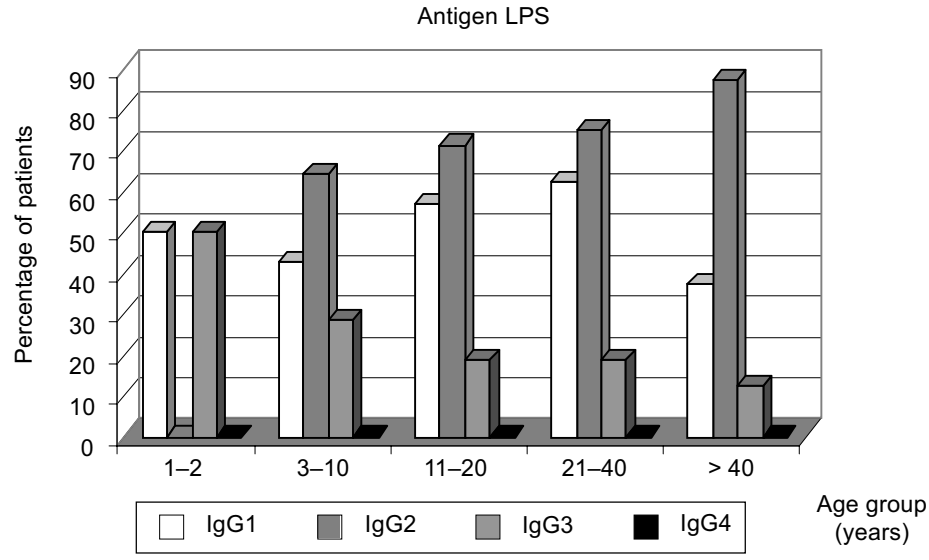

Fig. 2. Percentage distribution of specific anti-*Yersinia* IgG subclass responses to LPS antigen by age group.

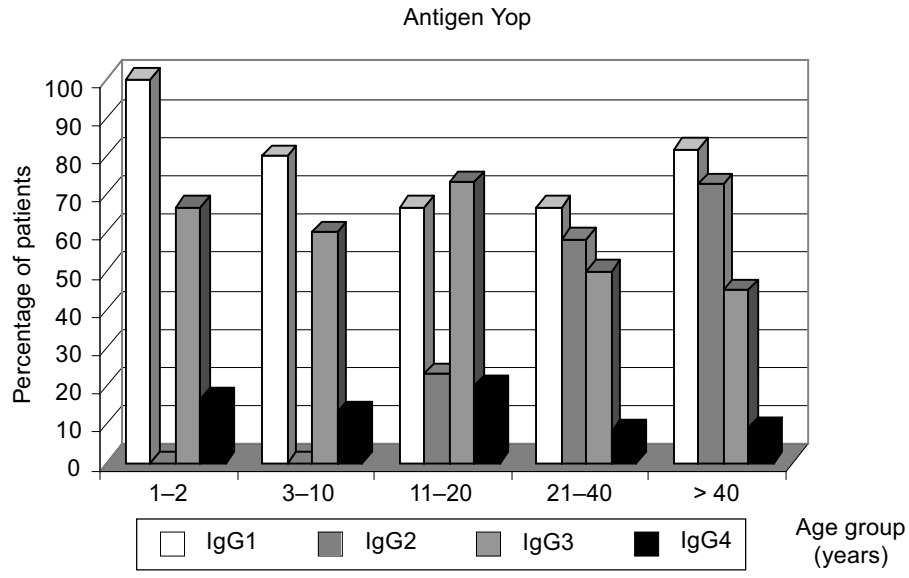

Fig. 3. Percentage distribution of specific anti-*Yersinia* IgG subclass responses to Yop proteins antigen by age group.

However, IgG4 antibodies to Yop proteins were detected in men statistically more frequently than in women ($\lambda^2 = 3{,}29$; $P < 0.05$).

Analysis of the frequency of occurrence of antibodies in particular subclass among patients of specific age group, it was found that IgG1 and IgG3 are the predominating IgG subclasses specific for LPS and Yop proteins of *Y. enterocolitica* among children of up to 2 years. The key finding is that the percentage of patients with IgG3 antibodies to LPS decreased with age and the percentage of patients with IgG2 antibodies to Yop proteins increased with age attained the peak among patients aged over 40 years (Fig. 2–3)

When comparing samples taken at an acute phase of yersiniosis, *i.e.* within 2 weeks after the onset of symptoms of diarrhea or abdominal pain, with those taken at a reconvalescence phase of the disease (a month later), the IgG3-positive samples were observed more frequently among the early samples, but not statistically significant (to LPS antigen 11.1% in acute phase and 4.8% in reconvalescence phase and to Yop proteins 69.2% and 39.3% respectively). On the other hand antibodies of IgG1 subclass were identified slightly more frequently in serum samples obtained at a reconvalescence phase of the disease, in comparison to an acute phase of disease (to LPS antigen 44.4% in acute phase and 57.1% in reconvalescence phase and to Yop proteins 69.2% and 78.6% respectively). Practically no changes in IgG2 and IgG4 subclass frequency were seen during the course of the

disease. The IgG subclass patterns of anti-LPS antibodies were similar in both groups of serum samples (IgG2>IgG1>IgG3>IgG4). The IgG subclass pattern for the anti-proteins Yop antibodies was different than against LPS, showing the orders IgG1 = IgG3>IgG2 >IgG4 at earlier time points and IgG1>IgG3>IgG2 >IgG4 at reconvalescence phase of disease.

Discussion

This paper reports, for the first time, the results of analyses of serum IgG subclass responses against *Y. enterocolitica* LPS and Yop proteins in patients with abdominal pain and reactive arthritis. We have shown that all four subclasses of IgG antibodies are produced during yersiniosis. In general, the IgG subclass pattern found against LPS and protein antigens is in line with that found in other bacterial infections (Chethamarakshan *et al*., 2001; Schenk and Michaelsen, 1987; Shackelford *et al*., 1987; Sieber *et al*., 1980). The isotypic restriction of antibodies is correlated with the biochemical nature of antigens: most antibodies against proteins are of IgG1 and IgG3 isotypes, while in those against LPS IgG2 is overrepresented. However, surprisingly, in our studies the differences in levels and frequency of detection the antibodies at diagnostically significance titers were not as distinct as expected. Moreover, in some cases of yersiniosis the humoral response differed from the general pattern presented. In some patients a high level of IgG1 and IgG3 antibodies to LPS was detected, while no IgG2 antibodies were detected. Similarly, in some persons the level of IgG2 antibodies to YOP proteins exceeded many times the IgG1 and IgG3 antibodies levels. Such disproportions may be caused by the differences in the activity of immune system of individual patients and not excluded that by deficiencies in some persons one or more of the IgG subclass.

IgG4 antibodies are known to be of limited importance in viral and bacterial infections (Hamilton, 1987; Whitney *et al*., 1992; Widhe *et al*., 1998; Lundkvist *et al*., 1993). Generally, our data is in line with this finding, since IgG4 antibodies directed against LPS were below the detection level and to Yop proteins in majority of cases were diagnosed in low titres with an one exception of a 16-years old boy with abdominal pain who had a greater level of IgG4 antibodies than any other of the three 3 subclass of IgG antibodies.

It has been previously shown using the solid-phase radioimmunoassay that arthritic patients (but no nonarthritic patients) with yersiniosis had a pronounced IgG2 response (Mattila *et al*., 1985). Similarly, one of the most interesting observations of our study is that the frequency of detection of IgG2 antibodies to Yop proteins, but not to LPS, was much higher in patients with arthritis than in patients with uncomplicated yersiniosis. However, because the reactive arthritis is more common among adults than among children we decided to analyse the percent of the distribution of specific anti-Yersinia IgG subclass responses to the age of patients with yersiniosis. Our study has revealed that IgG2 antibodies to Yop proteins were not detected in sera obtained from children below 12 years and the prevalence of these antibodies increased with age attaining peak among patients aged over 40 years. Furthermore, for 35 patients with arthritis 6 patients were below 20 years old (but older than 10 years) among them IgG2 antibodies in diagnostically significance titre were not detected. On the other hand, among 23 patients with uncomplicated yersiniosis of the age 11–20, there were 5 patients with elevated IgG2 antibodies. In our study patients above 20 years old with uncomplicated yersiniosis were not included but we suppose that the percentage of these patients with IgG2 is similar to patients with arthritis. Thus, the results obtained showed that the presence of IgG2 antibodies is connected with the age of patients not with the clinical symptoms. In our opinion, the fact that IgG2 antibodies were missing in small children with yersiniosis is important but not surprising. The data correspond with widespread opinions about the much slower maturation of the IgG2 subclass in humans (Gregorek *et al*., 1994; Berkel *et al*. 1994). It has been reported that serum IgG1 and IgG3 concentrations reached values characteristic for adults earlier than IgG2 and IgG4. In particular, it has been shown that IgG1 and IgG3 levels increase rapidly, reaching the mean adult values by 1.5 to 2 years of age, whereas the amount of IgG2 remains lower than 50% of the adult value, having not reached the normal concentration at the age of 9 to 12 years (Toptygina *et al*., 2005).

Another important point of our investigatations is founding that the prevalence of antibodies IgG4 to Yop proteins was significantly higher among males than among females. We were not able to explain this phenomena but our results are in agreement with an earlier study showing that a higher proportion of males than females harbour IgG4 in some diseases (Satoh *et al*., 2004; Haarbrink *et al*., 1999).

The duration of the disease seems to be of importance for the IgG subclass distribution. In the presented study we tested paired serum specimens obtained from 34 patients. Among sera obtained in the acute phase of yersiniosis the IgG1 and IgG3 antibodies to Yop proteins were observed with identical frequency, while among the sera obtained in the reconvalescence phase IgG1 were twice as common as IgG3. This difference may be caused by the fact

that the IgG3 antibodies have a much shorter half-life (7 days) in comparison to the IgG1 (21 days).

According to Mattila *et al*. (1985) IgG2 antibodies to *Y. enterocolitica* serotype O3 increased from the first sample to the second when the second was taken during the first 3 months of the disease. However in our study we did not observe significant differences in the frequency of IgG2 as well as IgG4 antibodies dependant on the disease phase.

In conclusion, IgG1 and IgG3 antibodies to Yop protein and IgG2 to LPS are the predominating IgG subclass in yersiniosis. However, the pattern of IgG subclass might change during the course of infection and is dependent of the age and, in the case of IgG4, of the gender of the patients.

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