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Recognition of core-derived epitopes from a novel HBV-targeted immunotherapeutic by T-cells from patients infected by different viral genotypes

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ABSTRACT

Hepatitis B virus (HBV) infects millions of people worldwide and is a leading cause of liver cirrhosis and hepatocellular carcinoma. Current therapies based on nucleos(t)ide analogs or pegylated-interferon-α lead to control of viral replication in most patients but rarely achieve cure. A potential strategy to control chronic hepatitis B is to restore or induce functional anti-HBV T-cell immune responses using HBV-specific immunotherapeutics. However, viral diversity is a challenge to the development of this class of products as HBV genotypes display a sequence diversity of up to 8%. We have developed a novel HBV-targeted immunotherapeutic, TG1050, based on a non-replicative Adenovirus vector encoding a unique and large fusion protein composed of multiple antigenic regions derived from a HBV genotype D sequence. Using peripheral blood mononuclear cells from 23 patients chronically infected by five distinct genotypes (gt A, B, C, D and E) and various sets of peptides encompassing conserved versus divergent regions of HBV core we have measured ability of TG1050 genotype D core-derived peptides to be recognized by T-cells from patients infected by various genotypes. Overall, PBMCs from 78% of genotype B or C- and 100% genotype A or E-infected patients lead to detection of HBV core-specific T-cells recognizing genotype D antigenic domains located both in conserved and variable regions. This proof-of-concept study supports the clinical development of TG1050 in large patient populations independently of infecting genotypes.
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1. Introduction

Infection by hepatitis B virus (HBV) is one of the major public health challenges worldwide. While the availability of a preventive vaccine has reduced the number of new HBV infections, it does not benefit the 370 millions of chronic hepatitis B (CHB) patients already infected by the virus. Approximately one third of these chronically infected individuals will die from serious liver disease, such as cirrhosis, hepatocellular carcinoma and liver failure [1]. Current CHB therapies include nucleos(t)ide analogs and pegylated-IFNα but despite their ability to control HBV replication in the great majority of patients and to improve liver histology, complete HBV cure is achieved in only 3–5% of patients [1].

Immune correlates of control of viral replication have been described in cohort studies of patients resolving infection and involve mainly the development of broad, robust and polyfunctional CD8+ and CD4+ T-cell responses targeting multiple HBV antigens among which the critical core antigen [2–4]. In CHB patients, the breath and the magnitude of these immune responses are reduced and the antigen specificity is narrowed [5]. Because of such strong interplay between T-cell immunity and control of viral replication, efforts at developing HBV-specific immune-based interventions are gaining attention. These have for objective to induce and/or recall immune responses similar to those found in HBV resolvers. The development of this novel class of agents, also referred to as immunotherapeutics or therapeutic vaccines [6] meets with a number of challenges: choice

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Table 1
Characteristics of patients.

Patient	Viral load*	HBV status	Genotype
1	7100	IC	B
2	3700	CONT	B
3	2900	CONT	C
4	<2000	CONT	C
5	<70	CONT	C
6	3852	IC	C
7	7800	IC	C
8	<2000	CONT	D
9	>1.10^8	AC	D
10	1 .3 10^6	AC	D
11	1932	IC	D
12	593	IC	D
13	1594	IC	D
14	11,400	CONT	A
15	23,000	IC	A
16	1129	IC	A
17	105	IC	A
18	8200	CONT	A
19	<2000	CONT	E
20	28,400	CONT	E
21	4700	IC	E
22	17,483	IC	E
23	19,736	IC	E

* Viral load at the time of PBMC sampling (copies/mL).
HBV status: CONT: chronic HBsAg carrier with control of viral replication (spontaneously or under therapy). AC: chronic HBsAg carrier with high viral load and chronic active viral hepatitis. IC: inactive HBsAg carrier with chronic hepatitis.

of broad and most appropriate antigens, capacity of the vaccine vector to induce sustained T-cell based immunity in face of virus-specific T-cell exhaustion typically found in the CHB carriers, generation of cytolytic-based mechanisms to eliminate infected cells and associated covalently closed circular DNA without exacerbating liver inflammation, finally accounting for the genetic diversity of HBV viruses (10 different genotypes) [7]. TG1050 is a novel immunotherapeutic product based on a non-replicative Adenovirus 5 vector encoding a unique and large fusion protein composed of modified HBV core and polymerase and selected domains of the Env proteins. TG1050, based on a genotype (gt) D HBV sequence was evaluated in a variety of HBV-naïve mouse lines as well as in HBV-persistent mouse models including the model described by Dion et al. [8], in which TG1050 was shown to display antiviral activities [9]. The core antigen is highly immunogenic and described to contain epitopes associated with resolution of infection [10], it thus represents a key antigen in TG1050. Hence, it is important to assess whether TG1050-encoded core has the capacity to induce broadly reactive T-cells recognizing epitopes present in CHB patients infected by non-genotype D isolates in order to support a broad application of TG1050 in the clinic. We report here a human cohort study designed to analyze the capacity of TG1050 encoded core peptide epitopes to stimulate and recall functional T-cell responses in CHB patients infected by genotype A, B, C, D and E.

2. Methods

2.1. Patients and samples

HBV chronically infected patients (Table 1) from previous cohorts [11,12] in whom HBV was genotyped and with frozen peripheral mononuclear cells (PBMC) available were selected. The ethics committee of the Hospital Cochin (Paris, France) approved the study and all patients gave informed, written consent for participation, in line with French ethical Guidelines.

2.2. Sequence analysis of HBV core gene

The genomic region covering the core gene was amplified and sequenced from eight patients (3 gt D, 2 gt B/C, 1 gt E, 2 gt A) for whom sera was available. Viral DNA was extracted with QIAgen DNA Blood kit (Qiagen) according to manufacturer's protocol. A 700-bp fragment was amplified in a two-step nested polymerase chain reaction using HBV-specific primers [13]. Polymerase chain reaction products were directly sequenced and the obtained sequences were analysed using Serial cloner 2.6 software (serialBasics.com) and aligned with Clustal software (EMBL-EBI).

2.3. Core sequences and peptide pools

Core sequences spanning from amino-acids (aa) 1–148 of the TG1050-encoded core protein derived from a gt D sequence (GenBank No. Y07587) as well as from consensus sequences representative of gt A, B, C and E were aligned (Fig. 1). Consensus sequences were obtained after alignment, for a given genotype, of 100 Core sequences downloaded from GeneBank. The consensus sequence from gt B and C were identical and thus assimilated to B/C sequence. Sequences were used to derive genotype-specific peptide libraries composed of 29 peptides (>75% purity, ProImmune, Oxford) consisting of 15 aa (except for peptide 17 and homologue peptide 46—supplementary Table 1) overlapping by 11 aa (full pool (FP) Nos. 1, 2, 6 and 7 spanning respectively aa 1-127 of gt B/C, D, A and E). Additional peptide pools were constituted to gather either peptides conserved (CP Nos. 5, 8, 9) or divergent (DP Nos. 4, 10, 12) between each genotype i.e. gt A, B/C, E and the gt D sequence (Supplementary Table 1A). Peptides were solubilized in 20% acetonitrile 80% water at a concentration of 10 mg/mL. Peptide pools were then generated and diluted in RPMI medium at a 1 mg/mL final concentration and aliquots frozen.

2.4. PBMC expansion and ELIspot assay

PBMC were first *in vitro* expanded to generate short-term cell lines. Briefly, after washing and an overnight rest in complete RPMI medium containing 10% human sera AB, cryo-preserved PBMC were washed and suspended at 2 to 3.10^6 cells/mL in complete medium with 20 ng/mL IL-7 (Cytheris SA, Issy les Moulineaux, France) and pool of peptides. Half of the medium was replaced every 2–3 days with a complete medium supplemented with recombinant IL-2 (50 IU/mL) (Roche, Meylan, France) as described [12,14,15]. After 10 days of culture, HBV core-specific-IFN-γ-producing cells were quantified by ELISpot assays [16]. Briefly, sterile nitrocellulose HA 96-well plates (Millipore Bedford, MA, USA) were coated with anti-IFN-γ mAb and incubated overnight at 4 °C. The coated wells were filled in triplicate with *in vitro* stimulated cells (2.10^5/well) with the appropriate HBV pools of peptides (1 μg/mL of each peptide), or with medium and solvent alone as a negative control, with phorbol myristate acetate (12.5 ng/mL)/ ionomycin (1 μg/mL) or the pool of 32 control peptides derived from common pathogens (CEF, Anaspec, San Jose, CA, USA) (62.5 ng/mL) as positive controls. A BioCys ELISpot automatic counter was used to score the number of spots. Responses were scored positive if: (1) the median number of spots obtained for cells cultured with peptides was twice the median number of spots obtained for cells cultured with medium alone and (2) if five specific spots were found per well. Only positive results are shown as the median value of the specific IFN-γ-producing cells (median number of spots observed with medium alone subtracted) obtained for triplicate wells, related to 10^6 cells.

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