Hepatitis B virus causes mixed cryoglobulinaemia by driving clonal expansion of innate B-cells producing a VH1-69-encoded antibody

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ABSTRACT

Objective. *To investigate the expression of a VH1-69-encoded idiotype, and the phenotypic and functional features of monoclonal B-cells from patients with type II mixed cryoglobulinaemia (MC) secondary to chronic hepatitis B virus (HBV) infection.*

Methods. *B-cell immunophenotype and expression of a VH1-69-encoded idiotype were investigated by flow cytometry. B-cell proliferative responses to stimuli were investigated by the CFSE dilution assay.*

Results. *Two out of five patients with chronic HBV studied had massive monoclonal expansion of VH1-69-expressing B-cells.*
These cells had the peculiar CD21^{low} phenotype and low responsiveness to stimuli typical of the VH1-69-expressing B-cells commonly expanded in MC secondary to hepatitis C virus (HCV) infection. In both patients, anti-HBV therapy led to the regression of MC and of VH1-69^{+} B-cell expansion.

Conclusion. *VH1-69-encoded antibodies are known to preferentially recognise a variety of viral proteins including HCV E2, influenza A virus haemagglutinin and HIV gp41/gp120, and may serve as innate first line antiviral defense. Thus, like HCV, HBV*
may cause MC by protracted antigenic stimulation of VH1-69-expressing B-cells.

Introduction

Type II mixed cryoglobulinaemia (MC) is a monoclonal lymphoproliferative disorder of marginal zone (MZ) B-cells producing a natural poly(auto)-reactive IgM antibody endowed with rheumatoid factor activity. Immune complexes formed by this antibody precipitate at t° less than 37°, causing small-vessel vasculitis characterised by purpura, arthralgia and involvement of other organs, and ranging from mild to life-threatening (1). MC is secondary to chronic HCV infection in more than 90% of cases (1); in a minority of cases, MC appears to be associated with HBV infection (2). Over time, chronic stimulation of B-cells by these viruses may give rise to genetic changes and evolution to overt lymphoma (3). A direct role for HCV and HBV in the pathogenesis of MC is supported by the observation that the forms associated with HCV regress upon successful interferon-based therapy (1), whereas the forms associated with HBV regress after therapy with telbivudine, a drug selectively active on this virus (2).

The monoclonal B-cells expanded in HCV-associated MC typically express an idiotype encoded by the VH1-69 heavy chain variable gene (4). These VH1-69^{pos} B-cells have a peculiar phenotype characterised by low levels of CD21 (CD21^{low} B-cells), high levels of the inhibitory receptors FCRL4, CD22 and CD95 and of the homing receptor CD11c, and low levels of CD62L (5, 6). In addition, these cells appear to be functionally anergic since they fail to proliferate in response to stimulation of the B-cell receptor (BCR) and of Toll-like receptor-9 (TLR9) (5, 6).

We report here two patients with HBV-associated MC who had a massive expansion of VH1-69-expressing B-cells; these cells displayed phenotypic and functional features identical to those of the VH1-6^{pos} B-cells expanded in patients with HCV-associated MC. Both MC and VH1-69^{pos} B-cell expansion regressed in these patients after successful antiviral therapy. These findings indicate that both HCV and HBV can cause cryoglobulinaemia by driving the clonal expansion of MZ B-cells producing a VH1-69-encoded natural antibody.

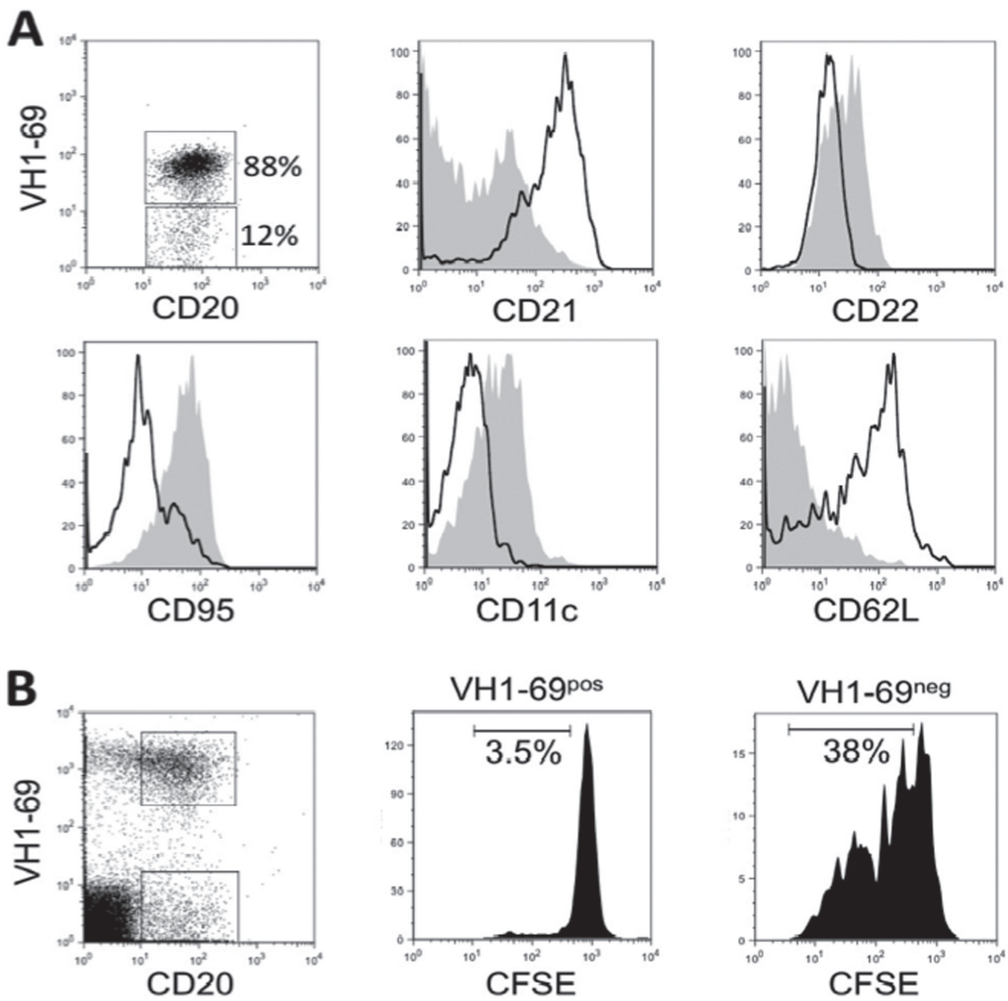

Fig. 1. Phenotype and function of VH1-69^{pos} B-cells from patient 1. **A**: CD20^{pos}VH1-69^{pos} and CD-20^{pos}VH1-69^{neg} B-cells are electronically gated within PBMC (dot plot) and analysed for surface receptor expression. VH1-69^{pos} B-cells (grey histograms) express lower levels of CD21 and differentially express homing (CD11c and CD62L) and inhibitory (CD22 and CD95) receptors compared to VH1-69^{neg} B-cells (open histograms). **B**: CD20^{pos}VH1-69^{pos} and CD-20^{pos}VH1-69^{neg} B-cells are electronically gated within CFSE-labelled PBMC stimulated with CpG for 5 days (dot plot). CFSE dilution analysis (histograms) reveals a strikingly lower percent of divided cells in VH1-69^{pos} compared to VH1-69^{neg} B-cells.

Materials and methods

Two HBV-infected patients with MC vasculitis were investigated by virologic and immunologic assays. For the determination of the cryocrit blood samples were drawn with pre-warmed syringes and tubes, allowed to clot and centrifuged at 37°C for serum separation. Sera were kept at 4°C for 7 days and cryocrit was expressed as the percentage of serum volume after centrifugation. The HBV DNA load in serum was determined by real-time PCR (COBAS® AmPliprep; Roche Diagnostics) with a limit of detection of 6 IU/mL. The HCV RNA load in serum was determined by qualitative real-time PCR (COBAS® Amplicor HCV Test, v2.0; Roche Diagnostics) with a limit of detection of 50 IU/mL. Blood samples were processed at 37°C to avoid the possible loss of HBV or HCV virions caused by co-precipitation with cryoglobulins.

Immunophenotyping and B-cell proliferation studies were performed as previously described (4, 6). Briefly, peripheral blood mononuclear cells (PBMC) were obtained by density-gradient centrifugation and stained with combinations of fluorochrome-labelled monoclonal antibodies (mAb), all from Becton-Dickinson. CD20^{+} B-cells were electronically gated, and VH1-69^{pos} cells were identified using the G6 mAb (kindly provided by R. Jefferis, Birmingham, UK), which binds an epitope of the VH1-69 gene product (4). Cell proliferation was measured after 5-days stimulation with the TLR9 ligand CpG (Sigma Genosys The Woodlands, TX; 2.5 μg/mL) by the carboxyfluorescein di-acetate succinimidyl ester (CFSE) dilution assay, as described (6). The number of cells entering proliferation (percent of divided cells) was calculated using the FlowJo software (Tree Star, Ashland, OR).

Results

Patient 1

A 37-year-old woman with purpura and arthralgia had a diagnosis of type II MC, with a serum cryoglobulin level (cryocrit) of 78%, low levels of C4 and positive RF. Immunofixation of the cryoprecipitate identified a double component of monoclonal IgM and polyclonal IgG. Renal function was normal and the patient did not complain neurological symptoms such as dysesthesia and paresthesia. ANA, anti-ENA, ANCA, AMA, ASMA and anti-CCP were undetectable. Anti-HCV antibodies and HCV RNA were repeatedly negative in the cryoprecipitate and in plasma, prepared at 37°C to avoid false-negative results for co-precipitation of HCV antibodies and RNA with cryoglobulins. HIV serology was negative. She had persistently normal ALT levels, and was HBsAg-positive, anti-HBsAg negative, anti-HBcAg

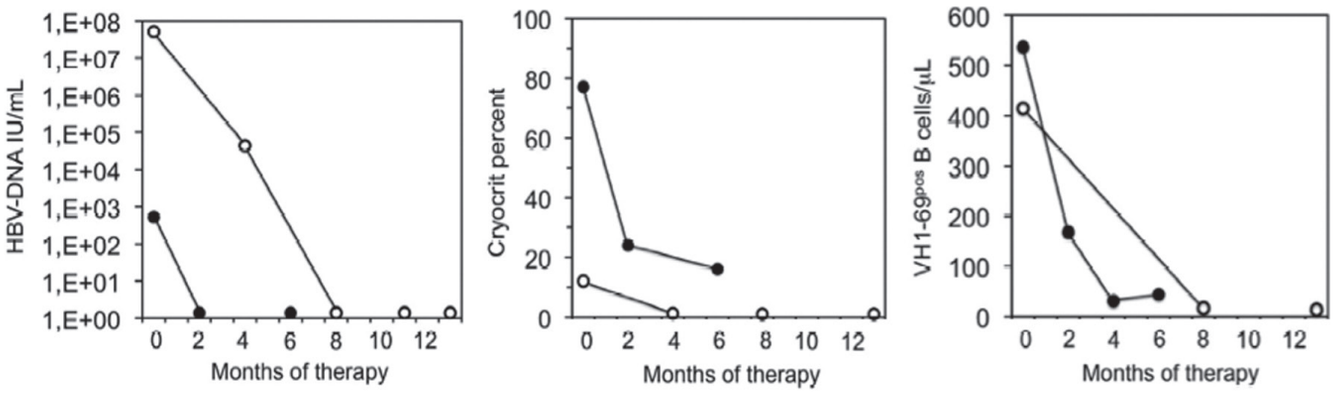

Fig. 2. Changes of HBV-DNA, cryocrit level and number of circulating VH1-69^{pos} B-cells after anti-HBV therapy. Closed circles denote patient 1, and open circles patient 2.

IgG positive, anti-HBcAg IgM negative, HBeAg negative and anti-HBeAg positive; HBV-DNA was 527 IU/ml immediately before therapy. Flow cytometry revealed a population of monoclonal B-cells, resembling human MZ B-cells by the co-expression of IgM and CD27, which expressed an idiotype encoded by the VH1-69 gene (Fig. 1A) associated with ϰ-chain. These VH1-69^{pos} B-cells accounted for 88% of circulating B-cells compared to less than 6% in normal individuals (5), and their absolute number was 537 cells/µL compared to less than 18 cells/µL in normal individuals. Detailed immunophenotyping (Fig. 1A) revealed that this patient's VH1-69^{pos} B-cells displayed the peculiar phenotype of VH1-69^{pos} B-cells from patients with HCV-associated MC, namely reduced expression of CD21 and of CD62L, and increased expression of CD11c, CD22 and CD95 (5, 6). A further similarity with the CD21^{low} B-cells of patients with HCV-associated MC was anergy (5, 6), since like those cells patient's VH1-69^{pos} B-cells failed to proliferate in response to TLR9 stimulation with CpG (Fig. 1B). The patient was treated with pegylated interferon alfa-2b (Pegintron, 1.5 µg/kg/week). Since ALT levels were normal in four three-monthly measurements during the year before treatment, and HBV viraemia was low, off-label use of pegylated interferon alfa-2b was requested to and approved by the Ethics Committee of Policlinico Umberto I, Sapienza University of Rome; written informed consent was obtained from the patient. After 6 months of therapy HBV-DNA was undetectable, purpura had cleared, the cryocrit had decreased to 16%, whereas C4 levels and RF remained stable. The absolute number of circulating VH1-69^{pos} B-cells had decreased to 44/µl (Fig. 2).

Table I. Patients' characteristics and B cell phenotype.

Pt. no.	Age / sex	HBV status	Total B cells (/µl)	VH1-69^{+} B cells (/µl)	B cell phenotype
1	37/f	Chronic hepatitis HBV DNA pos	610	537	CD27^{+}IgM^{+}κ^{+}CD21^{low}(98%) CD11c^{+}(30%) CD62L^{neg}(88%)*
2	62/m	Chronic hepatitis HBV DNA pos	628	415	CD27^{+}IgM^{+}κ^{+}(99%) CD21^{low}(60%) CD11c^{+}(35%) CD62L^{neg}(70%)*
3	54/m	Chronic hepatitis HBV DNA pos	178	0	CD27^{+}(40%) IgM^{+}(70%) κ^{+}(70%) CD21^{low}(65%)**
4	58/f	Chronic hepatitis HBV DNA pos	57	0	CD27^{+}(60%) IgM^{+}(80%) κ^{+}(67%) CD21^{low}(35%)**
5	88/f	Chronic hepatitis HBV DNA pos	32	0	CD27^{+}(73%) IgM^{+}(74%) κ^{+}(63%) CD21^{low}(75%)**
6	65/f	Resolved infection HBV DNA neg	90 1	57	CD27^{+}IgM^{+}κ^{+}(95%) CD21^{low}(76%) CD11c^{+}(43%)*

*percent of VH1-69^{+} B cells. **percent of total B cells.

Patient 2

A 62-year-old man with purpura, arthralgia and peripheral neuropathy had a diagnosis of type II MC with 12% cryocrit. Electromyographic indices confirmed a sensory peripheral polyneuropathy and laboratory analysis indicated C4 hypocomplementemia and positive RF. Immunofixation of the cryoprecipitate identified a double component of monoclonal IgM and polyclonal IgG. ANA, anti-ENA, anti-CCP, ANCA, AMA and ASMA were negative and renal function was normal. HCV antibodies and RNA were repeatedly negative; HIV serology was negative. He had persistently increased ALT levels, and was HBsAg-positive, anti-HBsAg negative, anti-HBcAg positive, HBeAg negative and anti-HBeAg positive; HBV-DNA was 51 x 10^6 IU/ml immediately before therapy. Flow cytometry disclosed a population of monoclonal VH1-69^{pos} κ-positive B-cells, accounting for 66% of circulating B-cells and with an absolute number of 415 cells/µL. Patient's VH1-69^{pos} B-cells were mostly CD21^{low} and expressed the same pattern of inhibitory and homing receptors seen on VH1-69^{pos} B-cells of patient 1 (not shown). No functional studies of anergy were done in this case. The patient was treated with tenofovir 245 mg/day; after 13 months of therapy, HBV-DNA was undetectable, purpura had cleared, cryoglobulins and RF were undetectable and C4 levels remained low. The absolute number of circulat-

ing VH1-69^{pos} B-cells had decreased to 14/μl (Fig. 2).

Discussion

MC secondary to HBV infection is rare (2). We reviewed the prevalence of HCV and HBV infection in 153 MC patients consecutively enrolled at our referral center. Chronic HCV infection was documented in 136 patients (89%), 23 of which (17%) were co-infected with HBV. In these patients clonal expansion of VH1-69^{pos} κ-positive B-cells was found in 32% of cases (44/136). Of 17 HCV-negative patients, 11 had apparently idiopathic MC, whereas 6 had evidence of active (5 pts) or resolved (1 pt) HBV infection. Clonal expansion of VH1-69^{pos} B-cells was seen in two out of five HBV-DNA-positive patients; in the other three patients, expanded populations of VH1-69^{neg} putative monoclonal B cells with an IgM^{+}k^{+}CD21^{low} phenotype were detected (Table I). Interestingly, one patient with resolved HBV infection (HBsAg and HBV DNA negative, HBsAb and HBcAb positive) presented clonal expansion of VH1-69^{pos} B-cells that accounted for approximately 30% of total circulating B cells. No data were available to determine the timing of HBV infection and healing in this case. Long-term persistence of exhausted monoclonal VH1-69^{pos} B-cells in patients with HCV-associated MC after the eradication of the infection has been previously observed (7); thus, it is possible that in this patient significant proportions of VH1-69^{pos} B-cells survived after the driving stimulus induced by HBV was withdrawn.

The VH1-69^{pos} B-cells accumulating in our patients with HBV-associated MC were identical, concerning CD21^{low} phenotype and anergy, to the VH1-69^{pos} B-cells found in patients with HCV-associated MC (6, 7).

In some patients, MC appears to be sustained by occult HCV infection (8). Thus, it could be argued that in the patients described here MC and VH1-69^{pos} B-cell expansion were caused by occult HCV rather than by the HBV infection. A compelling argument against this possibility is the response of patient 2 to tenofovir, a drug that, to the best of our knowledge, is not active on HCV. Cryoglobulins became undetectable at month 4 in the patient treated with tenofovir, although serum HBV DNA was still detectable at that time point; by contrast, in the patient treated with pegylated interferon cryoglobulins and circulating monoclonal B cells were still present at month 6, although reduced by 80% and 90% respectively, despite she had negative HBV viraemia since month 2. It is likely that incomplete clearance of cryoglobulins and clonal B cells in the latter patient depended on their very high baseline levels rather than on differences in their dependence on active infection. Indeed, persistence of_significant cryoglobulin levels for up to six months after the end of therapy with pegylated interferon alfa-2b and ribavirin was observed by Cacoub *et al*. (9) in patients with virologic response but high initial cryocrit.

The nature of the repertoire of antibodies encoded by the VH1-69 gene is of remarkable interest for human pathology. Antibodies directed to the E2 envelope glycoprotein of HCV are preferentially encoded by the VH1-69 gene (10). Recently, it has been shown that VH1-69 also preferentially encodes antibodies directed to the influenza A virus haemagglutinin stem (11), to the gp120 envelope glycoprotein of HIV (12), and to the cytomegalovirus phosphoprotein pUL 32 (13). Importantly, stereotyped VH1-69-encoded antibodies are expressed in 25% to 30% of unmutated CLL (U-CLL) and in a relatively large proportion of normal B-cells, which are believed to be the precursors of U-CLL cells (14). It has been suggested that this restricted repertoire of VH1-69-encoded IgM is evolutionarily shaped to recognise common pathogens, whose antigenic pressure may drive the development of U-CLL (14). Indeed, VH1-69-encoded antibodies derived from some U-CLL have broad cross-reactivity with HIV gp41, HCV/E2, influenza viruses, intestinal commensal bacteria and double-stranded DNA (15). Collectively, these data suggest that human VH1-69-encoded antibodies, like the natural poly(auto)-reactive antibodies produced by murine MZ B-cells (20), may serve as innate first line defense against several microorganisms posing, however, the risk of infection-driven lymphoproliferative disorders (16).

In conclusion, our findings indicate that HBV, like HCV, can cause MC through the activation of VH1-69-expressing B-cell clones. The similarity between HCV-associated and HBV-associated MC is strengthened by the observation that the VH1-69^{pos} B-cells expanded in these disorders share a peculiar memory phenotype (CD27+IgM+IgD+) characterised by the expression of CD11c, low expression of CD21 and CD62K, and larger cell size (4). In addition, VH1-69^{pos} B-cells from HCV- and HBV-associated MC share the functional features of anergy (5, 6). Because of the frequent usage of the VH1-69 gene in natural responses to several viruses, it is tempting to speculate that recognition of an HBV antigen by VH1-69-expressing B-cells underlies lymphoproliferation in HBV infection.

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