Haplotype HLA-B8-DR3 Confers Susceptibility to Hepatitis C Virus-Related Mixed Cryoglobulinemia

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Our aim was to investigate whether host genetic factors are involved in the onset of hepatitis C virus (HCV)-related mixed cryoglobulinemia (MC). We studied 25 consecutive patients presenting with a full-blown clinical picture of MC by physical examination, blood chemistry, assessment of cryoglobulins and their composition, nonorgan-specific autoantibodies, antibodies to HCV, serum HCV RNA, and HLA polymorphism. Biopsies of liver, bone marrow, and minor salivary glands were also performed in a number of patients. HLA results were compared with those of normal controls and patients with chronic HCV infection without MC and negative for autoimmune phenomena (pathological controls). Type II MC was found in 14 of 25 patients (56%), and type III MC was found in the remaining 11 (44%). All patients were positive for antibodies to HCV and/or serum HCV RNA. HLA-B8 was found in 40% (10 of 25) of patients compared with 10.1% (38 of 377) of normal controls (P = .00003, $P_{corrected}$ = .0005, relative risk [RR] 5.9) and 6.7% (2 of 30) of pathological controls (P = .007, $P_{corrected}$ = not significant). As for class II HLA molecules, only DR3 was significantly more frequent in MC patients (40%, 10 of 25) than in normal controls (15.1%, 57 of 377; P = .003, $P_{corrected}$ = .03, RR 3.7). Odds ratio (OR) for the risk of developing MC was calculated in patients positive for B8 and/or DR3, and the highest OR (8.2) was observed in individuals possessing both. The results suggest that the development of HCV-related MC is associated with HLA-B8 and DR3 markers.

MIXED CRYOGLOBULINEMIA (MC) is characterized by the presence of purpura, arthralgia and weakness associated with circulating immunoglobulins that precipitate when cooled and exhibit rheumatoid factor (RF) activity.[1] Based on the observation of frequent liver involvement in MC patients, hepatitis viruses have been investigated as possible etiologic agents. An initial report associating MC with hepatitis B virus infection[2] failed later to be confirmed,[3] whereas observations from different groups support a close association between MC and hepatitis C virus (HCV) infection.[4-6] HCV is a lymphotropic virus and persistent stimulation of the immune system by the virus seems to be responsible for the appearance of MC in a proportion of HCV-infected patients. Clinical signs of MC in HCV-infected patients are rather rare[7] in Mediterranean countries notwithstanding the high prevalence of HCV.[8] This would suggest that either viral or host genetic factors may be required for disease occurrence. To date, no significant association has been found between any HCV genotype and MC.[9] Likewise, the few attempts to associate host HLA profiles and MC[10-13] have been inconclusive. Therefore, we have analyzed HLA class I and II polymorphisms in patients with full-blown HCV-related MC.

MATERIALS AND METHODS

Patients. Twenty-five consecutive patients with MC took part in the study. Admission criteria were the presence of the typical cryoglobulinemic syndrome (purpura, arthralgia, and weakness associated with evidence of circulating cryoglobulins). Only one patient had a past history of blood transfusion, 11 of 25 had been treated with steroids and plasma-exchange, and none had been previously treated with interferon.

Thirty consecutive untreated patients with HCV-related chronic liver disease were prospectively enrolled as pathological controls. They all presented no signs of MC and were negative for nonorgan-specific autoantibodies and cryoglobulins tested at least twice at an interval of 2 months. The diagnosis of HCV-related chronic liver disease was based on clinical, serological, and histological criteria. Liver histology was available in all and was consistent with chronic hepatitis with mild to moderate activity in 23 and cirrhosis in 7. In both MC patients and pathological controls the apparent duration of disease was assessed on the basis of the appearance of MC signs or symptoms or the first detection of alanine aminotransferase (ALT) abnormality and/or anti-HCV positivity.

Clinical evaluation and laboratory studies. MC patients and pathological controls were evaluated at presentation by history, complete physical examination, and routine blood chemistry including proteinuria, serum transaminase, and creatinine levels.

The presence of cryoglobulins was evaluated in both groups as follows: 20 mL of blood was kept at 37°C for 2 hours in a glass tube. Serum was cleared by centrifugation at 2,000*g* for 15 minutes at room temperature and stored at 4°C for 7 days and examined daily. Samples were considered to be positive for cryoglobulins when a cryocrit of at least 0.5% was found and the heat resolubility of the cryoprecipitate was checked. Mixed cryoglobulins were classified as type II when a monoclonal component with RF activity was identified in the cryoglobulins by immunofixation using IgG, IgM, and κ and λ light chain monospecific antisera (Paragon IFE; Beckman Analytical, Milan, Italy). In addition serum RF was tested at 37°C by nephelometry using a commercial assay that detects IgM RF (RHF; Beckman Analytical) and antibodies to HCV were investigated by means of a second generation enzyme-linked immunosorbent assay (ELISA; Ortho Diagnostic System, Milan, Italy). A second-generation recombinant immunoblot assay (RIBA; Chiron Ortho Diagnostic System, Milan, Italy) was used as confirmatory test. All sera were tested for circulating HCV RNA by ''nested'' polymerase chain reaction (PCR) using primers derived from the highly conserved 5′ noncoding region of the HCV genome.[14] Nonorgan specific and double-stranded DNA autoantibodies were tested by indirect immunofluorescence on cryostat sections of rat liver, kidney and stomach, HEp-2 cell cultures (Kallestad, Austin, TX), and Crithidia luciliae cultures (Medic, Turin, Italy) at a serum dilution of

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1:40. Each positive serum was titrated to extinction. The presence of antiextractable nuclear antigen antibodies (anti-ENA) and antiactin antibodies were tested by counterimmunoelectrophoresis using a freshly prepared saline extract of lyophilized rabbit thymus acetone powder (Pel-Freez Biologicals, Rogers, AR) and purified actin, respectively. In 15 of the 25 MC patients C3 and C4 levels were measured by nephelometry using antisera to human complement factors (Behringwerke AG, Marburg, Germany).

Liver biopsy specimens were obtained in 15 MC patients who accepted to undergo the procedure. Similarly, a bone marrow biopsy was performed in 9 patients and a minor salivary gland biopsy in 3. Liver involvement was defined as alanine aminotransferase levels more than twice the upper normal limit for longer than 6 months. Kidney involvement was defined as urinary protein excretion greater than 0.15 g/dL and/or abnormal values of serum creatinine. Peripheral neuropathy was diagnosed on the basis of numbness, paresthesia, weakness, and loss of osteotendineous reflexes and confirmed by electromyography in four cases. Lymphoma was diagnosed by bone marrow biopsy. Sicca syndrome was diagnosed on the basis of xerophthalmia, xerostomia, abnormal Shirmer's test, and positive histology of minor salivary glands.[15]

HLA typing. The standard microcytotoxicity assay[16] was performed to determine HLA class I and II antigens using a panel of antisera from the 11th International Histocompatibility Workshop and several commercial antisera (Onelambda Inc, Los Angeles, CA). Results of MC patients were compared with those obtained by the same antisera in 377 healthy subjects (normal controls) and in the above described 30 pathological controls. Patients and controls were all Italian and their geographic origin had been carefully checked to compare subjects from the same areas.

Statistics. Statistical analysis was performed using χ^2 analysis with Yates' correction, Fisher's exact test, and Wilcoxon rank sum test when appropriate; probability (P) values were corrected ($P_{corrected}$) by the number of comparisons made (ie, depending on the number of variants and the number of groups tested) using Bonferroni's inequality method.[17] The strength of association between HLA antigens and MC was estimated by calculating the relative risk (RR) using the methods of Holdane.[18] Odds ratio (OR) for the risk of developing MC was calculated from 2×2 contingency tables, according to Svejgaard and Ryder.[19]

RESULTS

The median age of patients with MC was 59 years (range 43 to 75) with a median disease duration of 87 months (range 26 to 240). Both age and apparent duration of disease were not significantly different from those of pathological controls (median age 56 years, range 33 to 73; disease duration 102 months, range 30 to 294). Purpura, arthralgia, and weakness were present in all patients because they were required for admission into the study. As shown in Table 1 type II cryoglobulinemia was found in 14 patients (56%) and type III in 11. Liver involvement was found in 21 of 25 (84%): liver histology was available in 15 and consistent with chronic hepatitis with mild to moderate activity in 11 cases and cirrhosis in 4; in another 2 patients the diagnosis of cirrhosis was based on clinical and biochemical signs, and in 4 more patients there were ALT abnormalities but no histology was available.

Peripheral neuropathy was found in 15 of 25 patients (60%) and confirmed by electromyography in 4. The lower limbs were mostly involved. None of the patients had received a diagnosis of lymphoma before that of MC but in 9 of them (7 with type II MC) a bone marrow biopsy showed evidence of low-grade B-cell lymphoma according to the Updated Kiel Classification[20]

Table 1. Main Features of 25 Patients With Mixed Cryoglobulinemia (MC)

Case	Age, Sex	HLA B8/DR3	Cryo Type	Liver Involvement*	Peripheral Neuropathy	Lymphoma	Kidney Involvement	Sicca Syndrome
1	62F	–	III	+	+	nd	–	–
2	68F	–	II	+	+	nd	–	–
3	65F	–	III	+	+	nd	–	–
4	61F	–	III	+	–	nd	–	–
5	64F	B8	II	–	+	+	+	–
6	60F	–	II	+	–	+	–	–
7	49F	–	II	+	–	+	–	–
8	67F	B8-DR3	III	+	–	nd	–	–
9	43F	B8-DR3	II	+	–	nd	–	–
10	55M	–	II	+	–	+	–	–
11	54M	DR3	III	+	–	+	–	–
12	63F	B8	II	+	–	+	+	–
13	58F	–	III	+	–	nd	–	+
14	75F	–	III	+	+	nd	–	–
15	52F	B8-DR3	II	–	+	+	–	–
16	70F	B8-DR3	III	–	+	+	–	+
17	63F	–	III	+	+	nd	–	–
18	50F	DR3	II	+	+	nd	–	–
19	57M	–	III	+	+	nd	–	–
20	70F	B8-DR3	II	+	+	nd	–	–
21	50F	B8-DR3	III	–	+	nd	–	–
22	54F	–	II	+	+	nd	–	–
23	44F	–	II	+	+	+	–	+
24	72F	B8-DR3	II	+	+	nd	+	–
25	44F	B8-DR3	II	+	–	nd	–	–

Abbreviation: nd, not done.

*Cirrhosis in 6 (no. 2, 14, 17, 22, 24, 25); chronic hepatitis in 11, abnormal ALT without biopsy in 4 (no. 4, 6, 10, 20).

and the Revised European-American Lymphoma Classification (REAL).[21] Three patients (12%) showed signs of renal involvement and 3 of sicca syndrome.

All but 2 MC patients had a positive serum IgM RF. Antibodies to HCV were detected in 24 (96%) of the 25 patients. Serum HCV RNA was positive in 24 patients (96%), including the one who was anti-HCV negative. One patient was anti-HCV positive and HCV RNA negative. Nonorgan-specific autoantibodies were found in 5 (20%) cases. Four patients were positive for anti–smooth-muscle antibody with nonactin specificity (titer ranging from 1:40 to 1:160) and the remaining one had both anti–smooth-muscle antibody (antiactin, titer 1:320) and antinuclear antibody with homogeneous pattern (titer 1:320). All cases were negative for double-stranded DNA and antibodies to extractable nuclear antigens. In the 15 MC patients tested, normal levels of C3 were found, whereas C4 levels were below the lower limit of the normal range (15 to 45 mg/dL) in 10 (median 8 mg/dL, range 2 to 90).

HLA class I and class II allele frequencies in MC patients compared with pathological and normal controls are shown in Tables 2 and 3. Class I HLA-B8 was found in 40% (10 of 25) of patients compared with 10.1% (38 of 377) of normal controls ($P = .00003$, $P_{corrected} = .0005$, RR 5.9) and 6.7% (2 of 30) of pathological controls ($P = .007$, $P_{corrected}$ not significant [NS]). HLA-B18 was also more frequent in MC patients versus normal controls (32%, 8 of 25 *v* 13.3%, 50 of 377; $P = .017$, $P_{corrected}$ = NS). As for class II HLA antigens, only DR3 was significantly more frequent in MC patients (40%, 10 of 25) than in normal controls (15.1%, 57 of 377, $P = .003$, $P_{corrected} = .03$, RR 3.7), whereas no significant difference was found between MC patients and pathological controls. The frequency of the B8-DR3 phenotype was significantly higher in MC patients (32%, 8 of 25) than in healthy controls (6.1%, 23 of 377, $P = .00001$, $P_{corrected} = .0003$, RR 7.2) and pathological controls (6.7%, 2 of 30, $P = .03$, $P_{corrected}$ = NS). None of the MC patients were homozygous for B8/DR3 haplotype. No significant difference in HLA frequencies was found between pathological and normal controls.

Because both HLA-B8 and DR3 genetic markers were found to be associated with MC, we have calculated the OR for the risk to develop the disease in individuals positive for either or both HLA alleles (Table 4). The highest OR (8.2) was observed in subjects positive for both B8 and DR3, whereas B8-positive/DR3-negative or DR3-positive/B8-negative patients showed an OR of 3.1 and 1.3, respectively.

MC patients who were positive for B8 and/or DR3 did not show any significant clinical or biochemical difference in comparison with their negative counterparts (Table 1). How-

Table 2. HLA Class I Allele Frequencies in MC Patients Compared With Control Groups

Alleles	Patients (%) n = 25	Pathological Controls (%) n = 30	Normal Controls (%) n = 377	RR	p_cI	p_cII	p_cIII
A1	24.0 (6)	16.7 (5)	22.5 (85)	1.1	NS	NS	NS
A2	60.0 (15)	56.7 (17)	46.1 (174)	1.7	NS	NS	NS
A3	12.0 (3)	23.3 (7)	23.3 (88)	0.4	NS	NS	NS
A9	36.0 (9)	30.0 (9)	28.6 (108)	1.4	NS	NS	NS
A10	12.0 (3)	3.3 (1)	10.6 (40)	1.1	NS	NS	NS
A11	0.0 (0)	16.7 (5)	9.3 (35)	0.2	NS	NS	NS
A19	36.0 (9)	23.3 (7)	33.4 (126)	1.1	NS	NS	NS
A28	4.0 (1)	0.0 (0)	5.3 (20)	0.7	NS	NS	NS
B5	24.0 (6)	33.3 (10)	27.3 (103)	0.8	NS	NS	NS
B7	8.0 (2)	16.7 (5)	12.2 (46)	0.6	NS	NS	NS
B8	40.0 (10)	6.7 (2)	10.1 (38)	5.9	0.0005	NS	NS*
B12	16.0 (4)	13.3 (4)	15.4 (58)	1.0	NS	NS	NS
B13	4.0 (1)	3.3 (1)	6.1 (23)	0.6	NS	NS	NS
B14	16.0 (4)	3.3 (1)	6.9 (26)	2.6	NS	NS	NS
B15	20.0 (5)	13.3 (4)	8.5 (32)	2.7	NS	NS	NS
B16	8.0 (2)	13.3 (4)	9.0 (34)	0.9	NS	NS	NS
B17	0.0 (0)	10.0 (3)	8.5 (32)	0.2	NS	NS	NS
B18	32.0 (8)	13.3 (4)	13.3 (50)	3.1	NS†	NS	NS
B21	0.0 (0)	6.7 (2)	8.5 (32)	0.2	NS	NS	NS
B22	4.0 (1)	0.0 (0)	4.8 (18)	0.8	NS	NS	NS
B27	0.0 (0)	0.0 (0)	2.4 (9)	0.8	NS	NS	NS
B35	12.0 (3)	36.7 (11)	30.2 (114)	0.3	NS	NS	NS
B37	0.0 (0)	3.3 (1)	1.6 (6)	1.1	NS	NS	NS
B41	0.0 (0)	0.0 (0)	1.3 (5)	1.3	NS	NS	NS
B42	0.0 (0)	0.0 (0)	0.5 (2)	2.9	NS	NS	NS
B47	0.0 (0)	0.0 (0)	0.5 (2)	2.9	NS	NS	NS
B53	0.0 (0)	0.0 (0)	0.5 (2)	2.9	NS	NS	NS

p_cI = p_c patients versus normal controls; p_cII = p_c pathological controls versus normal controls; p_cIII = p_c patients versus pathological controls. RR was calculated comparing patients to normal controls. NS indicates Pc values less than 0.05. Symbols indicate all the differences that were significant before *P* values were corrected for the aggregate HLA antigens tested (Pc).

*$P = .007$.

†$P = .017$.

Table 3. HLA Class II Allele Frequencies in MC Patients Compared With Control Groups

Alleles	Patients % n = 25	Pathological Controls % n = 30	Normal Controls % n = 377	RR	p_cI	p_cII	p_cIII
DR1	12.0 (3)	16.7 (5)	16.7 (63)	0.7	NS	NS	NS
DR2	24.0 (6)	40.0 (12)	22.8 (86)	1.1	NS	NS	NS
DR3	40.0 (10)	23.3 (7)	15.1 (57)	3.7	0.03	NS	NS
DR4	0.0 (0)	6.7 (2)	15.9 (60)	0.1	NS	NS	NS
DR5	56.0 (14)	36.7 (11)	39.5 (149)	1.9	NS	NS	NS
DR6	24.0 (6)	30.0 (9)	26.3 (99)	0.9	NS	NS	NS
DR7	8.0 (2)	13.3 (4)	29.7 (112)	0.2	NS*	NS	NS
DR8	4.0 (1)	0.0 (0)	7.7 (29)	0.5	NS	NS	NS
DR9	0.0 (0)	0.0 (0)	1.1 (4)	1.6	NS	NS	NS
DR10	0.0 (0)	0.0 (0)	1.9 (7)	1.0	NS	NS	NS
DQ1	48.0 (12)	66.7 (20)	61.8 (233)	0.6	NS	NS	NS
DQ2	40.0 (10)	33.3 (10)	43.5 (164)	0.9	NS	NS	NS
DQ3	60.0 (15)	46.7 (14)	56.5 (213)	1.1	NS	NS	NS
B8/DR3	48.0 (12)	23.3 (7)	19.1 (72)	3.9	0.04	NS	NS
B8, DR3	32.0 (8)	6.7 (2)	6.1 (23)	7.2	0.0003	NS	NS*

*$P = .03$.

ever, type II MC was more frequent in the former than in the latter (67% *v* 46% , 8 of 12 *v* 6 of 13, P = NS).

DISCUSSION

Recent evidence[4-6] supports the ethiopathogenetic role of HCV in MC. However, it is noteworthy that cases of full-blown MC seem to be rare in comparison with the high prevalence of the virus as observed by Lunel et al[7] who have reported clinical signs of MC in 14% of their patients with HCV infection. This discrepancy does not appear to depend on viral factors, because multiple HCV genotypes have been detected in association with MC and the high prevalence of genotype II reported by Pozzato et al[9] mirrors the overall distribution of that same genotype in the same geographic area. Therefore, we have investigated whether, in the presence of HCV infection, host genetic factors may predispose to the onset of MC, as suggested by Agnello.[22]

We have found a significant association with class I HLA-B8 and class II HLA-DR3 alleles. Although the association was stronger with HLA-B8 than with DR3, it is remarkable that 8 of 25 (32%) MC patients were positive for HLA-B8 and DR3 and that the calculated OR (8.2) was higher for the haplotype B8-DR3 than for each single specificity (3.1 and 1.3, respectively). HLA typing of pathological controls did not differ from that of normal controls, confirming the lack of genetic predisposition to HCV-related liver disease.[23]

The significantly increased frequency of HLA-B8 in our patients does not seem to depend on an inappropriate selection of normal controls because they were matched with patients for geographic origin (Northern and Central Italy). Moreover, the 10.1% prevalence of B8 observed in our controls is in accordance with the North (20%) to South (8%) gradient of B8 frequency reported in our country.[24] Furthermore, our results cannot be attributed to the enrollment of autoimmune patients. In fact, markers of autoimmunity and sicca syndrome, which are both known to be associated with B8 and DR3, were present only in a minority of our cases. Of the five positive for nonorgan-specific autoantibodies, only one was B8 positive and only one out of three with sicca syndrome was B8-DR3 positive.

Table 4. Odds Ratio for the Risk of HCV-Related MC in Patients Positive for HLA-B8 and/or DR3

HLA		Patients	Controls	
B8	DR3	(n = 25)	(n = 377)	OR
−	−	13 (52%)	305 (80.9%)	
+	−	2 (8%)	15 (3.9%)	3.13
−	+	2 (8%)	34 (9.0%)	1.38
+	+	8 (32%)	23 (6.1%)	8.20

Before HCV was discovered, an early Italian attempt[10] to investigate genetic predisposition to MC failed to detect any significant association of the disease with either class I or class II HLA molecules. However, it should be pointed out that in our cohort HCV positivity was 100% but unknown in theirs; these conflicting results are, thus, not easily explained. Both Migliorini et al[10] and we studied patients from the same geographic area, selected on the basis of the same criteria and compared with controls showing similar frequency of HLA-B8 and DR3. Recently Ossi et al[11] have reported an increased frequency of HLA A9/A24, B51, and B35 in 16 patients with HCV-related MC. The design of their study is superimposable to ours in terms of both selection of patients and controls but unfortunately the investigators do not give any statistical evaluation of their data. The hypothetical protective role of DR7 is, similarly, statistically unproven. We have not found a positive association between MC and A9/A24, B51; B35 as well as DR7 were less frequent in patients than in controls. Of these, only DR7 showed a $P < .05$ value before correction. A further study by Congia et al[12] on Sardinian patients, selected among a polytransfused thalassemic anti-HCV population, reports a protective role for the DR2 subtype DRB1*1601 for HCV infection. Our data do not confirm this observation but are consistent with the reported increase in frequency of DRB1*0301 in patients with extrahepatic features. Similarly an increased frequency of DR3 in HCV infected patients with cryoglobulins was preliminarily reported by Hwang et al.[13]

It is known from HLA population genetics that a strong

linkage disequilibrium exists between B8 and DR3 alleles and that both belong to the extended haplotype B8,C2C,BSF, C4AQ0,C4B1,DR3,DQ2. The close association between the loci included in this haplotype makes it hard to say whether one or multiple loci have a role in susceptibility to disease. This extended haplotype is known to be overrepresented in several autoimmune diseases in which different primary associations have been proposed. In celiac disease[25] and insulin-dependent diabetes mellitus[26] the strongest association is reported with DQ molecules, in autoimmune hepatitis DRB1 and DRB3 loci are primarily involved,[27] whereas a deletion of C4A (described at proteinic level as C4AQ0) seems associated with systemic lupus erythematosus.[28] Such deletion results in a partial insufficiency of C4, which impairs the clearance of viruses and immunocomplexes.[29] Patients with C4 deficiency are thus susceptible to develop immunocomplex diseases,[30] and animal models with genetic C4A deletion share features of MC, ie, IgM rheumatoid factors and immunocomplexes.[31] Our data suggest that the genetic predisposition to MC is associated with the phenotype B8-DR3, the strongest association being with B8, and that HLA-B8 and DR3 may be considered risk factors for HCV-related MC in addition to cirrhosis.[7] Moreover, it is possible that patients exhibiting the B8-DR3 phenotype may carry the C4A deletion and that the low C4 levels measured in a proportion of our MC patients may be secondary to both complement consumption by the disease process itself and C4A deletion. This view is also supported by preliminary results on C4 allotyping in MC patients (data not presented) that showed an increased frequency of C4AQ0 phenotype, thus suggesting a possible role of class III molecules in MC susceptibility.

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