Part I.

Proposal Name: Effects of long-term interferon-alpha therapy on maintenance of T lymphocyte replicative potential

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HALT-C PI: Herbert Bonkovsky, M.D.

Funding Agency and Review Body:

I agree to follow HALT-C Policies and Procedures when conducting this study. I acknowledge the data obtained from this study will belong to the NIH and will be placed in the HALT-C database for use by other investigators. I understand that I cannot begin experiments using HALT-C specimens/data until I receive approval from the HALT-C Steering Committee and funding from the Scientific Review Body for my proposal. I also understand that the data analysis for this proposal will be performed by NERI (unless approved by the HALT-C study) and that Protocols approved by the HALT-C Steering Committee will be placed on the HALT-C Restricted Website.

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Proposal Principal Investigator Date

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HALT-C Principal Investigator Date

Protocol Part II:

1. Aims/hypothesis:

Sustained interferon therapy is hypothesized to result in accelerated erosion of telomeres in memory and naive T lymphocyte sub-populations.

Aim: Determine if peripheral blood T lymphocyte populations show statistically significant differences in changes in telomere lengths between hepatitis-C virus (HCV) non-responders receiving the 24-week course of high-dose followed by long-term interferon therapy and those receiving only the shorter, 24-week course of interferon.

2. Background/rationale:

In all normal eukaryotic cells, the end signal for chromosomes is established by a 6-base long nucleotide sequence (TTAGGG in mammals) repeated many hundreds to thousands of times, termed the telomere. Through still poorly understood structural mechanisms, these telomere repeats provide the unique signal which forms a DNA-protein structural cap (the so called "T loop" with a host of associated proteins) at both ends of every chromosome. This chromosome-protective DNA-protein structure has recently been named "shelterin" (1). The shelterin complex is believed to prevent DNA damage sensing and repair pathways from recognizing the chromosome ends as strand breaks, which would initiate chromosome-destabilizing end-joining reactions (2). Normal loss of telomere length, termed telomere erosion, occurs due to the difficulty of replicating the 5' ends of linear DNA molecules. Each cell division leads to the loss of 50–100 bp of telomeric DNA due to the inability of DNA polymerase to fully replicate the ends of the chromosomes. Accordingly, in the absence of a mechanism to specifically elongate and restore telomere length, telomere erosion is firmly established as creating a limit on replicative potential in normal human somatic cells (3, 4). Telomerase, along with a ribonucleic acid (RNA) template, is the ribonucleoprotein which slows telomere length losses during the rapid expansion of lymphocytes during their activation-induced expansion and homeostatic replication.

Most human somatic cells do not express detectable levels of telomerase. Human T and B cell lymphocytes, however, are firmly established as some of the few cells besides stem cells that are able to express telomerase (5, 6). Lymphocytes express telomerase at very low to near-undetectable levels during homeostatic maintenance and at high levels during the proliferative response to activation. Thus, these cells are able to reduce telomere erosion during proliferation. Maintenance of telomeres may be critical to protective memory T cell responses. A recently published study of $CD8^+$ T lymphocytes from chronically HIV-infected patients found that shortened telomere lengths contributed significantly to CD8 T cell dysfunction and replicative senescence, and that telomere length stabilization by the induction of telomerase extended the cells' replicative and functional capacities (7, 8).

Among interferon's many known pleiotropic effects, previously published *in vitro* experiments have firmly established that the type-I interferons inhibit telomerase in lymphocytes (9, 10). However, the association of long-term interferon therapy, e.g., in

patients with chronic hepatitis C (CHC), with alterations in lymphocyte telomere lengths has not been explored. Since T cell-mediated immunity is likely critical to sustained immune responses to HCV and other viruses to which these subjects are exposed, and since T lymphocytes are mediators of liver immunopathology in chronic hepatitis C, effects of interferon-alpha on telomere lengths in T lymphocytes could be important to both desirable or undesirable effects of long-term therapy. Furthermore, since these effects would likely affect all T lymphocytes, they could potentially influence natural and vaccine-induced immunity to other viruses, including influenza and hepatitis A viruses. The HALT-C trial, in which patients received up to 4 years of therapy with interferon-alpha, represents an ideal cohort to address this scientific question.

Currently several methods exist for measuring telomere lengths in human peripheral blood lymphocytes. The Southern Blot technique, using restriction enzyme digests of extracted DNA, is being used less today by researchers as it suffers from low resolution and provides only a gross average of telomere length from many millions of cells. Many researchers have now turned to the telomere flow cytometry-based fluorescent in-situ hybridization (flow-FISH) assay as a method for sensitive detection of single cell averaged telomere lengths. This assay uses a fluorescently-tagged, peptide nucleic acid (PNA) oligo-probe to hybridize to telomeric repeats, and the resulting fluorescent signal from each cell, proportional to telomere length, is then read by a flow cytometer (11). Due to conditions of the flow-FISH hybridization step, which denatures many protein epitopes and fluorochromes, the amount of information that can be obtained on different lymphocyte sub-populations is somewhat limited. Nevertheless, the UMMS laboratory has been developing techniques to evaluate multiple PBMC subpopulations based on expression of CD8, CD45RA, CD11a, and CD20. The combination of CD45RA and CD11a markers will allow discrimination of naïve, effector, and memory T cell subsets, while CD20 allows discrimination of B cells within the flow cytometry lymphocyte gating schema (12).

3. Relation to aims of HALT-C study

A major objective of the HALT-C study has been to understand the immunologic effects of long-term interferon-alpha therapy and their possible contribution to positive and negative clinical outcomes of therapy. The proposed Ancillary Study (AS) would extend those studies to characterize a novel potential effect of long-term interferon-alpha therapy. Additionally, there exists controversy within the clinical community concerning the optimal length of interferon therapy for a given HCV genotype to achieve a sustained virologic response (13, 14). This AS should provide additional data regarding how the in vivo T cell telomere lengths may be responding to different durations of type I IFN therapy. These data may assist in understanding possible implications for non-HCV related vaccination/re-vaccination protocols and the maintenance of long-term cellular immunologic function in the patients with CHC upon extended IFN therapy.

4. Study design, experimental groups.

Peripheral blood mononuclear cell (PBMC) samples from the two groups of patients are needed for this study, i.e. those who have received continuous peg-interferon

therapy (peg-IFN treatment) during the trial period, and those patients who have not received interferon therapy (no therapy). For each subject, PBMC would be studied from the following time points: screening (S00), intermediate (M21) time point, and final (M45) sample point. The screening time point (S00) would form the internal, baseline measurement for each patient to which that patient's subsequent PBMC sample time point results would be compared. Using this approach, the effects of therapy prior to screening would be accounted for in each individual's results.

5. Methods, data usage:

PBMC samples would be kept cryo-preserved until immediately before analysis using the flow-FISH protocol. This protocol, in brief, would consist of antibody surface staining and cross-linking for a limited set of cell surface markers, followed by the PNA probe hybridization protocol using a heated water bath with the samples in a 70% formamide buffer. Following PNA probe hybridization, post-hybridization staining and DNA staining are applied, and the samples transferred to standard flow cytometry analysis tubes. Each sample's fluorescence measurements will be made with a BD Aria flow cytometer. Flow cytometer data files will be analyzed using Flowjo (Treestar) software. The resulting telomeric fluorescence values from each sample will be calculated.

Serial PBMC samples from the same subject will represent a matched set that will permit a calculation of the loss of telomere length over time. Calculated telomere lengths at each time point, as well as the intra-patient change in telomere length over time, will be compared between the two treatment groups using standard statistical tests such as Student *t*-test, in coordination with the DCC. Likely covariates for telomere length, such as age, will be incorporated into the statistical analysis. The resulting data and conclusions will be submitted for publication in a peer-reviewed journal (e.g., immunology and/or therapeutics) with the approval of the HALT-C Publications Committee.

6. Anticipated Results:

If we assume that the changes in telomere lengths in the control subjects will be similar to those reported in normal adults, the decrease in telomere lengths of different T lymphocyte subjects between the S00 and M45 samples will be ~200bp. Our hypothesis is that the decrease in telomere lengths in the corresponding T lymphocyte subsets in patients receiving continuous peg-interferon therapy will be at least twice as great as in the control group. Assuming a normal distribution of values and standard deviation of ~200bp, using an unpaired *t*-test with $\alpha = 0.05$, a sample size of n = 16 in each group is needed to achieve 80% power. In addition to the start (S00) and terminal (M45) time points for HALT-C tracked peg-IFN therapy, use of patient PBMC samples from the intermediate time point (M21) should allow additional conclusions to be inferred concerning the kinetics of interferon-induced telomere length changes in the HALT-C cohorts.

7. Statistical support:

The specimens will be tested without knowledge of treatment assignment on the part of the laboratory staff. The HALT-C DCC will assist with the statistical analysis once the data on telomere lengths has been obtained. Alternatively, if the data on age and treatment assignment are provided to the investigator, we can perform the statistical analyses using standard software packages (e.g., SPSS or SYSTAT).

8. HALT-C samples to be used in the study:

HALT-C samples requested are peripheral blood mononuclear cell (PBMC) samples from three HALT-C time points: S00, M21, and M45. Approximately 10 $x10^6$ PBMC from each patient per time point will allow replicate analysis of each sample.

9. Financial issues:

Laboratory work with these samples would be funded through existing research grants in the Rothman laboratory.

10. References.

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Protocol Part III: Sample Requirements.

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount **PBMC**
Screen 1					
Screen 2 **(S00)**					15 pts/group*, 10^7 PBMC (30 samples)
Baseline					
Lead in Week 4					
Week 8					
Week 12					
W16					
Week 20					
Week 24					
Randomized Month 9					
Month 12					
Month 15					
Month 18					
Month 21 **(M21)**					15 pts/group*, 10^7 PBMC (30 samples)
Month 24					
Month 27					
Month 30					
Month 33					
Month 36					
Month 39					
Month 42					
Month 45 **(M45)**					15 pts/group*, 10^7 PBMC (30 samples)
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

* Samples from both peg-IFN therapy group patients and the no-therapy group patients, in donor-matched sets (same donors) for each of the three time points.