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Published in:
Digestive Diseases and Sciences

DOI:
10.1023/A:1014783815433

Publication date:
2002

Document Version
Post-print: The final version of the article, which has been accepted, amended and reviwed by the publisher, but without the publisher's layout.

Digestive Diseases and Sciences, Vol. 47, No. 4 (April 2002), pp. 760–766 (© 2002)

IgA Against Gut-Derived Endotoxins
Does It Contribute to Suppression of Hepatic Inflammation in Alcohol-Induced Liver Disease?

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Endotoxins of intestinal origin are supposed to play an important role in the development of alcoholic hepatitis in man. To estimate the role of immunoglobulin response to gut-derived endotoxin in the development of alcohol-induced liver disease, serum levels of IgA and IgG against fecal endotoxin, endotoxin, and acute-phase proteins were measured in patients with different stages of alcoholic liver disease and in healthy controls. Antibodies of type IgA, but not IgG, against fecal endotoxins were significantly increased in patients with alcohol-induced liver disease. IgA antibodies against fecal endotoxin were found to be closely correlated with the plasma concentrations of alanine aminotransferase, γ-glutamyl transferase, and C-reactive protein in patients with alcoholic liver disease. In conclusion, as IgA located in body tissue was shown to suppress the inflammatory process, enhanced production of IgA against endotoxin of intestinal origin may contribute to inactivation of this compound, thereby reducing its damaging effect on the liver.

KEY WORDS: alcohol abuse; alcohol-induced liver disease; IgA; IgG; fecal endotoxins; lipopolysaccharides.

Alcohol abuse is the leading cause of chronic liver disease in most industrialized countries. The formation of antigenic epitopes from protein adducts with acetaldehyde (1) or hydroxyethyl radicals (2) and the destruction of cell organelles by reactive oxygen species (3) with the subsequent activation of the immune system have been suggested to induce the inflammatory process found in alcoholic liver disease. These models, however, fail to explain the fact that acute and chronic administration of alcohol does not induce inflammatory infiltrates and fibrosis in the liver in animals with the exception of primates (4), despite the fact that the metabolic changes induced by alcohol including the formation of protein adducts are nearly identical.

Endotoxins (lipopolysaccharides, LPS) are glycolipids constituting the outer membrane of gram-negative bacteria. Contact of immunocompetent cells with LPS even at picomolar concentrations results in the formation of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) which were found to be increased in alcohol abusers with mild and severe liver disease (5, 6).

Chronic alcohol abuse leads to changes in the ultrastructure of enterocytes in the small intestine of animals (7) and man (8–10). These changes result in an impaired barrier function, enabling macromolecules to permeate through the intestinal barrier (11) and to enter the systemic circulation (12). In view of the increased intestinal permeability to macromolecules, gut-derived endotoxins may be regarded as inducers of the inflammatory cascade in the liver of patients chronically abusing alcohol. This holds especially true as the upper part of the small intestine,

Manuscript received April 19, 2001; accepted September 21, 2001.

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which is populated by only small numbers of bacteria in healthy subjects, is overgrown in alcohol abusers (13), leading to increased endotoxin concentrations in the small intestine. In line with the hypothesis that the coincidence of bacterial overgrowth and increased intestinal permeability in alcohol abusers leads to systemic endotoxemia are studies showing increased endotoxin levels after both acute and chronic alcohol intake. This endotoxemia is transient in healthy subjects (14) and in recently drinking alcoholics with early forms of alcohol-related liver damage, such as fatty liver or mild alcoholic hepatitis (15). Furthermore, the removal of the intestinal flora by antibiotics prevents alcohol-induced liver injury in rats induced by intragastric feeding in the model of Tsukamoto and French (16), emphasizing the role of bacterial toxins in the development of ALD (17).

The lipid A moiety of LPS is a strong inducer of inflammation and might work as an adjuvant (18), leading to increased antibody production against epitopes of the lipopolysaccharide chain. The likelihood of antibody induction is particularly high after chronic alcohol consumption. In accordance with this hypothesis, an increased level of antibodies against lipid A was found in alcoholics (19).

This study was performed to further elucidate the role of IgA-LPS antibodies in ALD. Three specific questions should be answered: (1) At which stage of ALD does the formation of antibodies against endotoxins of intestinal origin occur? (2) Is IgA or IgG the preferred class of antibodies that is produced against these endotoxins? (3) Are there correlations between pathological parameters specific for ALD and antibody expression?

MATERIALS AND METHODS

Forty-seven subjects participated in the study. Thirty patients were chronic alcohol abusers with an alcohol consumption of at least 60 g alcohol per day over a period of at least three years. Patients with ALD were classified into three groups, ALD1–ALD3, according to the severity of liver damage (Table 1). In patients with alcohol-induced liver cirrhosis (group ALD3), diagnosis was confirmed either by liver biopsy or, in the case of pronounced coagulation defects or biopsy refusal, by meeting at least two of the following criteria: the presence of ascites, esophageal varices, and characteristic findings on ultrasound of the liver. Alcohol abusers not meeting these criteria of liver cirrhosis were assigned to group ALD2, if AST (aspartate aminotransferase) in serum was >35 units/liter and bilirubin >1.5 mg/dl, whereas group ALD1 comprised patients with AST and bilirubin values below these limits. Exclusion criteria for groups ALD1–ALD3 were abstinence from alcohol for more than three days prior to admission and any evidence of liver disease of other than alcohol-induced origin. Patients with diabetes, malignancies, spontaneous bacterial peritonitis and/or other infections, and recent intake of antibiotics or corticosteroids were also excluded from the study. Details concerning anthropometric data and clinical parameters of the patient groups are given in Table 1. Eleven healthy subjects consuming less than 12 g alcohol per day served as controls.

All subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Robert Bosch Hospital and followed the ethical guidelines of the 1975 Declaration of Helsinki.

Venous blood samples were taken after an overnight fast between 7:30 and 9:00 AM on the first or second day of

Table 1. Clinical Characteristics and Laboratory Findings in Patients with Mild (ALD1), Moderately Severe (ALD2), and Severe (ALD3) Alcoholic Liver Disease on Admission (Values Are Means ± sem, if Not Indicated Otherwise)

	Healthy controls	*All ALD patients*	*ALD1*	*ALD2*	*ALD3*
Number	11	30	10	9	11
Male/female	3/8	22/8	9/1	7/2	6/5
Age [yr, Median (range)]	41 (30–56)	49 (26–69)	40 (26–64)	52 (27–59)	54 (26–69)
Smokers*	5	14	6	5	3
BMI (kg/m^2)†	22.5 ± 0.9	22.9 ± 0.6	23.4 ± 1.1	22.4 ± 0.79	22.8 ± 1.3
Ascites	0	8	0	0	8
Esophageal varices	0	11	0	0	11
Av. alcohol intake (g/day)	4.8 ± 1.3	150 ± 18	191 ± 35	135 ± 17	108 ± 32
γ-GT‡ (units/liter; <28)§	10.2 ± 3.3	375 ± 65.6	137 ± 48.9	604 ± 146	404 ± 92.5
AST‡ (units/liter; <18)§	11.3 ± 0.8	46.4 ± 6.1	21.9 ± 2.2	64.6 ± 10.9	53.7 ± 11.4
ALT‡ (units/liter; <24)§	14.4 ± 0.8	31.6 ± 4.1	23.3 ± 3.3	51.1 ± 10.2	23.1 ± 3.7
Bilirubin (mg/dl; <1.5)	0.91 ± 0.13	2.5 ± 0.8	1.4 ± 0.2	1.7 ± 0.2	4.3 ± 2.0
Prothrombin time (INR¶)	1.03 ± 0.04	1.13 ± 0.05	1.02 ± 0.02	1.06 ± 0.04	1.29 ± 0.10
Albumin (g/dl; >3.5)§	4.6 ± 0.1	4.2 ± 0.1	4.6 ± 0.2	4.4 ± 0.2	3.7 ± 0.2

*>10 cigarettes/day.
†Body-mass index.
‡γ-Glutamyltransferase; AST; aspartate aminotransferase; ALT; alanine aminotransferase.
§Nonpathogenic range.
¶International standardized ratio.

admission. Endotoxin concentrations were measured with a modified chromogenic *Limulus* lysate assay using intraindividual standardization (15). For the determination of antibodies against endotoxins, the method of Scott and Barclay (20) with small modifications was applied. In brief, microtiter plates (Maxisorp by Nunc, Wiesbaden, Germany) were coated with a polymyxin B (Sigma, Deisenhofen, Germany; 1 mg/ml)–endotoxin (20 μg/ml) complex, which was dialyzed overnight for the removal of excess polymyxin. The endotoxins were from bacteria of fecal origin, which were raised from eight different feces samples. The bacteria were bred in a 24-hr culture in a 10-liter fermenter with Standard Nutrient Broth (Becton Dickinson, Heidelberg, Germany) at 37°C. The bacteria were killed at the stationary growth phase by addition of 1% phenol. Endotoxins were extracted according to the method of Westphal and Jann (21). The dialyzed polymyxin–endotoxin solution (20) was diluted with hydrogencarbonate buffer (pH 9.6, 1:16) and used for overnight-coating of microtiter plates. Excess polymyxin–endotoxin solution was removed by washing the plates thoroughly with 0.1 M phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (Sigma, Deisenhofen, Germany). To avoid nonspecific binding, cavities were blocked by a 1-hr incubation with 5% gelatin from pork skin (Fluka, Heidelberg, Germany). Plasma samples were diluted 1:200 in PBS and incubated in the coated plates for additional 2hr. Human antibodies were detected by polyclonal horseradish-conjugated rabbit antibodies against human IgG or IgA, respectively (Dako, Hamburg, Germany). After incubation with a citrate-buffered (pH = 5.5) o-phenylenedimine–hydrogenperoxide solution, optical density was measured with a computer-guided microtiter plate reader (SLT, Crailsheim, Germany) at 492 nm. The ELISA was standardized by the application of a pooled serum from 4000 healthy blood donors, which was a kind gift from Chromogenix (Mölndal, Sweden). The IgA and IgG content in this serum was assumed to correspond to 100 median units (MU). Other values mentioned in this study were measured in the Department of Clinical Chemistry of the Robert Bosch Krankenhaus according to standard procedures. Average alcohol consumption and energy intake of the patients and controls volunteers were determined by a computer-aided diet history (EBIS) (22). For calculation of mean daily alcohol consumption, the following alcohol concentrations (v/v) were assumed: beer 4%, wine 11%, and hard liquor 40%.

Statistics. If not indicated otherwise, values are given as means ± standard error of the mean (SEM). Significances were calculated by ANOVA analysis (Statistica for Windows, release 5.1, StatSoft Inc., Tulsa, Oklahoma USA), if the variances were homogenous, as controlled by Bartlett's test. Statistically significant differences between the single groups were checked by the LSD *post-hoc* test. If an inhomogeneity of variances was detected, the values were transformed (logarithmic or square-root transformation) and consequently analyzed by ANOVA. Correlations were checked with Spearman's rank sum correlation test.

RESULTS

Endotoxin concentrations in peripheral venous blood were increased significantly in all patient groups with alcohol-induced liver disease (ALD) (Table 2). In patients with only minor signs of ALD (ALD1), as well as both of the other patient groups, the concentration of immunoglobulins of type IgA against fecal endotoxins was increased, but not that of IgG against the same kind of antigen (Table 2). Total concentrations of IgG were elevated only in alcohol abusers with the most severe stage of ALD (ALD3). Ferritin concentrations were also increased in all patient groups, especially in patients with severe liver disease (ALD2, ALD3).

IgA against *LPS* of intestinal origin was found to correlate closely with the calculated alcohol intake

Table 2. Concentrations of Acute-Phase Proteins, Endotoxins, and Total IgA and IgG Antibodies Against Fecal Endotoxins in Serum of Patients with Mild (ALD1), Moderately Severe (ALD2), and Severe (ALD3) Stages of Alcoholic Liver Disease*

	Healthy controls	*All ALD patients*	*ALD1 patients*	*ALD2 patients*	*ALD3 patients*
Endotoxin concentration (pg/ml)	2.4 ± 0.9	8.6 ± 1.4 $P = 0.009$	8.1 ± 1.6 $P = 0.009$	8.7 ± 3.5 $P = 0.029$	8.9 ± 2.3 $P = 0.012$
α1-Antitrypsin (mg/l)	191 ± 7	258 ± 13 $P = 0.001$	254 ± 16 $P = 0.004$	223 ± 23 N/S	301 ± 22 $P < 0.001$
C-reactive protein level > 0.5 mg/dl	0/9	17/30 $P = 0.003$	3/10 N/S	4/9 $P = 0.023$	10/11 $P < 0.001$
Ferritin (μg/l)	58 ± 17	481 ± 66 $P < 0.001$	295 ± 79 $P = 0.002$	698 ± 96 $P < 0.001$	435 ± 124 $P < 0.001$
IgA against fecal endotoxins (MU)	118 ± 29	248 ± 19 $P < 0.001$	211 ± 24 $P = 0.040$	276 ± 42 $P = 0.001$	260 ± 32 $P = 0.002$
IgG against fecal endotoxins (MU)	113 ± 22	75 ± 9.0 N/S	70 ± 16 N/S	80 ± 20 N/S	75 ± 12 N/S
Total IgA (mg/dl)	201 ± 36	605 ± 58 $P < 0.001$	366 ± 45 $P = 0.003$	554 ± 77 $P < 0.001$	864 ± 92 $P < 0.001$
Total IgG (mg/dl)	1370 ± 80	1669 ± 83 N/S	1368 ± 65 N/S	1576 ± 161 N/S	2019 ± 113 $P = 0.001$

*All values are means ± SEM, if not indicated otherwise; *P* values are given for comparisons to the group of healthy controls.

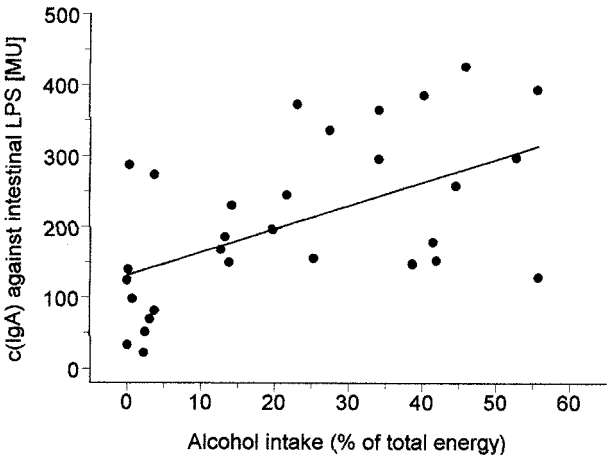

Fig 1. Correlation between the average relative alcohol intake (calculated as % of total energy intake) and the plasma concentration of IgA recognizing endotoxins deriving from fecal flora in healthy controls and patients with ALD. The solid line gives the linear correlation function ($y = 3.28 \times x + 131$; Pearson's correlation coefficient: $R = 0.5454$, $P < 0.001$).

(Figure 1), to AST activity in blood, and to C-reactive protein (CRP) (Table 3). Total IgG against LPS from the intestine, on the other hand, did not show a correlation to alcohol intake, AST, or γ-GT, but correlated negatively with the increase of CRP in plasma. Furthermore, the elevation of ferritin concentration in patients with ALD showed an association with increased levels of total IgA and IgA against fecal endotoxin (Table 3).

DISCUSSION

The elevation of total IgA in the plasma of patients with alcohol-induced liver disease (ALD) has been recognized for a long time (23, 24), and ALD has therefore been attributed to the IgA-associated disorders (25). IgA levels are increased in hepatic diseases of other origin also, but are related to more advanced stages of liver disease, especially cirrhosis. As can be derived from this and other studies (26, 27), the elevation of total serum IgA occurs even in the case of mild histopathological changes of alcohol-induced liver disease and can be closely related to daily alcohol consumption (25).

A reduced clearance of circulating IgA by hepatic tissue (26) and circulating monocytes (28) as a result of the toxic action of alcohol has been suggested as a possible reason for its elevated concentration, but the impaired catabolic clearance of IgA has been cast in doubt (29). These doubts are supported by the data of the present study, as IgA levels are significantly increased even in patients with minor hepatic changes (ALD1), in whom a nearly unchanged liver function can be assumed as shown by normal bilirubin and albumin concentrations in the plasma. Furthermore, in view of the normal jejunal IgA secretion in patients with ALD (30), the elevation of both monomeric and polymeric IgA in serum is unlikely to result from an impaired transport of this compound through the intestinal mucosa.

IgA-producing cells isolated from venous blood of alcohol abusers have been shown to proliferate spontaneously more frequently and to produce more IgA (31, 32). In addition, the increased serum concentration of IgA has been assumed to correspond to a higher number of circulating cells secreting IgA, whose number increases about threefold in alcohol abusers (31). This order of magnitude parallels the elevation of total serum IgA found in this study.

An elevated concentration of proinflammatory cytokines such as TNF-α and IL-1 has been measured in alcohol abusers (5), and endotoxins are suspected to contribute to the stimulation of cytokine release by the monocyte/macrophage system (6). This immunostimulation seems to be counteracted by a parallel increase of IL-6, which forms a positive secretory feedback loop with IgA in these patients (33). Both IgA and IL-6 were assumed to contribute to the limitation of inflammatory response (34, 35) and to the IgA commitment of B cells (36). In accordance with this hypothesis is the accumulation of IgA de-

TABLE 3. CORRELATION COEFFICIENTS OF ALCOHOL INTAKE (CALCULATED AS % OF TOTAL ENERGY INTAKE), ACTIVITY OF LIVER ENZYMES IN BLOOD, AND C-REACTIVE PROTEIN WITH CONCENTRATIONS OF ENDOTOXIN AND ANTI-LPS IMMUNOGLOBULINS

	Alcohol (% energy intake)	*AST*	*ALT*	*γ-GT*	*CRP*	*Ferritin*
LPS	0.5542†			0.3580*		
IgA (LPS)	0.5636‡	0.4505†	0.3590*	0.4012*	0.4281†	0.4431†
IgG (LPS)					−0.4561†	

*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

posits in hepatic sinusoids (26), as serum IgA has been shown to prevent the activation of the complement system and to inhibit phagocytosis and cellular cytotoxicity (37, 38). These findings lead to the assumption that IgA may bind antigens without induction of an inflammatory response, possibly ameliorating the inflammatory effect of gut-derived endotoxins on the liver after alcohol abuse.

Numerous antigens were suggested to play an important role as targets of IgA formed in patients with ALD. Components of the liver cell membrane (39), phospholipids (40), cytokeratin filaments (41), acetaldehyde adducts of proteins from the liver (42), dietary antigens (43), and bacterial compounds of intestinal origin were suspected to deliver epitopes for immunoglobuline production. Among bacterial products, *Escherichia coli* antigens (44), enterobacterial common antigen (45), and lipid A (19) were suggested to play a pivotal role in triggering lymphocyte activation. The importance of such antigens in the induction of IgA elevation in plasma of patients with ALD became evident as the level of this specific IgA decreased after alcohol withdrawal (44).

Bacterial overgrowth occurs in the upper gastrointestinal tract of alcohol abusers (13, 46). In the duodenum of these patients, gram-negative rods were found to be markedly more prevalent (13), implicating a higher load of endotoxins in the small intestine of alcoholics. Additionally, both the morphology and the function of the intestinal barrier is impaired by alcohol abuse (10) so that macromolecules can translocate through the intestine (12). The role of the molecular mass of the transmigrating endotoxins seems to be of special importance, since LPS with short carbohydrate chains or lipid A alone are not able to induce the release of comparable amounts of cytokines the way complete LPS does (47). The coincidence of these events, along with a decreased number of mucosal macrophages (48), may lead to endotoxemia (15) with the described onset of the inflammatory process in the liver. As lipopolysaccharides are able to induce a specific humoral immune response directed against the O-specific chain (49), formation of immunoglobulins against LPS can be expected as a consequence of systemic endotoxemia in patients with ALD. As evident from the data presented, the onset of IgA formation against gut-derived endotoxin occurs even in patients with mild forms of ALD (group ALD1). This finding underscores the importance of the immunological response to endotoxin challenge for the development of ALD, which is further corroborated by the close correlation between clinical markers specific for ALD and production of IgA specific for gut-derived LPS.

In conclusion, the results of this study and earlier investigations support the assumption that endotoxins derived from intestinal bacteria trigger the production of IgA, but not IgG, specific for these endotoxins in patients with ALD. Together with other factors such as the elevated release of IL-6, the enhanced production of IgA against gut-derived LPS might contribute to the limitation of inflammatory changes occurring in these patients, which can be assumed to be of crucial importance for the development of ALD.

ACKNOWLEDGMENTS

The authors are indebted to Ms. E. Feil for her technical assistance and Dr. J. Landig for sample collection. Kits for endotoxin measurement and the standard serum from 4000 individuals were a kind gift from Chromogenix, Mölndal, Sweden.

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