Multiplex Cytokine Profiling of Initial Therapeutic Response in Patients with Chronic Hepatitis C Virus Infection

HARLAN WRIGHT, MD,* 1 PHILIP ALEX,†‡ 1 THUAN NGUYEN,† 1 TEDDY BADER, MD,* 1 AHMET GURAKAR, MD,* ANTHONY SEBASTIAN, MD,* LIBERTY GONZALES,† GEMMA WALLIS, MS,† MARK NAYLOR, MD,†‡ IGOR DOZMOROV, PhD, DSci,† MICHAEL CENTOLA, PhD,†‡ and BAKR NOUR, MD*

Currently available prognostic tools are inadequate to discern the molecular basis of the heterogenic response in hepatitis C virus (HCV)-infected patients treated with the current standard of therapy. The expression and biological function of immune mediators have been shown to be critical in all phases of the immune response to HCV infection and likely therefore influence host response. Herein, a biometric multiplex serum cytokine assay was utilized to characterize the immunomodulatory effects of host response in 10 HCV patients. Serum levels of 17 cytokines were compared before and after 1 month of treatment and against controls. Overall serum cytokine levels were significantly higher in patients (P < 0.05) than controls. Additionally, viral titers decreased in all patients after 1 month of therapy, as did overall serum cytokine levels in the cohort (P < 0.05). To assess relationships between changes in cytokine levels and changes in viral titer, the cohort was divided into three statistically distinct subgroups based on changes in viral titers. Specific sets of cytokines decreased in each group: decreases in CCL4, interleukin (IL)-2, CXCL8, and IL-1β correlated with the greatest drops in viral titer, decreases in IL-5, granulocyte colony stimulating factor (G-CSF), and CCL4 correlated with moderate drops in viral titer, and only CCL2 correlated with the lowest drops in viral titer. Interestingly, decreases in CCL4 levels correlated with decreases in viral titers in all patients. CCL4 controls leukocyte influx and thus propagates inflammation. In conclusion, these data raise the possibility that characteristic changes in host response modulate the therapeutic response, demonstrating the prognostic power of serum cytokine profiling in chronic HCV.

KEY WORDS: cytotoxic and humoral cytokines; chemokines; pegylated interferon α; ribavirin.

Hepatitis C virus (HCV) 1 infection is a worldwide epidemic that is among the leading infectious causes of morbidity and mortality (1–3). Population-based studies indicate that 40% of chronic liver disease is HCV-related, becomes chronic in about 55%–85% of patients (4), may progress to cirrhosis (5), and is also a major risk factor for the development of hepatocellular carcinoma (6, 7). The current therapy for HCV infection includes pegylated interferon (IFN-α) plus ribavirin; the sustained virological response (SVR) rates with this combination therapy were improved to more than 40%, compared to less than 20% with IFN-α monotherapy (8, 9). Patients infected with
HCV genotypes 2 and 3 have been estimated to be curable at a rate of approximately 80% with this combination, whereas pegylated IFN-α-2b and ribavirin combination therapy helped achieve SVR rates of up to 72% in HCV relapers after treatment for 48 weeks (10–12).

Given the clinical and economic costs of hepatitis C infection, surprisingly little is known regarding the molecular mechanisms governing therapeutic response and, more importantly, nonresponsiveness to standard treatments. Evidence elucidating the progression of the pathogenic portal inflammatory process through septal fibrosis, interface hepatitis, and lobular necro-inflammatory lesions in hepatitis C has demonstrated the importance of host immune response in HCV pathogenicity (13, 14). However, the immunomodulatory mechanism involved in the pathogenic progression of HCV-related liver disease is poorly characterized due in part to the insufficiency of evidence and the lack of suitable animal models (15). A defect in the ability to mount an efficient cellular immune response has been proposed to be primarily inducive for the establishment of chronicity (15). Cytokines are critical in all phases of the immune response to HCV infection, from early onset of liver damage to chronic infection, viral persistence, tissue damage, and fibrosis. The immune response in HCV is mediated with a predominantly Th2/Th0 response in the periphery (16, 17) and a Th1 response in the liver (18). Elevated serum levels of Th2 cytokines interleukin (IL)-4, IL-10, and IL-2 have been observed in chronic HCV patients (19). Cytokines produced by both CD4+ and CD8+ cells play an important role in both inhibiting viral replication and causing liver injury. Macrokinase (WF10), a novel macrophage-regulating agent, has been shown to downregulate elevated levels of tumor necrosis factor (TNF)-α, IL-1β, and CCL4 (MIP-1β) in a dose-dependent fashion in nonresponsive patients with HCV infection (20). Cytokines therefore play a critical role in coordinating the response of the immune system to viral infection.

Furthermore, the control of cytokine production is highly complex and multifactorial and the effects of cytokines are mediated through multiple regulatory networks. The intricate complexity of both cytokine networks clearly conceals the role that a single cytokine may play in the pathogenesis of the disease. It is therefore informative to investigate the immunopathogenesis of the disease process by analyzing multiple cytokines. Utilizing a broad-spectrum bead-based multiplex immunometric assay, we have effectively characterized the serum levels of cytokines that mediate humoral and cellular immunity and inflammation, correlated these serum cytokine levels with disease activity, and characterized the immunomodulatory effects of therapy 1 month posttreatment. This has provided us with a better understanding of the role of cellular, humoral, and chemotactic immunity at a critical time in the treatment course of HCV infection, when the most significant changes in viral titer are observed.

SUBJECTS AND METHODS

Study Population. The study population consisted of 10 adult patients (2 men and 8 women; mean age, 44.40 [95% CI, 39.60–49.20]) with chronic hepatitis C who had not previously undergone treatment with a combination of pegylated IFN-α and ribavirin or patients who had previously failed to respond to interferon-α therapy. Ten healthy controls were also enrolled in the study. The protocol was approved by the Institutional Review Board at Integris Baptist Medical Center and all patients provided written informed consent prior to enrollment.

Treatment Protocol. Patients received standard treatment based on weight dosing, which included a dose of 1.5 g/kg/week of pegylated IFN-α-2b and a weighted dose (10–15 mg/kg) of ribavirin. Patients who had been diagnosed with hepatitis C genotype 2 and 3a received the combination treatment for 48 weeks, whereas patients diagnosed with genotype 1 received the treatment for 24 weeks. Routine blood biochemical and histological tests were performed regularly for the duration of treatment.

Sample Collection and Processing. HCV genotype was evaluated in all sera from subjects. Genotype was determined by Taqman reverse transcription–polymerase chain reaction (RT-PCR) and TMA (transcription-mediated amplification) using type-specific primers (Quest Diagnostics, Teterboro, NJ) and classified according to the criteria of Simmond and colleagues (21). Venous whole blood from hepatitis C patients was collected from each patient prior to and 1 month following IFN-α and ribavirin therapy. Blood was collected in endotoxin-free silicone-coated tubes without additive. The blood samples were allowed to clot at room temperature for 30 min before centrifugation (3000 rpm, 4°C, 10 min) and the serum was removed and stored at −80°C until analyzed.

Multiplex Cytokine Assay. A multiplex biometric enzyme-linked immunosorbent assay (ELISA)-based immunometric assay, containing dried microspheres conjugated with a monoclonal antibody specific for a target protein, was used according to the manufacturer’s instructions (Bioplex Human Cytokine Assay; BioRad Inc., Hercules, CA). Cytokines measured were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-10, IL-12 (p70), IL-13, IL-17, granulocyte colony stimulating factor (G-CSF), granulocyte–monocyte colony stimulating factor (GM-CSF), CCL2 (monocyte chemoattractant protein [MCP]-1), CCL4 (macrophage inflammatory protein [MIP]-1β), and TNF-α. Briefly, serum samples were diluted 1:4 and incubated with antibody-coupled beads. Complexes were washed, then incubated with biotinylated detection antibody and, finally, with streptavidin–phycoerythrin prior to assessing cytokine concentration titers. Concentrated human recombinant cytokine was provided by the vendor (BioRad, Inc.). A broad range, 1.95–32,000 pg/ml of standards was used to establish standard curves to maximize the sensitivity and dynamic range of the assay. Cytokine levels were determined using a Bio-Plex array reader (an automated flow-based microfluidics device that uses a dual-laser fluorescent detector with real-time digital signal
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TABLE 1. CHARACTERISTICS OF PATIENTS AT BASELINE AND 4 WEEKS POSTTREATMENT

|                             | Demographics          | Controls          |
|-----------------------------|-----------------------|------------------|
| Gender (female/male)        | 8/2                   | 7/3              |
| Age, years (mean ± SD)      | 44.40 ± 6.7           | 41.70 ± 7.83     |

**Pretreatment pathology scores and genotype**

| Pretreatment Posttreatment |
|---------------------------|
|                           |
| Inflammatory activity grade (mean ± SD) | 1.75 ± 0.89           |
| Stage/degree of fibrosis (mean ± SD)    | 1.5 ± 0.76             |
| Knodell score (mean ± SD)               | 5.5 ± 0.7              |
| Viral genotype (1a/1b/2/3)              | 6/1/1/2                |

**Response levels**

|                   | Pretreatment | Posttreatment |
|-------------------|--------------|---------------|
| ALT ([×ULN] mean ± SD) | 2.4 ± 1.5 | 0.9 ± 0.5     |
| Serum HCV RNA (copies ± SD) | 6.22 × 10^4/ml ± 16.2 | 3.3 × 10^2/ml ± 0.23* |

**Response rates**

|                | Group A | Group B | Group C |
|----------------|---------|---------|---------|
| EOT, n(%)      | LF      | 4 (100) | 4 (100) |
| SVR, n(%)      | LF      | 3 (87.5%) | 4 (100) |

Note. ALT, alanine aminotransferase; ULN, upper limit of normal; HCV, hepatitis C virus; EOT, end of treatment; SVR, sustained viral response; LF, lost to follow-up.

*Posttreatment serum HCV RNA titer was undetectable in four patients.

RESULTS

**HCV Genotyping and RNA Titer.** For this study, a cohort of 10 adult patients (2 men and 8 women; mean age, 44.4 [95% confidence interval (CI), 39.60–49.20]) with chronic hepatitis C infection were followed prospectively during treatment with a combination of pegylated INF-α-2b and ribavirin. Liver biopsies and peripheral blood samples were obtained prior to therapy and blood samples were collected at 1 month following therapy for viral testing and assessment of clinical parameters (Table 1). Viral subtyping demonstrated that four different HCV subtypes were present within this cohort: subtypes 1a, 1b, 2, and 3a. Viral titers in peripheral blood samples were estimated by quantitative PCR. Viral titers decreased in all patients after 1 month of combined therapy highlighting the efficacy of this treatment modality (Table 1, Figure 1A). Titer dropped below detectable levels in four patients, depicting a complete remission. In the remaining patients the response to therapy was variable (Table 1, Figure 1A). At the end of treatment (EOT), 8 of 10 patients were viral negative (2 patients were lost to follow-up). The SVR was determined in these eight patients, of whom seven remained viral negative and one relapsed (Table 1).

To refine our understanding of the heterogeneity of therapeutic response, patients were classified into three statistically distinct groups based on quantitative changes...
Assessment of therapeutic response. (A) Decrease in viral titers 4 weeks posttreatment are represented for all patients in the cohort. (B) The average changes in viral titers for three statistically distinct subgroups of patients subdivided based on changes in viral titers 1 month posttherapy.

in viral titer at 1 month posttreatment and these groups were used for further analysis. Three distinct groups were denoted: a high-responsive group, group C, which included four patients (P7–P10); a moderate-responsive group, group B, which also included four patients (P3–P6); and a low-responsive group, group A, which included only two patients (P1 and P2) (Figure 1B). Though viral titers dropped below detectable levels in one patient (P5), this patient was not included in group C. This is due to the fact that the pretreatment viral titer values in this patient were low, the lowest of the cohort, so the overall change in viral titer at 1 month was not as high as for the other individuals in group C. Also of note, not all individuals in the high-response group had undetectable levels of virus at 1 month, but they had in common the highest quantitative drop in viral titer at 1 month. The decline in viral titer in group C was significantly higher than in group B ($P = 0.0266$) or A ($P = 0.03199$), and the decline in viral titer in group B was significantly higher than in group A ($P = 0.045076$).

Multiplex Serum Cytokine Assays. Both HCV and its treatment modulate the immune response of the host. The heterogeneity of therapeutic response may therefore be due to differences in host response to virus, to therapy, or to a combination of the two. Inflammation and immunity are mediated in part by the action of cytokines. We used a multiplex cytokine immunosay to assess therapy-induced changes in the overall host response of these patients. This broad-spectrum cytokine panel includes mediators of humoral and cell-mediated immunity and inflammation and thereby provides a means of discerning both gross and subtle differences in the inflammatory and immunologic state of the individuals in this cohort.

Serum cytokine levels were assessed for patients prior to initiation of therapy (denoted “baseline levels”). An intriguing aspect of these data was that they provided evidence that one patient, P10, was likely experiencing a dramatic inflammatory episode prior to treatment. The majority of cytokines in this individual (14 of 17), including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, G-CSF, GM-CSF, and CCL2 (MCP-1), were substantially increased, with some cytokine levels over two orders of magnitude higher than the levels observed in the remaining patients (Figure 2A). While HCV titers dropped to below detectable levels in this patient after 1 month of therapy, cytokine levels increased, suggesting that the inflammatory response of this patient was not due directly to HCV infection. Moreover, cytokine levels were high in this patient prior to therapy, suggesting that this response was not due to a reaction to IFN-α or ribavirin treatment. Because cytokine levels remained extremely high in this patient for over a month (Figure 2B), they may be indicative of an unrelated chronic infection or of an autoimmune disease such as HCV-induced rheumatic disease.

Interestingly, this patient experienced both arthralgia and myalgia and cytokine levels characteristic of patients with inflammatory arthritis prior to and after treatment (22–25), suggesting that this patient may have also had a preexisting musculoskeletal disease. We therefore assessed the levels of several biochemical markers of autoimmunity. Serum levels of rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (diagnostic markers of rheumatoid arthritis), antinuclear antibodies (a diagnostic marker of systemic lupus erythematosus, Sjögrens syndrome, polymyositis, and scleroderma), and
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C-reactive protein (CRP; inflammatory marker) were assessed prior to and post therapy. Of these markers, only RF and CRP were elevated above normal levels in this patient, which may also occur during HCV infection. Given the uniqueness of the cytokine response in this patient, they were excluded from further analysis.

In the remaining nine patients the overall cytokine levels measured prior to therapy ranged from 8.96 to 49.80 pg/ml (95% CI). These levels were significantly higher than those observed in the control population ($P = 0.0498$), which ranged from 3.46 to 41.29 pg/ml (95% CI). After 1 month of therapy, overall serum cytokine levels decreased in patients ($P = 0.0436$), with values ranging from 5.96 to 17.32 pg/ml (95% CI), suggesting that assessment of serum cytokine levels may provide clinically relevant information with regard to HCV infection and therapeutic response.

**Correlation of Cytokine and Therapeutic Responses.** Changes in individual serum cytokine levels were assessed and correlations between these levels and changes in viral titer were determined. If the average levels of a given serum cytokine decreased more than three standard deviations after therapy, the change was considered statistically significant. In group C, the group with the most significant drop in viral titer, decreases were observed in the levels of CCL4 (MIP-1β), IL-2, CXCL8 (IL-8), and IL-1β after 1 month of combination therapy (Figure 3). Patients with moderate drops in viral titer in

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Fig 3. Changes in serum cytokine levels in patient subgroups. Average serum cytokine changes after 1 month of therapy in patient subgroups with a high (A), medium (B), and low (C) response to therapy are shown. Error bars represent standard deviations.
response to therapy (group B) had significantly decreased levels in only three cytokines, IL-5, G-CSF, and CCL4 (MIP-1β) (Fig. 3B). In weakly responsive patients (group A) only CCL2 (MCP-1) decreased significantly posttreatment (Figure 3B). No other cytokines had statistically significant decreases at 1 month posttreatment in any of the three groups (Figures 3A–C). The only statistically significant elevations in serum cytokine levels were observed in group A, where IL-7, IL-13, and IL-17 increased at 1 month posttreatment (Figure 3A). These data demonstrate that patients can be differentiated by their inflammatory and immune responses and that these differences correlate with therapeutic response.

When all nine patients were grouped and the average levels of all cytokines compared, CCL4 (MIP-1β) was found to be the most significantly decreased after treatment ($P = 0.029$) (Figure 4). When assessed on a patient-by-patient basis, changes in CCL4 (MIP-1β) levels were significantly correlated with decreases in viral titer for all nine patients. Decreased serum CCL4 (MIP-1β) levels were observed after 1 month of therapy in all high and moderate responders and remained unchanged or were increased in patients with the weakest response to therapy (group A; specifically patients P1 and P2).

Characterization of Cytokine Response to Therapy by Correlational Clustering. As clear correlations were established between cytokine levels and therapeutic response, we assessed the potential of multiplex cytokine monitoring to define likely therapeutic response. A multivariate analytical method, correlational clustering (26, 27), was used to group patients with similar changes in cytokine levels after 1 month of therapy. The results of these analyses were represented in a graphical output, denoted a cluster mosaic, that can be used for assessment of the results by visual inspection. Based on cytokine changes, three distinct clusters of patients were obtained. Interestingly, patients with strong to moderate responses to therapy (i.e., those with significant drops in viral titer) were clustered in one group and the two patients with the weakest response to therapy (P1 and P2) clustered separately into two minor clusters (Figure 5), demonstrating the expected result that therapeutic response has a characteristic effect on systemic cytokine levels and therefore on the immune/inflammatory response.

**DISCUSSION**

The introduction of new agents and regimens for the treatment of chronic hepatitis C, such as pegylated IFNs and combination therapy with ribavirin, has resulted in substantial improvements in SVR rates (16, 17). However, treatment remains a challenge, particularly for certain patient populations, because several virus-related and individual patient-related factors are associated with a poor therapeutic response. Ten adult patients with HCV infection were followed prospectively over 4 weeks during combination therapy with pegylated IFN and ribavirin. Significant changes in viral titer were observed in the majority of patients after 1 month of treatment, with no virus detected in 40% of patients and a greater than 10-fold decrease in viral titer observed in the remaining patients, illustrating the significance of this phase of therapy with regard to modulation of infection. Interestingly, five of the six partially responsive patients were infected with genotype 1 HCV, further confirming previous findings of lower response rates in patients with this viral genotype (28–30) and demonstrating the complexity of therapeutic outcome.
Importantly, cirrhosis is projected to occur in 10%–20% of patients in whom chronic infection persists (6, 7). Defining the bases of nonresponse is therefore crucial for defining more efficacious treatments. Host response plays a key role in response to virus. The expression and biological function of cytokines are therefore critical in defining host response, as these mediators are principal regulators of the immune response. To assess the role of host response in therapeutic outcome, we used a multiplex cytokine immunoassay to measure the therapy-induced changes among 17 serum cytokines from this cohort. Serum cytokine levels were assessed in patients prior to initiation of therapy and posttherapy.

A distinct set of cytokines characteristic of autoimmune disease (22–25) was grossly upregulated in one patient (P10) relative to the other individuals in the cohort and remained high despite the absence of detectable virus after 1 month of therapy. HCV has been shown to have the potential to induce autoimmune disease including inflammatory arthritis and Sjögren’s syndrome (31–34). The relatively high cytokine levels and the persistence of significant arthralgic and myalgic symptoms in the absence of detectable virus may suggest the need for anti-inflammatory therapy in this patient. While diagnostic biochemical analyses failed to identify a likely cause of the musculoskeletal symptoms, serum cytokine profiling provided a likely biochemical basis and evidence for the systemic inflammatory symptoms, demonstrating a utility of cytokine profiling as a screening tool. In the remaining nine patients serum cytokine levels measured prior to therapy were significantly higher than those observed in the control population ($P < 0.05$). After 1 month of therapy, overall serum cytokine levels decreased in these patients ($P < 0.05$). In group C, the group with the most significant drop in viral titer (high-responsive group), significant decreases were observed in the levels of CCL4 (MIP-1β), IL-2, CXCL8 (IL-8), and IL-1β after 1 month of combination therapy. Patients with moderate drops in viral titer in response to therapy (group B) had significantly decreased levels in only three cytokines, IL-5, G-CSF, and CCL4 (MIP-1β) while in weakly responsive patients (group A), only CCL2 (MCP-1) significantly decreased posttreatment. It was therefore demonstrated that patients can be further differentiated by their immune responses and these differences categorically correlate with therapeutic response.
Using correlational clustering, three distinct clusters of patients were obtained. Intriguingly, clusters correlated well with viral titer data, the largest cluster (strong to moderate responders) contained only patients from groups B and C, while group A patients (weak responders) were present in two separate minor clusters. These results demonstrate that serum cytokine levels can be effectively used to distinguish weak responders from moderate and strong responders. These data also suggest that the heterogeneity of therapeutic response is in part mediated by differences in host response among individuals in this cohort.

CCL4 (MIP-1β) was found to be the most significantly decreased cytokine posttreatment when all the patients were grouped together. Changes in CCL4 (MIP-1β) levels were significantly correlated with decreases in viral titer for all nine patients when the data were assessed on a patient-by-patient basis. Decreased serum CCL4 (MIP-1β) levels were observed after 1 month of therapy in all high and moderate responders. However, serum CCL4 (MIP-1β) levels remained unchanged or were increased in patients with the weakest response to therapy (group A; specifically patients P1 and P2). The association between a drop in CCL4 (MIP-1β) levels at 1 month and response to virus at 1 month may be of prognostic merit, as a drop in CCL4 (MIP-1β) levels at 1 month also correlates with response at end of treatment (100%) and SVR (87.5%).

Liver-infiltrating T cells express high levels of CCR5: the receptor for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 A1 (RANTES) (35). Because these CCR5 ligands are constitutively expressed on portal vessels, they provide a mechanism for the recruitment of CCR5 memory T cells to portal areas in normal liver during immune surveillance, as well as for recruiting T cells to portal areas in inflammatory liver diseases. CCR5 has preferential activity for Th1 lymphocytes (36), triggering the activation of IL-2 and IL-1β. Furthermore, liver injury mediated by CD8+ T cells in graft-versus-host disease is CCR5-dependent (37). The decrease in serum levels of CCL4 (MIP-1β) and CXCL8 (IL-8) posttreatment suggests that a decrease in the transendothelial migration of leukocytes occurs in responsive patients, which would preclude the retention and the survival of lymphocytes in the liver and, thereby, ameliorate tissue damage and fibrosis. Accordingly, concomitant decreases in CCL4 (MIP-1β) and the Th1 cytokines IL-2 and IL-1β were observed in patients with the highest response rates, which would be expected given the predominantly Th1 immune response in the liver HCV patients (19).

Intracellular immunity, influenced by IFN-γ is imperative for defense against viral infections. CCL4 (MIP-1β) mediated T cell infiltration is essential for the delivery of IFN-γ to mediate downstream protective responses against HCV infection in the liver. It has been shown recently from intrahepatic gene expression profiles of chimpanzees that MIP-1β was upregulated in acute infection at the time of viral clearance, but not in those who failed to eradicate the virus (38), and previous studies have demonstrated that HCV-infected individuals have a diminished response to CCL4 (MIP-1β) in the liver (39). These data and the fact that serum CCL4 (MIP-1β) levels correlated with therapeutic response in these nine patients suggest that the action of CCL4 (MIP-1β) part influences an appropriate treatment-induced host response to HCV.

It is also of interest to note that the correlation between CCL4 (MIP-1β) levels and therapeutic efficacy is also consistent with an opposing hypotheses, that chronic CCL4 (MIP-1β)-mediated inflammation limits the effects of antiviral therapy and is a prime contributor to morbidity by inducing liver damage. This somewhat antithetical conjecture is consistent with recent observations that mortality in SARS virus infection is due in part to the Th1-biased inflammation induced in lungs by the virus and the fact that mortality can be decreased by treating SARS patients with a combined course of immunosuppressants (corticosteroids) and antivirals (ribavirin) late in the infection cycle at a time when the host response is causing maximal damage to the infected tissue (40). If this provocative hypotheses is valid, then treating nonresponsive HCV patients with a combination of immunosuppressants and antivirals may improve response rates and targeted anti-CCL4 (MIP-1β) therapy could be of interest in future clinical trials.

A defect in the ability to mount an appropriate cellular immune response is responsible for the establishment of chronicity in HCV infections. The mechanisms that regulate the induction of an integrated cellular and humoral response in chronic HCV have not been fully elucidated. Cytokines have been shown herein to be effective noninvasive markers of sustained response and potential prognostic surrogates for therapeutic outcome. Assessing a cytokine profile containing chemotactic and humoral inflammatory cytokines helps elucidate the pathogenic processes on an individual basis and may be helpful in understanding the nature of the immune process in HCV patients overall, thereby assisting with prognostication and treatment decisions.

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