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Serum Immunoglobulin G Subclass Responses in Different Phases of Hepatitis E Virus Infection

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To investigate the specific immunoglobulin (Ig) G subclass responses in patients with hepatitis E virus (HEV) infection, an open reading frame 2 (ORF2) protein based enzyme-linked immunosorbant assay was used to measure antibody levels in sera obtained at different phases of infection. Sera were collected at 2–31 days and at 6 months after the onset of symptoms corresponding to the acute (n = 48, 100% IgM-positive) and convalescent (n = 17/48, 53% IgM-positive) phases of infection, respectively. IgM-negative sera from 61 individuals infected at least ≥6 months ago (prior exposure) were also tested. IgG1, IgG2, IgG3, and IgG4 antibodies were detected in 100%, 6%, 56%, and 4% of acute phase sera, respectively, and in 100%, 0%, 0%, and 65% of convalescent phase sera, respectively. IgG1 antibody levels were significantly higher than those of the other detectable subclasses of IgG in the acute and convalescent sera ($P < 0.05$). The IgG3 antibodies in six acute phase patients were replaced by IgG4 antibodies in the convalescent phase of infection. Patients with prior exposure to HEV had low total IgG antibody titers and decreased IgG1 seropositivity compared with those in the acute and convalescent phases. IgG1 was the only major subclass of antibody to be detected in all the three phases of infection. Other than IgG1 antibodies, the subclass antibody response was restricted to IgG3 and IgG4 antibodies in the acute and convalescent phases of infection, respectively. ***J. Med. Virol. 85:828–832, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: hepatitis E; IgG subclass; IgM; ORF2

INTRODUCTION

Hepatitis E is a major public health problem in developing nations and a noteworthy disease in developed nations [Fukuda et al., 2007]. Hepatitis E virus (HEV) is responsible for sporadic and epidemic forms of enterically transmitted acute hepatitis in adults [Emerson and Purcell, 2007] and is a non-enveloped, single-stranded, positive-sense RNA virus (~7.2 kb). The genome contains three open reading frames (ORFs) flanked by non-coding regions at the 5′ and 3′ ends. The genome is polyadenylated at the 3′ end. The nonstructural and structural proteins are encoded by ORF1 (~5 kb) and ORF2 (~2 kb), respectively. ORF3 (~0.342 kb) overlaps ORF2 and encodes a small phosphoprotein [Tam et al., 1991; Graff et al., 2006]. Although HEV is classified into four mammalian genotypes, only one serotype exists [Schlauder and Mushahwar, 2001; Emerson et al., 2004]. Until recently, HEV genotypes 1 and 4 continue to circulate in human and domestic swine populations, respectively, in India [Arankalle et al., 2002, 2003]. Cases of HEV genotype 4 infection seem to be very rare in India, although one report has documented this infection in a traveler from India [Rolfe et al., 2010].

Seroepidemiological, animal transmission, and candidate vaccine studies have revealed that the ORF2 protein (capsid) contributes significantly to the induction of humoral immune responses during HEV infection. Since HEV has limited growth in tissue culture, recombinant ORF2 antigens and/or synthetic peptides have been used to develop immunoassays [Yarbough et al., 1991; Dawson et al., 1992; Favorov et al., 1996; Li et al., 1997; Zhou et al., 2004; Arankalle et al., 2007; Deshmukh et al., 2007]. The detection of HEV using molecular techniques is restricted currently to the early acute phase of infection, and is therefore of limited clinical use. The diagnosis of HEV infection is confirmed by the serological detection of anti-HEV IgM antibodies, which indicates recent or ongoing infection. HEV-specific IgM and IgG antibodies are detectable in sera obtained during the acute phase of

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infection. A low IgG antibody titer together with the absence of IgM antibodies indicates prior infection [Herremans et al., 2007].

The human IgG antibodies have been classified into four subclasses: IgG1, IgG2, IgG3, and IgG4. This classification is based on the differences in physical, chemical, and biological properties of these antibodies. The physical differences include minor differences in their primary amino acid sequences, the length of the hinge region, the inter-chain disulphide bonds, the point at which the light chain attaches to the heavy chain, and the ratio of κ to λ light chains [Roux et al., 1998]. The IgG subclass antibodies show varied sensitivity to digestion with proteolytic enzymes, indicating differences in their chemical properties. The differences in the biological properties of the IgG subclass antibodies are based on their interactions with antigens (i.e., primary function), complement fixation, and binding to monocytes, neutrophils, platelets, and lymphocytes using different Fc receptors (i.e., secondary effector functions). The concentrations of IgG antibodies in sera from apparently healthy adults are highest for IgG1 antibodies, followed by the IgG2, IgG3, and IgG4 antibodies [Mayumi et al., 1983]. IgG antibodies take longer to synthesize and show the longest biological half-life in serum of all immunoglobulins. Regarding the IgG subclass antibodies, the half-life is shorter for IgG3 antibodies than the IgG1, IgG2, and IgG4 antibodies. During complement fixation by the classical pathway, IgG3 antibodies show the greatest binding capacity to the C1q complement factor followed by the IgG1, IgG2, and IgG4 antibodies [Bindon et al., 1988; Cavacini et al., 2003; Mehlhop et al., 2007]. The type of stimulus and cytokine response also influence the IgG subclass response, a possible biomarker for T helper cell function [Mayumi et al., 1983; Kawano et al., 1994; Isa et al., 2001; Gregorek et al., 2005]. IgG subclass response to several viral antigens has been demonstrated [Rath and Devey, 1988; Xu et al., 2005; Madsen et al., 2009]. However, the specific IgG subclass response during the clinical course of HEV infection is still not known. Therefore, the aim of the present study was to characterize the specific IgG subclass responses during the clinical course of HEV infection.

MATERIALS AND METHODS

Study Patients

Serum samples obtained from 48 hepatitis E patients with symptoms for 2–31 days (median, 10 days), and positive for anti-HEV IgM antibodies represented the acute phase of HEV infection. The blood samples were collected from patients during an outbreak that occurred in the Sangli district of Maharashtra State in 2009. Sera were obtained 6 months later, corresponding to the convalescent phase of HEV infection, from 17/48 patients. The anti-HEV IgM antibody titers were low in 9/17 sera at 6 months. Sera were also obtained from 61 apparently healthy individuals who were only positive for anti-HEV IgG antibodies, indicating prior exposure to HEV. The HEV-specific total IgG, IgG1, IgG2, IgG3, and IgG4 antibody titers were measured in 126 serum samples, across the three phases of HEV infection. The presence of genotype 1 HEV in acute phase sera was confirmed by PCR and phylogenetic analysis.

ELISA for Anti-HEV Antibodies

Enzyme-linked immunosorbant assay (ELISA) was performed according to the procedure described in previous reports [Arankalle et al., 2007; Deshmukh et al., 2007]. The procedure was as follows, the Sf9 insect cell lysate containing the recombinant ORF2 protein was diluted in 0.05 M carbonate buffer (pH 9.5) and was used to coat a 96 well microtiter plate (100 μl/well; Maxisorp; Nunc, Roskilde, Denmark). After antigen coating at 37°C for 2 hr, the contents of the wells were aspirated. Blocking solution was added to each well, and the plate was incubated at 37°C for 30 min. After washing, the sera were diluted in blocking solution and were added to each well (100 μl/well of twofold serially diluted sera starting from 1:100). The plate was then incubated at 37°C for 30 min. The positive and negative control sera were tested at 1:100 dilutions. Horseradish peroxidase-conjugated goat anti-human IgG (1:10,000 dilution; Sigma Chemical Co., St. Louis, MO) was used as the detector antibody. The enzymatic reaction with the substrates (*O*-phenylenediamine and urea peroxide) was stopped by the addition of 4 N H_2SO_4 and the optical density (OD) was measured at a wavelength of 492 nm. A serum sample was considered to be positive when the OD was greater than or equal to the sum of the mean OD value of negative controls and three times the standard deviation (ELISA cutoff) [Arankalle et al., 2007]. The reciprocal of the highest dilution that had an OD greater than or equal to the ELISA cutoff was taken as the HEV-specific total IgG antibody titer.

Detection of HEV-Specific IgG Subclass Antibodies

To detect the HEV-specific IgG subclass antibodies, mouse anti-human IgG subclass monoclonal antibodies (anti-human IgG1–4; Sigma Chemical Co.) were added to the wells after incubation with the test serum was completed. The monoclonal antibodies were used at the following dilutions: 1:6,000 (IgG1), 1:5,000 (IgG2), 1:6,000 (IgG3), and 1:10,000 (IgG4). The plate was then incubated at 37°C for 30 min. The optimal dilutions of the four monoclonal antibodies were determined by titrations against reference positive sera. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution; Sigma Chemical Co.) was used as the detector antibody. The reciprocal of the highest dilution that had an OD greater than or equal to the ELISA cutoff was taken as the HEV-specific antibody titer of the respective IgG subclass antibodies. The HEV-specific antibody titer $\log_{10}$ (total IgG and IgG1–

TABLE I. Seropositivity for HEV-Specific Class and Subclass Antibodies in Sera Obtained at Different Phases of HEV Infection

Phase of infection	Median age, years (range)	Time after onset of symptoms	Total samples (n)	No. of samples positive (% seropositivity, 95% CI)					
				IgM	Total IgG	IgG1	IgG2	IgG3	IgG4
Acute	31 (17–70) 7 females 41 males	2–31 days	48	48 (100)	48 (100)	48 (100)	3 (6, 0–13)	27 (56, 42–70)	2 (4, 0–10)
Convalescent	30 (16–64) 3 females 14 males	6 months	17	9 (53, 29–77)	17 (100)	17 (100)	0 (0)	0 (0)	11 (65, 42–87)
Prior	37 (13–95) 19 females 42 males	Not known	61	0 (0)	61 (100)	18 (30, 18–41)	0 (0)	0 (0)	5 (8, 1–15)

4) for negative sera from all of the groups was taken as 0.301, and was included in the analyses.

Statistical Analyses

Proportions were compared using the χ^2 test with Yate's correction for independent groups or the McNemar test for paired groups with OpenEpi software [Dean et al., 2009]. The mean log titers were compared using t tests for independent groups or paired t tests for paired groups with PASW Statistics 18 software. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The percent seropositivity of anti-HEV antibodies in the different groups of samples is shown in Table I. The HEV-specific IgG1, IgG2, IgG3, and IgG4 antibodies were detected in 100%, 6%, 56%, and 4% of acute phase sera, respectively (n = 48). Sera were available in both the acute and convalescent phases from 17 patients. In the acute phase samples of these 17 patients, HEV-specific IgG1, IgG2, IgG3, and IgG4 antibodies were detected in 100% (17/17), 0% (0/17), 59% (10/17), and 0% (0/17), respectively. In the convalescent phase samples of these 17 patients, HEV-specific total IgG, IgG1, IgG2, IgG3, and IgG4 antibodies were noted in 100%, 100%, 0%, 0%, and 65%, respectively. Significant decreases in IgM ($P < 0.05$) and IgG3 ($P < 0.05$) antibody seropositivity and an increase in IgG4 antibody seropositivity ($P < 0.05$) were noted in the convalescent phase of infection compared with the acute phase. IgG3 antibodies present in 10/17 patients (59%) in the acute phase were undetectable in the convalescent phase (0%). On the contrary, IgG4 antibody positivity rose from 0/17 in acute phase to 11/17 in the convalescent phase (65%). Of the 11 IgG4 antibody positive patients, 6 patients were seropositive for IgG3 antibodies in the acute phase, showing a subclass switch from IgG3 to IgG4 antibodies. The IgG1 and IgG4 antibodies were detected in 30% and 8% of sera, respectively, from 61 individuals with prior exposure to HEV. The IgG2 and IgG3 antibodies were not detected in any of these individuals (Table I).

In acute phase sera, the HEV-specific IgG subclass mean antibody titers were highest for IgG1 (4.1 ± 0.1) antibodies and significantly higher than IgG3 (1.6 ± 0.2), IgG2 (0.4 ± 0.1), and IgG4 (0.4 ± 0.0) antibodies (all $P < 0.05$; Fig. 1). The IgG3 antibody titer was significantly higher than those of IgG2 and IgG4 (both $P < 0.05$). Two of the three IgG2 antibody positive samples were also positive for IgG3 antibodies but were negative for IgG4 antibodies, whereas the two IgG4 antibody positive samples were negative for IgG2 and IgG3 antibodies (Table I). The 17 patients showed similar results in their acute phase except that none of them were positive for IgG2 or IgG4 antibodies. HEV-specific total IgG antibodies (4.8 ± 0.2) in the 17 acute phase sera were predominantly IgG1 antibodies (4.2 ± 0.2) followed by IgG3 antibodies (1.8 ± 0.3). The mean titers of the total IgG and IgG1–4 antibodies were not significantly different between all 48 sera and the 17 acute phase

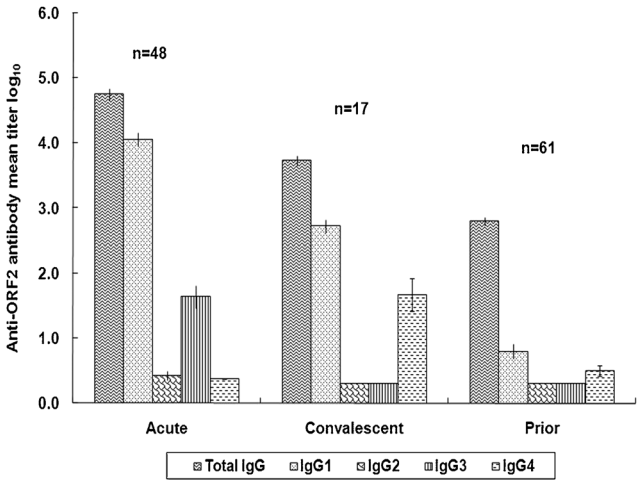

Fig. 1. HEV-specific total IgG and subclass antibody titers in sera from patients at different phases of HEV infection. Data are mean $\log_{10}$ concentrations.

sera (all $P > 0.05$). The mean titer of IgM antibodies in acute phase sera was 3.9 ± 0.1 (n = 48). Except for titers in three patients, the total IgG antibody titers in the acute phase sera were 2–128 times greater than the corresponding IgM antibody titers, irrespective of time after onset of symptoms.

During the convalescent phase, the titers of specific IgG1 (2.7 ± 0.1) antibodies were higher than IgG4 (1.7 ± 0.3) antibodies (Fig. 1). The IgG2 and IgG3 antibodies were undetectable. Notably, the mean IgG3 antibody titer of the 17 acute phase sera (1.8 ± 0.3) was comparable to the mean IgG4 antibody titer (1.7 ± 0.3) of the corresponding 17 convalescent phase sera ($P > 0.05$). The mean titer of IgM antibodies in convalescent phase sera was 1.4 ± 0.3 (n = 17).

The specific total IgG and IgG1 antibody titers were significantly lower in sera from individuals with prior infection (2.8 ± 0.1 and 0.8 ± 0.1) compared with that in convalescent phase (3.7 ± 0.1 and 2.7 ± 0.1), which, in turn, was significantly lower than that in the acute phase (4.8 ± 0.1 and 4.1 ± 0.1; all $P < 0.05$; Fig. 1). IgG1 and IgG4 antibodies were detected in 18/61 (30%) and 5/61 (8%) samples, respectively, while 2/5 IgG4-positive serum samples were also positive for IgG1 (Table I).

DISCUSSION

The present report describes for the first time the HEV-specific IgG subclass antibody responses during different phases of HEV infection. These results help to understand the humoral immune response induced during HEV infection.

The HEV-specific IgG1 antibodies predominated in all three phases of HEV infection although the titer decreased from the acute phase to prior exposure. Specific IgG3 antibodies also contributed to the IgG response in the acute phase of infection. Specific IgG1 and IgG3 antibodies are also associated with other acute/chronic viral infections, including acute hepatitis A virus infection [Rath and Devey, 1988; Joassin et al., 1989; Muller et al., 1990; Isa et al., 2001; Cavacini et al., 2003; Toptygina et al., 2005; Xu et al., 2005]. Other than IgG1 antibodies, the IgG subclass response was restricted to IgG4 antibodies in the convalescent phase of infection. A subclass switch from IgG3 to IgG4 antibodies was evident, as six IgG3 antibody positive and five IgG3 antibody negative acute phase patients switched to IgG4 antibody positivity in the convalescent phase. In four patients, IgG3 antibody negativity was not accompanied by the appearance of IgG4 antibodies in the convalescent phase. Notably, the mean titers of IgG3 and IgG4 antibodies in the acute and convalescent phases were similar. Negligible IgG2 antibody titers were noted in the acute phase only. IgG4 antibodies were detected in all three phases of infection with higher titers during convalescent phase. The IgG2 and IgG4 antibodies were reported to be directed against polysaccharide antigens/allergens and chronic antigenic stimulation, respectively, in other infections/conditions [Rath and Devey, 1988; Gregorek et al., 2005; Toptygina et al., 2005].

The anti-viral IgG subclass response is influenced by the type of antigen, clinical phase of infection, sensitivity of the detection system, and the age and physiological state of the patient [Rath and Devey, 1988; Isa et al., 2001; El Mubarak et al., 2004; Istrate et al., 2008; Madsen et al., 2009]. It would therefore be worthwhile, to study specific IgG subclass profiles in HEV infected pregnant women and fulminant hepatitis E patients. Further studies are also needed to establish the significance of the subclass switch (IgG3–IgG4) and its association with the T helper cell response. The ORF2 protein has three putative glycosylation sites [Zafrullah et al., 1999]. Therefore, the impact of the protein's glycosylation state on the IgG subclass response should also be investigated. The present results will help us to better understand the pathogenesis of HEV infection. Finally, studies should determine why less than 50% of the acute and convalescent phase sera were negative for anti-HEV IgG3 and IgG4 antibodies.

In conclusion, HEV-specific IgG1 and IgG3 antibodies were detected in sera obtained from patients in the acute phase of infection, while IgG1 and IgG4, but not IgG3, antibodies were detected during the convalescent phase.

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