SYNOPSIS OF RESEARCH REPORT PROTOCOL ML18675

Immunology Sub-Study of Study ML 18253

COMPANY: Roche NAME OF FINISHED PRODUCT: PEGASYS®, Ro 25-8310 NAME OF ACTIVE SUBSTANCE(S): pegylated interferon alfa-2a (40 KD)	(FOR NATIONAL AUTHORITY USE ONLY)

TITLE OF THE STUDY: / REPORT No.:	Longitudinal analysis of HBV-specific T cell responses in patients with HBeAg-negative chronic hepatitis B (CHB) treated with pegylated interferon alfa-2a (40 KD) (PEGASYS®, Ro 25-8310). Immunology sub-study for a selected subgroup of patients participating in study ML 18253	
DATE OF REPORT:	22nd June 2012	
INVESTIGATORS / CENTERS AND COUNTRIES:	Three principal Investigators/Three investigational study sites in Italy	
PUBLICATION (REFERENCE):	Not applicable	
PERIOD OF TRIAL:	Date first patient enrolled: 28 Jul 2005; Date last patient completed: 22 Aug 2007	CLINICAL PHASE: IIIB
OBJECTIVES:	This was an exploratory immunological investigation carried out to understand the extent and nature of the antigen-specific and non-specific immune defects in patients with HBeAg-negative CHB and their modulation by therapy with Pegylated-Interferon-α-2a (PEG-IFN) during the first 6 months of treatment in the progenitor study ML 18253. The objective of the study was to analyze the impact of the combined anti-viral and immunoregulatory functions of IFN-alpha treatment on the function and quantity of HBV-specific CD4 and CD8 response, using modern assays able to detect directly HBV-specific T cells *ex-vivo* and *in-vitro* and to analyze a wide profile of T-cell cytokine production.	
STUDY DESIGN:	Exploratory immunology single-arm sub-study in a selected subgroup of patients participating in study ML 18253. Patients who were randomized in study ML 18253 (a multicenter, randomized, open label national study) to receive PEG-IFN 180 µg qw for either 48 or 96 weeks were allowed to participate in the immunology sub-study	
NUMBER OF SUBJECTS	18 enrolled, 15 evaluable	
DIAGNOSIS AND MAIN CRITERIA FOR INCLUSION	Patients with HBeAg-negative CHB (HBsAg-positive and anti-HBs negative with HBeAg-negative and anti-HBe positive) meeting the selection criteria for inclusion in the main study (ML 18253) could be included in the immunology sub-study, provided they met the additional selection criteria highlighted by bold print below. Inclusion Criteria • **Male and female patients ≥18 and ≤55 years of age;** • Chronic hepatitis B: HBsAg-positive for >6 months, anti-HBs negative, HBeAg-negative, anti-HBe positive; • **ALT >ULN but ≤10 x ULN and HBV-DNA between 10^5 and 10^7 copies/mL or ALT >2.5 x ULN but ≤10x ULN and HBV-DNA >10^5 copies /mL;** • Patients with a histological diagnosis of cirrhosis (Ishak Fibrosis Score 6) and compensated liver disease (Child A, score 5) could be included; • Liver biopsy carried out within the preceding 18 months demonstrating liver disease consistent with chronic hepatitis with Ishak Fibrosis Score ≥2; • Negative urine or blood pregnancy test (for women of childbearing potential) documented within the 24-hour period prior to the first dose of study drug.	
TRIAL DRUG / STROKE (BATCH) No.	PEG-IFN used batches: PEG-IFN 135 µg: [redacted] PEG-IFN 180 µg: [redacted]	

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DOSE / ROUTE / REGIMEN / DURATION	PEG-IFN 180µg sc once weekly, subcutaneous injection for the entire duration of the treatment phase of the immunology sub-study (for both groups 1 and 2)
CRITERIA FOR EVALUATION	
ASSESSMENT OF IMMUNE RESPONSE:	CD4- and CD8-mediated immune responses

1. ELISPOT analysis of IFN-gamma production by T cells *ex-vivo* using recombinant HBV proteins and pools of overlapping 15-mer peptides covering the overall HBV sequence of genotype D to derive a representation of the global T cell reactivity against HBV.
2. Analysis of the role played by the PD-1 molecule on T cell activation and responsiveness by intracellular cytokine staining (flow cytometry) of IFN-gamma, IL-2 produced by both CD4 and CD8 T cells after 10 days of *in-vitro* expansion (with the same pools of peptides used for the ELISPOT) in the presence or absence of an anti-PDL-1 antibody.
3. Characterization of the cytokine profile after *in-vitro* expansion by testing culture supernatants for the presence of IFN-gamma, TNF-alpha, IL-10, IL-2, IL-4, IL5 produced by HBV-specific CD4+ and CD8+ cells. Briefly, PBMC of chronic patients were stimulated with peptide mixtures and after 10 days of culture the T cell lines were restimulated and supernatants were collected and analyzed with multiple cytokines assays kit ([redacted]) after 16 h of stimulation.
4. Proliferative response (7 days) upon stimulation with recombinant HBV envelope and nucleocapsid proteins.
5. Analysis of the CD8 response in HLA-A2 positive patients using pre-selected panels of HLA-A2 restricted peptides representing widely recognized epitopes to define:
   - Frequency of circulating CD8+ cells, phenotypic expression of CD127 and PD-1
   - Expansion capacity of tetramer+ T cells upon peptide stimulation assessed by comparing the CD8 frequency *ex-vivo* and after 10 days of *in-vitro* peptide stimulation in the presence or absence of an anti-PDL-1 antibody

   If HBV specific T cell expansion was detected, either in the presence or in the absence of anti-PDL-1 antibody, and when sufficient cells were available:
   - Analysis of cytokine production (IFN-gamma, IL2, IL4, IL5) by tetramer positive CD8+ T cells after 10 days of *in-vitro* peptide stimulation detected by intracellular cytokine staining
   - CD107a expression (by flow cytometry) to assess the degranulation capacity of HBV specific CD8 T cells perforin expression after 10 days of *in-vitro* peptide stimulation detected by flow cytometry.
   - Cytolytic activity after stimulation *in-vitro* with selected peptide panels.

Profile of proinflammatory cytokines

Modulation of pro-inflammatory cytokines (IL-1 beta, IL-6, IL-8, IL-10, IL-12, TNF-alpha) induced by treatment was analyzed directly in the serum of patients using multiple cytokines assays kit ([redacted]). These cytokines (IL-10, IL-6, IL-12) have known immunoregulatory functions or can inhibit IFN-alpha mediated anti-viral effect (IL-8). They can be triggered by IFN treatment and can have an impact on the quantity and function of HBV-specific T cells.

Analysis of HBV viremia

HBV viremia was assessed by quantitative PCR (centralized COBAS® TaqMan HBV Test) at the same time points as chosen for the immunological analysis in order to correlate the behavior of immune responses with changes in viral load.

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PHARMACODYNAMICS:	Not applicable
PHARMACOKINETICS:	Not applicable
SAFETY:	Not applicable for the sub study. The efficacy and safety results of patients included in this sub study are included in the entire database of the main study ML 18253.
STATISTICAL METHODS	This was an exploratory immunological study. The suggested sample size is typical for such an in-depth and extensive analysis using highly complex analysis methods. A patient's total exposure to the study drug (PEG-IFN during the duration of the sub-study (24 weeks) was calculated taking into account dose reductions or treatment interruptions. Samples from patients exposed to less than 60% of the scheduled PEG-IFN drug dose (i.e., a cumulative dose of PEG-IFN over 24 weeks of less than 2592 µg) were not analyzed. Statistical methods included descriptive statistics. For the various immunological parameters pre-treatment and on-treatment values were compared. Continuous variables were summarized by descriptive statistics (number of cases, mean, standard deviation, median, minimum, 25th percentile, 75th percentile and maximum). Categorical variables were summarized using counts of patients and percentages. Repeated measures analysis of covariance (MANCOVA) was applied on intracellular cytokine staining (ICS) and on all immunological parameters. The mean value of screening and pre-treatment values was used as covariate for ICS. The baseline value was used as covariate for all immunological parameters: if baseline values of immunological parameters were not available, the pre-treatment or screening values were used. Correlation analysis was applied on all immunological parameters at baseline and delta HBV-DNA at week 24.

EFFICACY RESULTS:

ELISPOT analysis of IFN-gamma production by T cells:
The intensity of IFN-gamma production, expressed by both the mean number and the mean ratio of specific forming cel was generally weak (36 determinations in total). The highest mean values of both mean delta spot (41.33 ± 48.3) and me ratio (11.13 ± 9.2) was higher at week 24 than at the other time points. There were no statistically significant visit effects visit by baseline value interactions in the MANCOVA model for both mean delta spot and mean ratio. The highest rates positive response (No. of positive tests/No. of determinations) were observed at week 8 (4.12%) and at week 18 (3.92% Data of other parameters to IFN-gamma were not generated.

Intracellular cytokine staining - CD4/CD8 T cell responses:
A total of 880 and 882 T cell lines were derived at ICS from PBMC stimulated with 8 different peptide pools, respectively in absence of a-PDL-1 (CTRL) and with a-PDL-1. CD4 cell lines were activated with greater extent compared to CD8 cell lines in absence of anti PDL-1 antibody, as well as for IL-2 lines in the presence of a-PDL-1, while there were no substantial differences between induction of CD4/IFN-gamma and CD8/IFN-gamma in the presence of a-PDL-1. The analysis of the mean values did not show substantial improvements of CD4 and CD8 reactivity from baseline up to week 24. The addition of an anti PDL-1 antibody to the T cell cultures had a positive but weak effect on both IFN-gamma and IL-2 production.

Analysis of the supernatant:
Data of supernatants were not reliable due to problems in collection.

PMBC proliferation:
The number of missing values did not allow a reliable assessment of changes from baseline to any post-baseline visit.

Analysis of the CD8 response in HLA-A2 positive patients:

Ex-vivo frequency and expansion capacity of HBV-specific, tetramer+ T cells
CD8+/tetramer+ cells were detectable *ex-vivo* in few time points for part of the examined epitopes (Core 18-27, Env 183-191, Pol 575-583), while CD8+ cells specific for the Env 335-343, Env 348-357 and Pol 816-824 epitopes were not identified at any time point in any of the 6 HLA-A2 positive patients. CD8+/tetramer+ cells were detectable following *in-vitro* stimulation in few time points only for the Core 18-27 epitope, while CD8+ cells specific for the other epitopes were not identified at any time point in any patient.

Ex-vivo phenotype and functional analysis of HBV-specific T cells
Data were not generated since the frequencies of antigen-specific T cells detected in the peripheral blood were too small to allow reliable analysis.

Functional analysis of cytokine production by intracellular cytokine staining of HBV core 18-27 specific T cell lines
After in-vitro stimulation only Core 18-27-specific T cell lines were expanded. Their frequency as well as any of the examined parameters (% tet+, % IFN-gamma, % IL-2 and % CD107), both in absence or presence of PDL-1, were low and did not changed from the screening visit to week 24.

HBV-DNA decline at week 24:

Treatment with PEG-IFN was associated with a marked decline of HBV-DNA from baseline to week 24: the median decrease at week 24 was 918147 IU/mL.

Serum cytokines:

Mean levels of IL-8, IL-6 and IL-12 levels decreased from baseline to week 24, while TNF-alpha levels increased from baseline to week 24, and IL-10 and IL-1-beta mean values remained stable from baseline to week 24. However, there were no statistically significant visit effects or visit by baseline value interactions in the MANCOVA model for any of the measured variables.

Correlation tests between immunological parameters at baseline and Delta HBV-DNA at week 24:

Serum cytokine and ELISPOT (ex-vivo):

A statistically significant inverse correlation was found between serum TNF-alpha level at baseline and HBV-DNA drop at week 24 (Pearson r correlation coefficient: -0.614, p = 0.015; Spearman r correlation coefficient: -0.569, p = 0.027). There were no other statistically significant direct or inverse correlations between Delta HBV-DNA at week 24 and any of the other tested parameters.

Intracellular cytokine staining:

ICS + CTRL (absence of anti PDL-1 antibody):
A statistically significant direct correlation with HBV-DNA drop was found for CD4 IFN pep-mix pol 11-12 (Spearman r correlation coefficient: 0.569, p = 0.027), while a statistically significant inverse correlation was found for CD4 IFN pep-mix X-1-2 (Pearson r correlation coefficient: -0.761, p = 0.001) and CD8 IFN pep-mix core 3-4 (Pearson r correlation coefficient: -0.902, p <0.001). There were no other statistically significant direct or inverse correlations between Delta HBV-DNA at week 24 and any of the other tested immunological parameters.

ICS + anti PDL-1 antibody:
A statistically significant correlation with HBV-DNA drop was found for CD4 IFN pep-mix core 3-4 (Spearman r correlation coefficient: 0.554, p = 0.032), while a statistically significant inverse correlation was found for CD8 IFN pep-mix Pol 13-14 (Pearson r correlation coefficient: -0.961, p<0.001; Spearman r correlation coefficient: -0.534, p = 0.041), CD4 IL2 pep-mix core 3-4 (Spearman r correlation coefficient: -0.676, p = 0.006) and CD8 IL2 pep-mix Pol 13-14 (Pearson r correlation coefficient: -0.613, p = 0.015). There were no other statistically significant direct or inverse correlations between Delta HBV-DNA at week 24 and any of the other tested immunological parameters.

There were no other statistically significant direct or inverse correlations between Delta HBV-DNA at week 24 and any of the other tested immunological parameters.

Exploratory analysis:

The analysis of the distribution of frequency of positive responses at baseline (defined as delta spot ≥ 10 and ratio ≥ 2) in the *ex-vivo* ELISPOT and the HBV DNA levels at week 24 (grouped by values < 6 and ≥ 6 IU/mL) showed a statistically significant effect (p = 0.017 in the Fisher's exact test). Of the 10 patients with HBV DNA > 6 IU/mL at week 24, only one (10%) had positive responses at baseline, while 4 out of 5 patients (80%) with HBV-DNA< 6 IU/mL at week 24 showed detectable positive responses at pre-treatment time points.

PHARMACODYNAMIC RESULTS:

Not applicable.

PHARMACOKINETIC RESULTS:

Not applicable.

SAFETY RESULTS:

Not applicable. The efficacy and safety results of patients included in this sub study are included in the entire database of the main study ML 18253.

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CONCLUSIONS:

The main results of this study have shown that:

- CD4 cell lines were activated in a greater extent compared to CD8 cell lines in absence of anti PDL-1 antibody, as well as for IL-2 lines in the presence of a-PDL-1, while there were no substantial differences between induction of CD4/IFN-gamma and CD8/IFN-gamma in the presence of a-PDL-1.
- The correlation tests between immunological parameters at baseline and Delta HBV-DNA at week 24 at ICS showed a weak degree of correlation.
- The analysis of HBV-specific CD8+ T lymphocytes in HLA-A2 positive patients by tetramer staining showed that HBV-specific CD8 cells were detectable both *ex-vivo* and after *in vitro* expansion only in a minority of the examined time points for part of the examined epitopes.
- There were no statistically significant changes from baseline to endpoint in serum cytokines level.
- The probability of achieving HBV DNA < 6 IU/mL at week 24 was significantly higher in patients with positive response at the *ex-vivo* ELISPOT at baseline than in those with negative response.
- With respect to tetramer and serum cytokine analysis, the small number of examined patients and of analyses may have contributed to the lack of significant results.