Selective Suppression of NF-kBp65 in Hepatitis Virus-Infected Pregnant Women Manifesting Severe Liver Damage and High Mortality

Bhupesh K Prusty,[1] Suresh Hedau,[1] Ajay Singh,[2] Premasis Kar,[2] and Bhudev C Das[1]

[1]Division of Molecular Oncology, Institute of Cytology and Preventive Oncology, Noida, India; [2]Department of Medicine, Lok Nayak Hospital, Maulana Azad Medical College Campus, Bahadur Shah Zafar Marg, New Delhi, India

Fulminant hepatitis in Asian pregnant women is generally caused by hepatitis E virus infection, and extremely high mortality is most common in them. Decreased cell-mediated immunity is considered a major cause of death in these cases, but what exactly influences decreased immunity and high mortality specifically during pregnancy is not known. We used electrophoretic mobility shift assays, immunoblotting, and immunohistochemical analysis to study the expression and DNA binding activity of NF-kB p50 and NF-kB p65 in pregnant fulminant hepatic failure (FHF) patients and compared them with their nonpregnant counterparts. In both PBMC and postmortem liver biopsy specimens the DNA-binding activity of NF-kB was very high in samples from pregnant FHF patients compared with those from nonpregnant women as well as pregnant women with acute viral hepatitis (AVH) without FHF. Further dissection of the NF-kB complex in supershift assays demonstrated complete absence of p65 in the NF-kB complex, which is formed by homodimerization of the p50 component in pregnant FHF patients. Western blotting and immunohistochemical analysis of the expression of p50 and p65 proteins both showed higher levels of p50 expression and a complete absence or a minimal expression of p65, indicating its nonparticipation in NF-kB–dependent transactivation in pregnant FHF patients. We suggest that the exclusion of p65 from the NF-kB transactivation complex seems to be a crucial step that may cause deregulated immunity and severe liver damage, leading to the death of the patient. Our findings provide a molecular basis, for developing novel therapeutic approaches.

Online address: http://www.molmed.org
doi: 10.2119/2007–00055.Prusty

INTRODUCTION

Viral hepatitis constitutes a major public health problem in developing countries, including India. In addition to parenterally transmitted hepatitis B and C viruses, which cause majority of hepatitis, enterically transmitted hepatitis E virus (HEV) infection is mainly responsible for sporadic as well as large waterborne hepatitis epidemics related to poor hygiene and sanitation. HEV-induced viral hepatitis is the most common cause of death in Indian pregnant women (1). Studies carried out in Iran, Africa, the Middle East, and other Asian countries have also found a high mortality due to fulminant hepatic failure (FHF) during pregnancy in women with HEV infection (2–5). In contrast, reports from the United States and Europe have failed to find any significant correlation between death during pregnancy and viral hepatitis (6). The mortality rate in pregnant women with FHF has been found to be specifically higher during second and third trimesters of pregnancy (1,2,7–9), which are associated with an altered status of hormones and immunity, but what exactly influences high mortality during pregnancy is not known.

NF-kB, a eukaryotic dimeric transcription factor formed by hetero- or homodimerization of proteins of the Rel family, is involved in a wide range of cellular effects, including immune and inflammatory responses, proliferation, cell survival, and apoptotic stimuli (10). Different members of the Rel family, such as p50, p52, p65, cRel, and RelB possess a *rel* homology domain that confers DNA binding and protein dimerization properties (11). NF-kB remains in an inactive form in the cytoplasm by binding to the labile cytoplasmic inhibitor IκB (12,13), which masks the RelA nuclear localization signal. The release of IκB in response to intracellular signals leads to nuclear translocation of the p50 and p65 subunits and subsequent activation of a whole set of NF-kB responsive effector genes. Disruption of the RelA locus in mice lacking the p65 subunit of NF-kB has been demonstrated to lead to embryonic lethality at 15-16 d of gestation due to massive degeneration of the fetal liver by programmed cell death (14). Studies done with p65 knock-out mice also indicated that p65 is indispensable for liver development and causes enhanced cell proliferation during embryonic development (9). This finding prompted us to investigate the probable role of NF-kB during the death of hepatitis virus infected

Address correspondence and reprint requests to *Bhudev C Das, Division of Molecular Oncology, Institute of Cytology and Preventive Oncology (ICMR), I-7, Sector 39, Noida 201301, India. Tel: + 91-120-2575838; Fax: + 91-120-2579437; E-mail: bcdas48@hotmail.com*
Submitted May 12, 2007; Accepted for publication July 9, 2007.

Table 1. p50 and p65 Expression of NF-kB Components and the Type of Hepatitis Virus Infection in Pregnant and Nonpregnant Fulminant Hepatic Failure Patients and Controls.

Sr. No.	Patient type and number	Total No.	Virus infection	Type of Hepatitis	[a]Status of p50	[a]Status of p65
1	Pregnant women 15		HEV	FHF	+ + +	-
	Pregnant Women 3	20	HBV	FHF	+ + +	-
	Pregnant Women 2		HCV	FHF	+ + +	-
2	Pregnant women (disease control) 5	5	HEV	AVH	+ + + / + +	+ + / +
3	Nonpregnant women 5	5	HEV	FHF	+ + +	+
4	Healthy pregnant women 10	10	Nil	Nil	+ +	+ + / +
5	Pregnant FHF patients recovered 2	2	Not detected	No symptoms	+	+

[a]Level of expression compared to normal controls on the basis of densitometric analysis data;+ + +, high expression; + +, moderate expression;+, low expression; -, no expression.

pregnant women with FHF. We report that the suppression of p65 expression appears to be associated with the breakdown of immunity and with severe liver degeneration leading to death of the patient.

MATERIALS AND METHODS

Study Subjects

Heparinized peripheral venous blood was collected from 25 female patients (age 20-35 years) with FHF comprising 20 pregnant and five nonpregnant women. Ten pregnant healthy women without liver disease and matched for age, parity, and trimester served as controls. An additional disease control group comprising five pregnant women with acute viral hepatitis (AVH) due to HEV infection was also included. All the women were in the third trimester of their pregnancy and were admitted consecutively for treatment of hepatic liver failure during the period from July 2004 to January 2005. Of the 20 pregnant women with FHF, 15 were infected with HEV and five were infected with either HBV or HCV, but all five nonpregnant FHF and five pregnant AVH patients were infected with HEV only (Table 1). Postmortem liver biopsies were collected from 18 patients who died during the study. Informed consent was obtained from all the patients and controls. For the postmortem biopsy, written consent was obtained from their immediate relations. The patients included were either admitted to Lok Nayak hospital, New Delhi, or followed up by the hospital's outpatient services. Only two patients who survived were kept on long-term follow-up until a clinical and biochemical recovery was achieved and the outcome of hepatitis virus infection was assessed. Only those patients who were found to be positive for HEV, HCV, or HBV and did not have any other serious diseases were recruited for the study.

All women recruited had undergone clinical assessment, a urine test for human chorionic gonadotropin, and pelvic ultrasound examination for diagnosis of pregnancy and duration of gestation. In pregnant women, a careful clinical assessment, hematological and biochemical investigations, and liver imaging were performed to further define whether liver disease unique to pregnancy could be the cause of acute liver failure (ALF). Fulminant hepatitis was considered in patients with no history of preexisting liver disease who suffered typical acute-onset hepatitis with severe liver injury and then became deeply jaundiced and went into hepatic encephalopathy within 8 wk of the onset of the disease with no past history of liver disease (15). AVH was considered in patients who had suffered acute self-limiting disease and a serum aminotransferases (ALT and AST) elevation of at least fivefold or jaundice, or both. We also obtained five normal liver biopsy specimens from patients who had undergone abdominal surgeries at Lok Nayak Hospital for conditions other than liver problems and had normal viral markers for hepatitis viruses and normal liver function tests. We obtained informed consent from these patients.

Serology and RT-PCR

The sera from approximately 10 mL of patient blood was separated and stored at –70 °C for subsequent viral assays. All sera collected during the acute phase of illness were tested for markers of HAV (IgM anti-HAV), HBV (HBsAg and IgM anti-HBc), and HCV (anti-HCV s generation) with commercially available enzyme-linked immunosorbent assay (ELISA) kits, strictly following manufacturer instructions. All sera were also tested for IgM and IgG antibodies to HEV with an ELISA kit that uses two recombinant HEV antigens corresponding to structural region of the HEV (Dignostic Biotechnology, Singapore) (16,17). DNA and RNA were extracted from the serum samples of all the patients and employed for detection of HEV and HCV RNA by RT-PCR and HBV DNA by PCR using standard procedures routinely followed in our laboratory (18). On the basis of the above viral markers, AVH was classified as hepatitis A (presence of IgM anti-HAV), or acute hepatitis B (presence of HBsAg and IgM anti-HBc). Hepatitis E was diagnosed by the presence of IgM anti-HEV (n = 11) and/or HEV RNA (n = 4) in acute phase sera or seroconversion to

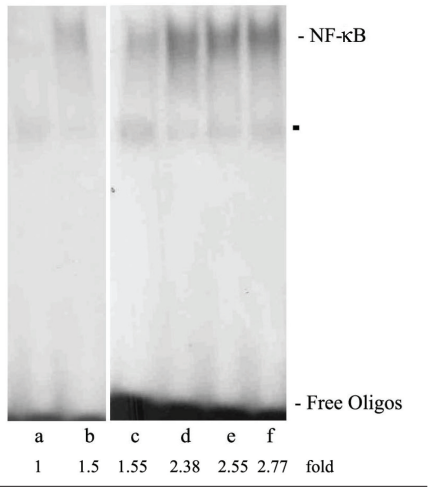

Figure 1. Gel shift analysis using nuclear extracts from PBMC proteins of different subjects with ^{32}P-labeled oligonucleotide probe harboring an NF-kB consensus sequence. Very low NF-kB binding activity was observed in the healthy pregnant women (lane a), a moderate level of binding activity was observed in disease control (pregnant women with AVH) (lanes b and c), while a very high binding activity was observed in the hepatitis virus infected FHF patients, both nonpregnant (lane d) and pregnant (lane e and f). The positions of the specific retarded bands are indicated. The squares mark the unspecific complexes. The extent of binding activity as revealed by densitometry analysis is indicated below each lane.

IgG anti-HEV alone in convalescent sera (16,17). Presence of anti-HCV (n = 1) and HCV RNA (n = 2) was taken as evidence of hepatitis C infection.

Preparation of Nuclear Extract and Western Blot Analysis

Frozen liver biopsies were minced finely and immediately processed for preparation of nuclear extract using previously described methods (19). The blood samples collected were also immediately processed for nuclear extract preparation using the same protocol as for tissue biopsies but with the exception that peripheral blood mononuclear cells (PBMCs) were first separated from whole blood using Histopaq solution (Sigma, St. Louis, MO, USA) and then processed for protein extraction. Western blot analysis was carried out as described earlier (19,20). The bands were visualized with an anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugated with horseradish peroxidase in a dilution of 1:10000, using the Santa Cruz Luminol detection kit. To confirm equal protein loading, Ponceau red staining was used and in addition, the filters were reincubated, after stripping, with a polyclonal actin antibody raised against a peptide mapping at the carboxy terminus of a normal actin gene of human origin in a dilution of 1:1000 (Santa Cruz Biotechnology, Cat No. sc-1615). The bands were visualized and quantitated using the BioRad Gel Doc 2000^{TM} gel documentation system with Quantity One Quantitation Software (BioRad Laboratories, Hercules, CA, USA). The densitometric analysis of fold increase in the level of expression was calculated in comparison to that of normal controls. The densitometric value of actin was used to normalize the signal. The blots were stripped of and reincubated with different antibodies when required. Stripping of antibody was done by the standard method of incubating the membranes in a buffer containing 2% SDS, 62.5 mM TrisCl, pH 6.8, and 0.1 M β-mercaptoethanol for 30 min.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was carried out using previously described techniques (19). For EMSA, the following oligonucleotides were used: an NF-kB consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and an Oct-1 consensus oligonucleotide primer 5′-TGTCGAATGCAAATCACTAGAA-3′. The antibodies used were: NF-kB p50 Ab, an epitope corresponding to the nuclear localization signal region of NF-kB p50 of human origin (Cat No. sc-114) and NF-kB p65 Ab, an epitope corresponding to the amino terminus of NF-kB p65 of human origin (Cat No. sc-7151, Santa Cruz Biotechnology). The dried gels were visualized and bands were quantitated using a BioRad Gel Doc 2000^{TM} gel documentation system with Quantity One Quantitation Software (BioRad).

Immunohistochemical Analysis

Immunohistochemistry was also carried out using the previously described protocol (21). Briefly, after deparaffnization and rehydration, the tissue sections were blocked in 3% bovine serum albumin for 30 min and incubated overnight at 37°C with the primary antibody. Immunoreactivity was visualized using an ABC Staining System Kit from Santa Cruz Biotechnology following the manufacturer's protocol. The slides were then counterstained regressively in Mayer's hematoxylin, dipped in methanol for a few seconds, cleared in xylene, and mounted in Permount. To assess the specificity of staining, sections were processed without primary or without secondary antibodies as controls. The same antibodies were used for the immunohistochemical study.

RESULTS

Of the 20 pregnant women with FHF, 15 were infected with HEV, three with HBV, and two with HCV, as revealed by both ELISA and PCR tests. The age of the patients ranged from 20-35 years. Postmortem liver biopsies were collected from 18 patients who died during the study. Two patients recovered following treatment. Ten healthy pregnant women constituted the control group, and an additional disease control group consisted of five pregnant women with AVH. The details of hepatitis viral infection, disease, and NF-kB p50 and p65 status are presented in Table 1.

Constitutive Activation and High Binding Activity of NF-kB in Pregnant Women with FHF

The DNA binding activity of NF-kB in FHF patients, as revealed by EMSA in nuclear extracts obtained from both PBMCs and postmortem liver biopsies demonstrated a very high binding activity of NF-kB in both pregnant and nonpregnant women (Figure 1; lanes d, e,

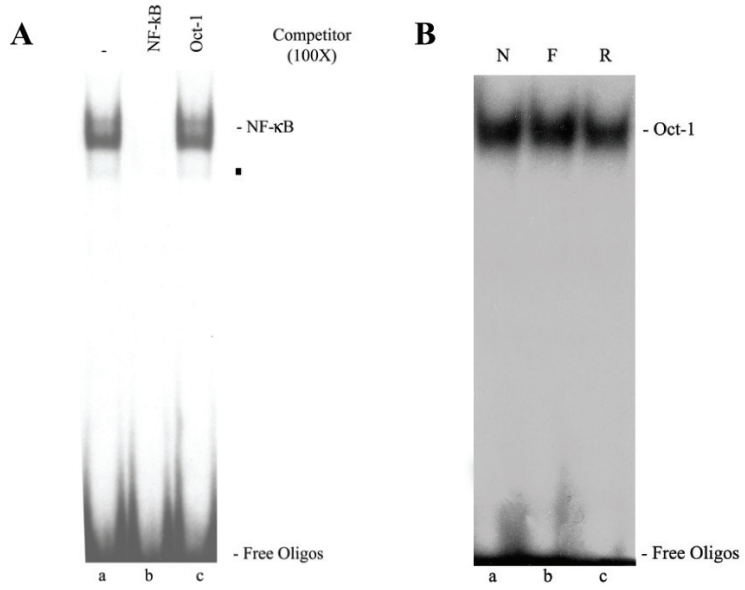

Figure 2. (A) EMSA using PBMC proteins from the FHF patients, with a ^{32}P-labeled oligonucleotide harboring an NF-kB consensus sequence. Binding specificity was evidenced by pre-incubation with a 100-fold molar addition of the homologous unlabeled oligonucleotide (lane b) in comparison with competition experiments using a heterologous consensus sequence of the Oct-1 transcription factor (lane c). The squares mark the unspecific complexes. (B) EMSA carried out with labeled oligos encompassing a consensus sequence of Oct-1 transcription factor showing uniform binding activity in PBMC proteins from different subjects. Lane a, normal healthy pregnant women as controls (N); lane b, hepatitis virus infected FHF pregnant patients (F); lane c, PBMC protein of patients who recovered back to normalcy (R). The positions of the specific retarded bands are indicated.

and f) with FHF due to hepatitis virus infection. In contrast, 8 of 10 women in the healthy control group showed very low binding activity (Figure 1; lane a). Two healthy controls, however, showed moderately higher NF-kB binding activity. Women in the disease control group (pregnant women with AVH) also showed a moderate level of NF-kB binding activity (Figure 1; lanes b and c). The extent of binding activity as revealed by densitometric analysis is indicated below each lane (see Figure 1). All of the pregnant women infected with HBV, HCV, or HEV showed very high NF-kB binding activity. The binding was found to be specific, because the retarded complex disappeared after competition with a 100-fold molar excess of a homologous (NF-kB), but not with a heterologous, cold probe containing the consensus sequence for the transcription factor Oct-1 (Figure 2A). The specificity of NF-kB binding was reconfirmed in a separate band shift assay in which we used labeled consensus sequence of Oct-1 as a probe (Figure 2B).

Absence of p65 Component in NF-kB Complex Formation in Pregnant FHF Patients

For further dissection of the NF-kB complex, band supershift experiments were carried out using antibodies raised against p50 and p65 components of NF-kB. In the normal healthy pregnant women who showed low or moderate levels of NF-kB binding activity, both p50 and p65 were involved in binding activity and complex formation, although p50 was the major component (Figure 3A). Most interestingly, we observed complete absence of p65 in dimer formation in pregnant females with FHF. It appears that p50 formed a homodimer, because all of the p50 but none of the p65 was supershifted (see Figure 3B, C). A similar pattern of differential binding activity of NF-kB was also observed in postmortem liver biopsy specimens from 18 pregnant women who died (Figure 4A). All cases of FHF in pregnant women revealed the absence of p65 in dimer formation in both PBMCs and liver tissues. In the two women who recovered, however, some p65 participation in the binding complex of NF-kB was observed, but to a much lesser extent (Figure 3 D, E).

SELECTIVE SUPPRESSION OF p65 PROTEIN EXPRESSION IN PREGNANT FHF PATIENTS

When we analyzed the expression profile of p50 and p65 at the protein level by immunoblotting, we observed that all pregnant women with FHF also showed a moderate to high level of p50 expression while p65 was completely absent or present in a negligible amount (Table 1, Figure 5). The same pattern of expression of NF-kB components was also observed in the postmortem liver biopsy specimens of FHF pregnant females who died after being admitted to the hospital (Figure 4B). To rule out the possibility of cytoplasmic localization of p65 in liver tissues of pregnant women with FHF, immunohistochemical analysis was carried out for in situ visualization, and it was very clear from the immunohistochemical staining that p65 expression was neither cytoplasmic nor nuclear in the postmortem liver biopsies (Figure 6). A complete absence of p65 expression was observed in all the postmortem liver biopsy specimens, and p50 expression was found to be both nuclear as well as cytoplasmic (Figure 6 C, D). In contrast, nuclear localization of both p50 and p65 was observed in normal liver biopsies (Figure 6 A, B).

Partial Participation of p65 in FHF Patients after Recovery

Although the binding activity of p65 as revealed by band supershift assays did not show significant differences be-

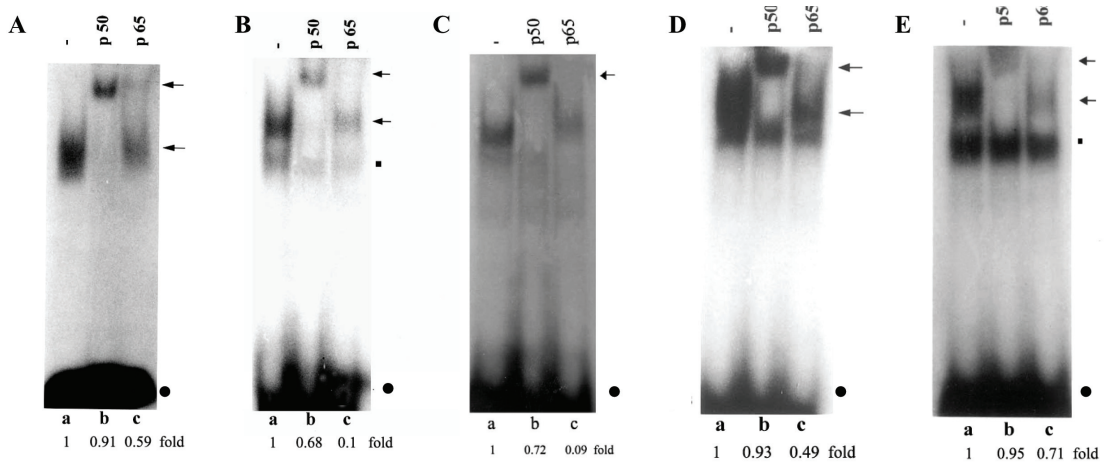

Figure 3. Electromobility supershift analysis using nuclear extracts from PBMC of (A) healthy pregnant women without hepatitis virus infection, (B, C) pregnant FHF patients infected with different hepatitis viruses, and (D,E) FHF patients after their recovery to normalcy, with ^{32}P-labeled oligonucleotides harboring an NF-kB consensus sequence showing differential binding activity of NF-kB components. PBMC nuclear extracts were incubated with specific antibodies (Abs) recognizing different members of the NF-kB family. Lane a, without Ab; lane b, addition of p50 Ab; lane c, addition of p65 Ab. The position of the NF-kB specific complex and the additionally retarded complexes is indicated with arrows. The squares mark the unspecific complexes. The dark circles mark the free oligos. The extent of supershifting as revealed by densitometry analysis are indicated below each lane and was derived in comparison to the non-shifted NF-kB complex in lane a.

tween pregnant and nonpregnant FHF patients, the expression of p65 protein in Western blotting did show low expression in nonpregnant FHF females (Figure 7, lane b) similar to that observed in FHF patients after recovery (Figure 7, lane c). Of 20 pregnant women with hepatitis virus-induced FHF, 18 died within 24 h of their hospital admission. Only two patients, one infected with HBV and the other with HEV, recovered following treatment. Interestingly, when we examined these women again one month after their complete recovery, we observed a moderately elevated level of p65 expression in these patients (Figure 7, lane c), although p50 still remained the major component (Figure 3D, E). A similar pattern of high binding activity of NF-kB but a low expression of p65 was observed in pregnant women with AVH who served as an additional control group (see Table 1 and Figure 7, lane d and e).

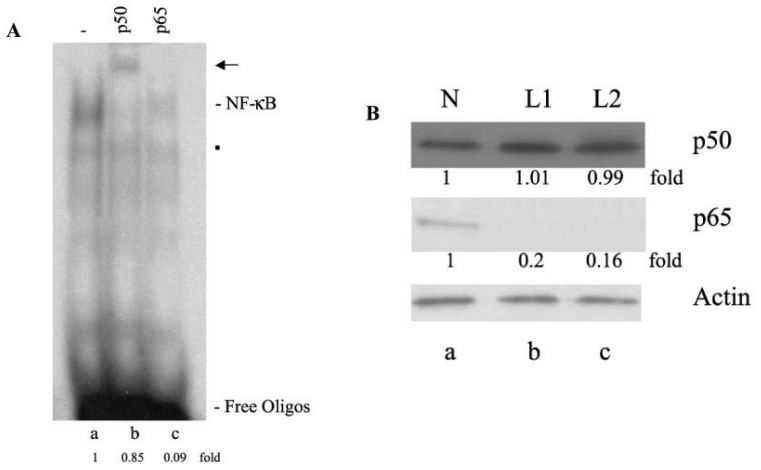

Figure 4. (A) Electromobility supershift analysis using nuclear extracts from liver biopsies of died pregnant FHF patient, with ^{32}P-labeled oligonucleotides harboring an NF-kB consensus sequence showing differential binding activity of NF-kB components. Liver biopsy extracts were incubated with specific antibodies (Abs) recognizing different members of the NF-kB family. Lane a, without Ab; lane b, addition of p50 Ab; lane c, addition of p65 Ab. The position of the NF-kB specific complex is indicated. The arrowheads mark the positions of additionally retarded 'supershift' complexes. The squares mark the unspecific complexes. The extent of supershifting as revealed by densitometry analysis are indicated below each lane and was derived in comparison to the non-shifted NF-kB complex in lane a. (B) Differential expression of p50 and p65 in liver biopsy from pregnant FHF patients who died after being admitted in the hospital; 30 μg protein from each case was separated in a 10% SDS-PAGE mini gel. After electrotransfer, the filters were consecutively incubated with p50 and p65 antibodies of NF-kB family. To confirm, equal protein loading, the filters were reincubated with a polyclonal Actin antibody. Lanes a, normal healthy pregnant women as disease controls (N); lane b and c, pregnant FHF patients who died. The increases in expression in comparison to the normal control as revealed by densitometric analysis are indicated below each lane.

DISCUSSION

HBV, HCV, and HEV are considered the principal etiologic agents for viral hepatitis and FHF, and extremely high mortality caused mainly by HEV infection occurs in Indian pregnant women

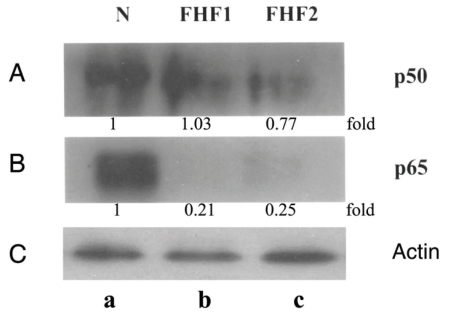

Figure 5. Differential expression of p50 and p65 in PBMC proteins from pregnant FHF patients (FHF1 and FHF2). From each patient, 30 μg of protein was separated in a 10% SDS-PAGE mini gel. After electrotransfer, the filters were consecutively incubated with p50 and p65 antibodies of NF-kB family. To confirm, equal protein loading, the filters were reincubated with a polyclonal Actin antibody. Lanes a, normal healthy pregnant women as disease controls (N); lane b and c, pregnant hepatitis virus infected FHF patients. The increase in expression in comparison to the normal control as revealed by densitometry analysis is indicated below each lane.

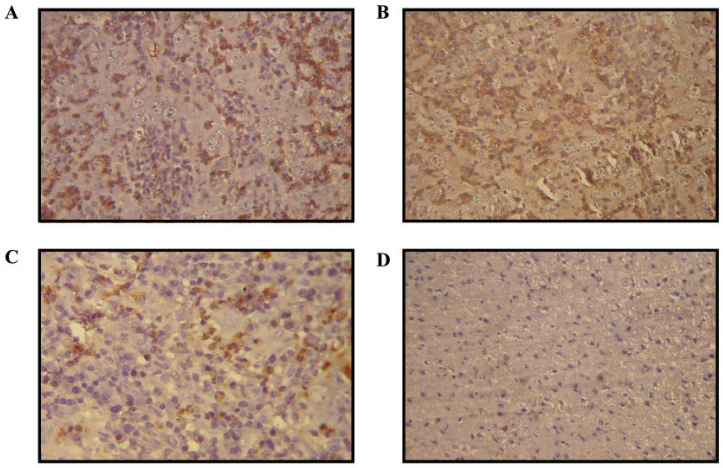

Figure 6. Immunohistochemical analysis of expression and localization of p50 and p65 in normal as well as post-mortem liver biopsies of FHF patients. Paraffin-embedded sections (4-5 μm thick) were immunolocalized with the Santa-Cruz antibodies specific for p50 and p65 of NF-kB. (A) IHC using p50 antibody in normal liver tissue; (B) IHC using p65 antibody in normal liver tissue; (C) IHC using p50 antibody in postmortem liver biopsy; (D) IHC using p65 antibody in postmortem liver biopsy.

compared with their nonpregnant counterparts (1,3). This high mortality was evident in the present study. Out of 20 pregnant FHF patients, 18 (90%) of which 14 (77.8%) were infected with HEV died within 24 h of their admission in the hospital, whereas none of five nonpregnant women with FHF died. We performed electrophoretic mobility supershift assays and immunoblotting using antibodies against p50 and p65 with the nuclear extracts prepared from blood samples as well as liver tissues of dying FHF patients along with controls. We observed a very high DNA binding activity of NF-kB in pregnant FHF patients compared with that of healthy controls, but further dissection of components in band-supershift assays revealed that in spite of high binding of NF-kB, the p65 always remained absent in NF-kB complex formation (Figure 3B, C). The complete absence of p65 expression is also confirmed by immunoblotting (Figure 5). Thus the major component showing high DNA binding activity and expression was p50, which was found to be forming a homodimer in absence of its canonical dimerization partner, p65. Interestingly, the two pregnant FHF patients who received treatment and returned to normal health showed reappearance of p65, although in a low amount, as a heterodimerization partner both in gel shift assay (Figure 3D, E) as well as in immunoblotting (Figure 7, lane c). Most interestingly, an inverse correlation between p65 expression and viral load of HEV has been observed in pregnant FHF patients who showed an extremely high

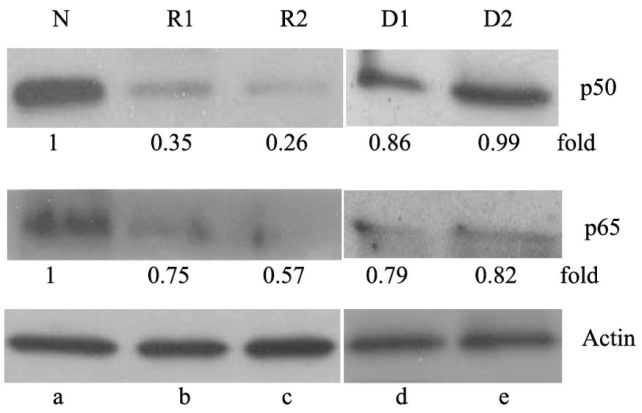

Figure 7. Comparison of differential expression pattern of p50 and p65 in PBMC proteins from a non-pregnant FHF (lane b) and a pregnant (lane c) FHF patient who recovered back to normalcy following treatment and 2 pregnant acute viral hepatitis (AVH) patient (disease controls) (lane d and e); 30μg proteins from each case was separated in a 10% SDS-PAGE mini gel. After electrotransfer, the filters were consecutively incubated with p50 and p65 antibodies of NF-kB family. To confirm, equal protein loading, the filters were reincubated with a polyclonal Actin antibody. Lanes a, normal healthy pregnant women as disease controls (N); lane b, non-pregnant FHF patients; lane c, recovered pregnant FHF patients. The increase in expression, in comparison to the normal control, as revealed by densitometry analysis is indicated below each lane.

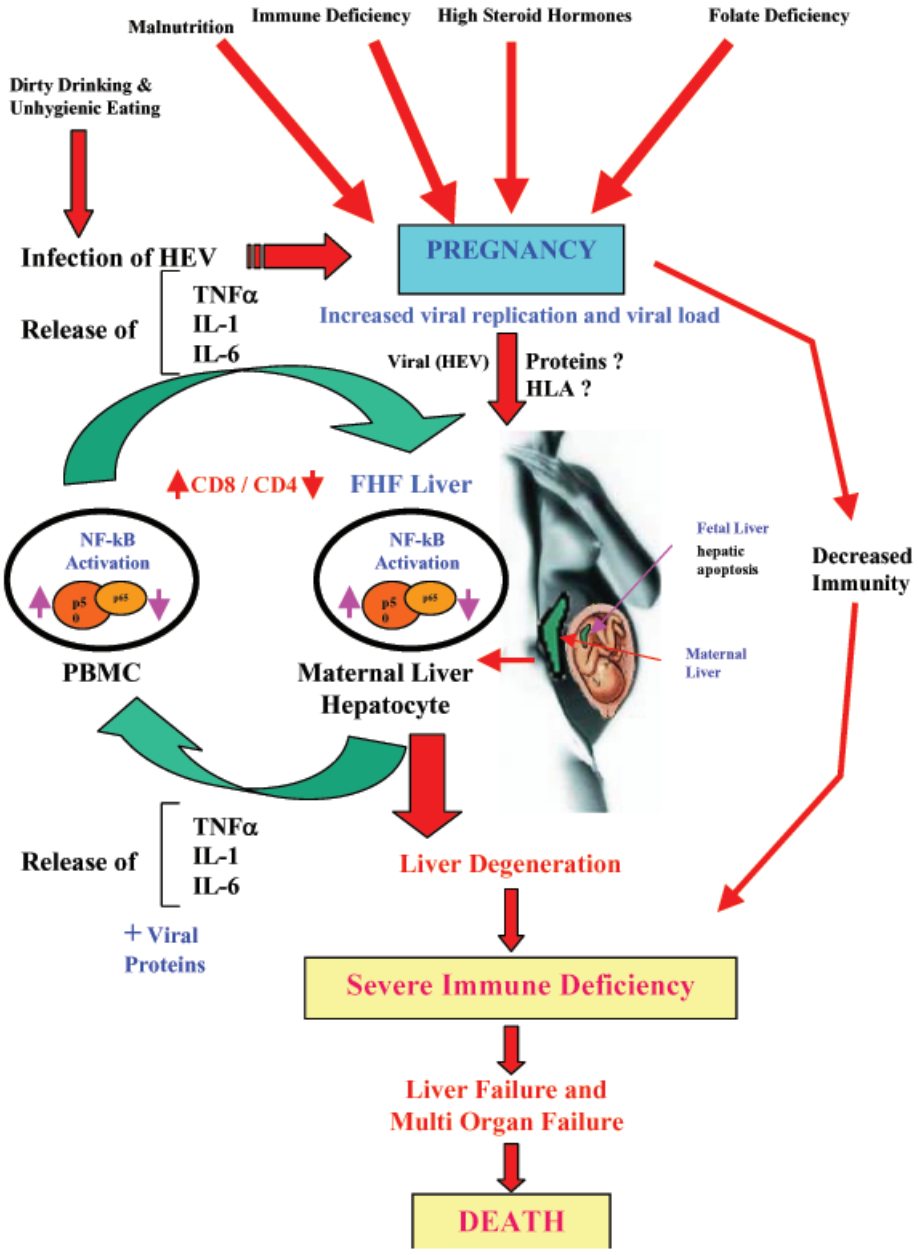

Figure 8. Diagrammatic representation of the possible signaling pathways involved in HEV-induced pathogenesis during pregnancy.

titer of viral load compared with that of pregnant women with AVH or nonpregnant women with FHF (Jilani et al, unpublished data).

In an ingenious experiment by Baltimore and his group [Beg et al. (14)], it has been demonstrated that mice lacking the p65 component of NF-kB show widespread hepatic apoptosis and die at 15-16 d of embryonic development. Also, experiments done with fibroblasts from 13-day-old embryos revealed that p65 deficiency interferes with inducible but not basal levels of NF-kB activity, and p65 has been shown to be essential for liver development, enhanced cell proliferation, and liver regeneration. Therefore, NF-kB is mostly activated after partial hepatectomy. Because the principal factors responsible for the poor clinical outcome and high mortality of FHF patients are severe liver damage, lack of liver regeneration, and impaired immunity, our observation of selective suppression of p65 and an increased homodimerization of p50 subunits leading to disruption of normal NF-kB complex and its function seems to play a crucial role during viral hepatitis and FHF. This hypothesis gains credence from our observation of partially upregulated expression of p65 in two pregnant FHF women who recovered after treatment. It is also important to note that although there is apparently no significant difference in increased NF-kB binding activity between pregnant and nonpregnant FHF patients, nonpregnant patients did show similarly moderate to low levels of p65 expression, as observed in patients who recovered. Also, an almost similar pattern of high binding activity of NF-kB but a low expression of p65 was observed in pregnant AVH patients who served as an additional disease control group (see Table 1 and Figure 7, lane d and e). Thus our results establish that the presence of p65 is most essential, and its absence is responsible for severe liver damage and high mortality in pregnant FHF patients. This conclusion gains further support from the recent observation that decreased expression of p65 causes liver fibrosis and liver damage in patients with HCV-induced chronic liver disease (22).

The expression of transcription factor NF-kB, which is generally downregulated during pregnancy, was recently demonstrated to play a pivotal role in regulating the maternal immune responses throughout gestation (21). NF-kB expression also regulates apoptosis of certain specific cell types through transcriptional control of cell-cycle regulatory and other protective genes (23,24), as is evident from the massive degeneration of liver by apoptosis leading to embryonic lethality in p65 knockout mice. Recently, McCracken et al (25) have showed that NF-kB is downregulated in T-cells during pregnancy, which in turn

increases the susceptibility of T-cells to apoptosis. We found very high NF-kB activity in pregnant FHF patients, which may be indicative of highly increased T-cell response leading to a severe imbalance in maternal immune responses. It is well established that T-cell-mediated immunity is highly decreased during pregnancy owing to marked reduction in T-cells and increased B cell counts (26–28). Recently, low CD4 and high CD8 T-cell counts and a decreased CD4/CD8 ratio leading to a low or loss of immunity in pregnant women was demonstrated (29). This finding indicates that HEV infection induces high production of CD8 cells, which appear to be recognized by specific viral proteins that are expressed specifically in liver cells (Figure 8). As is the case in HCV (30,31) and HBV (32) infection, the expression of specific HEV protein can also enhance NF-kB activation, which modulates immunoregulatory molecules and leads to production of serum and intrahepatic inflammatory cytokine including TNF-α (31). CD8 cells that kill hepatocytes may cause degeneration of liver and release of cytokines such as TNF-α, IL-1, and IL-6, which in turn activate NF-kB in PBMCs, leading to further release of the above cytokines (see Figure 8). It is also known that viral hepatitis and FHF can induce local production of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (33,34), which not only activate NF-kB but also exert effects on liver regeneration and hepatocyte apoptosis and liver necrosis (35). In addition, the decreased CD4/CD8 ratio causes severe immune deficiency in pregnant FHF women. Because the fetus is also infected through maternal circulation, similar phenomena occur in fetal liver, which is the site for development of the immune system. Therefore both mother and fetus are equally affected. The growing fetus is highly susceptible and affected first, and may die in utero or be aborted, or both the fetus and mother may die together. A schematic model showing the possible signaling pathways involved during hepatitis virus-induced FHF leading to death in advanced stage pregnant women is presented in Figure 8.

In contrast, immune function in the nonpregnant FHF patients was found to be much better than in their pregnant counterparts. A further deterioration in immune response during advanced pregnancy, when mortality due to FHF is significantly higher, may be contributed by selective suppression of p65 and/or disruption of normal NF-kB complexes.

The immunosuppressive functions of steroid hormones in lymphocytes are well documented (36) and are mediated through interactions between steroid receptors and NF-kB, leading to inhibition of NF-kB DNA binding activity. It is paradoxical, however, to find a high NF-kB binding activity but a low cellular immunity in pregnant FHF patients. The formation of NF-kB p50 homodimers, which also act as repressors of NF-kB–dependent transcription, and absence of p65 might explain this finding. Recently, a similar pattern of homodimerization of p50 subunits leading to functional inhibition of NF-kB was reported in laryngeal papilloma (37) and cervical (38) and oral carcinoma (39).

The level of sex steroid hormones, particularly progesterone and estrogens that are increased during later half of pregnancy, are also known to directly influence viral replication and viral gene expression through their effects on viral regulatory elements (40–42). Therefore pregnancy appears to be a potential risk factor for enhanced viral replication/expression and along with this extremely low immunity in Indian/Asian pregnant women, a vast majority of whom suffer from malnutrition and folate deficiency (43), which also contribute to liver injury, low immunity, and disease severity, leading to death (see Figure 8). Highly reduced immunocompetence is known to be associated with folate deficiency (43–45), which increases the multiple viral infection and/or increased viral load (43), including multifactorial disorders, in the Asian population (46,47). A selective high susceptibility of Asian pregnant women to viral hepatitis leading to liver failure could also be due to ethnicity-associated host genetic susceptibility, particularly associated with the genes of the major histocompatibility complex (MHC), which has a strong effect on immune response to viral antigens (48,49). Thus it is possible that a specific HLA allele(s) or haplotype(s) prevalent in Indian women might be associated with severe immune deficiency that influences the persistence HEV infection.

In conclusion, we suggest that the NF-kB signaling pathway is differentially regulated at the transcriptional level through upregulation and homodimerization of p50 subunits but selective suppression of its canonical dimerization partner p65. This suppression correlates with severe liver damage and complete breakdown of the immune system, which leads to multiple organ failure and death of both the mother and the fetus.

ACKNOWLEDGMENTS

The study was funded by Indian Council of Medical Research and Department of Biotechnology, Government of India, New Delhi. BKP received SRF funding from the Council of Scientific and Industrial Research, Government of India. Authors thank Dr. Alok C. Bharti for critical discussion.

REFERENCES

1. Khuroo MS, Teli MR, Skidmore S, Sofi MA, Khuroo MI. (1981) Incidence and severity of viral hepatitis in pregnancy. *Am. J. Med.* 70:252–5.
2. Balayan MS. (1997) Epidemiology of Hepatitis E virus infection. *J. Viral Hepatitis* 4:155–65.
3. Dilawari JB, Singh K, Chawla YK, *et al*. (1994) Hepatitis E virus, epidemiological, clinical and serological studies of a north Indian epidemic. *Indian J. Gastroenterol*. 134:44–8.
4. Arankalle VA, Chobe LP, Jha J, et al. (1993) Aetiology of acute sporadic non-A, non-B viral hepatitis in India. *J. Med. Virol*. 40:121–5.
5. Tsega E, Krawczynski K, Hansson BG, Nordenfelt E. (1993) Hepatitis E virus infection in pregnancy in Ethiopia. *Ethiop. Med. J*. 31:173–81.
6. Cahill KM. (1962) Hepatitis in pregnancy. *Surg. Gynecol. Obstet*. 11:545–52.
7. Purdy MA, Krawczynski K. (1994) Hepatitis E. *Gastroenterol. Clin. North Am*. 23:537–46.
8. Hamid SS, Jafri SM, Khan H, Shah H, Abbas Z, Fields H. (1996) Fulminant hepatic failure in

pregnant women: acute fatty liver or acute viral hepatitis? *J. Hepato*. 25:20–7.
9. Arankalle VA, Chadha MS, Dama BM, Tsarey SA, Purcell RH, Banerjee K. (1998) Role of immune serum globulins in pregnant women during an epidemic of hepatitis E. *J. Viral Hepat*. 5:199–204.
10. Siebenlist U, Franzoso G, Brown K. (1994) Structure, regulation and function of NF-kappa B. *Ann. Rev. Cell Biol*. 10:405–55.
11. Ghosh S, May MJ, Kopp EB. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Ann. Rev. Immunol*. 16:225–60.
12. Baeuerle PA, Baltimore D. (1998) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell* 53:211–7.
13. Baeuerle PA, Baltimore D. (1998) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242:540–6.
14. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376:167–70.
15. Trey C, Davidson C. (1970) The management of fulminant hepatic failure. *Prog. Liver Dis*. 3: 282–98.
16. Madan K, Gopalkrishna V, Kar P, Sharma JK, Das UP, Das BC. (1998) Detection of hepatitis C and E virus genome in sera of patients with acute viral hepatitis and fulminant hepatitis by their simultaneous amplification in PCR. *J. Gastroenterol. Hepatol*. 13:125–30.
17. Goldsmith R, Yarbough PO, Reyes GR, et al. (1992) Enzyme-linked immunosorbent assay for diagnosis of acute sporadic hepatitis E in Egyptian children. *Lancet* 339:328–31.
18. Katiyar S, Dash BC, Thakur V, Guptan RC, Sarin SK, Das BC. (2000) p53 tumor suppressor gene mutations in hepatocellular carcinoma patients in India. *Cancer* 88:1565–73.
19. Prusty BK, Das BC. (2005) Constitutive activation of transcription factor AP-1 in cervical cancer and suppression of human papillomavirus (HPV) transcription and AP-1 activity in HeLa cells by curcumin. *Int. J. Cancer* 113: 951–60.
20. Sambrook J, Russel, DW. (2001) Molecular cloning: a laboratory manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; p. 957–9.
21. McCracken SA, Drury CL, Lee HS, Morris JM. (2003) Pregnancy is associated with suppression of the nuclear factor kappaB/IkappaB activation pathway in peripheral blood mononuclear cells. *J. Reprod. Immunol*. 58:27–47.
22. Boya P, Larrea E, Sola I, Majano PL, Jiménez C, Civeira MP, Prieto J. (2001) Nuclear factor-kappa B in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. *Hepatology* 34: 1041–8.
23. La Rosa FA, Pierce JW, Sonenshein GE. (1994) Differential regulation of the c-myc oncogene promoter by the NF-kappa B rel family of transcription factors. *Mol. Cell Biol*. 14:1039–44.
24. Wu H, Lozano G. (1994) NF-kappa B activation of p53. A potential mechanism for suppressing cell growth in response to stress. *J. Biol. Chem*. 269:20067–74.
25. McCracken SA, Gallery E, Morris JM. (2004) Pregnancy-specific down-regulation of NF-kappa B expression in T cells in humans is essential for the maintenance of the cytokine profile required for pregnancy success. *J. Immunol*. 172:4583–91.
26. Purtilo DT, Hallgren HM, Yunis EJ. (1972) Depressed maternal lymphocyte response to phytohaemaglutinin in human pregnancy. *Lancet* 1: 769–71.
27. Lewis J Jr, Whang J, Negal B, Oppenheim JJ, Perry S. (1966) Lymphocyte transformation in mixed leukocyte cultures in women with normal pregnancy or, tumors of placental origin. A preliminary report. *Am. J. Obstet. Gynecol*. 96:287–90.
28. Hsia DY, Taylor RG, Gellis SS. (1952) Long-term follow-up study of infectious hepatitis in pregnancy. *J. Pediatr*. 41:13–17.
29. Jilani N, Das BC, Husain SA, et al. (2005) Hepatitis E virus infection and fulminant hepatic failure (FHF) during pregnancy. *J. Gastroenterol. Hepatol*. 22:676–82.
30. Liao QL, Ye LB, Timani KA, et al. (2005) Hepatitis C virus non-structural 5A protein can enhance full-length core protein-induced nuclear factor-kappa B activation. *World J. Gastroenterol*. 11: 6433–9.
31. Hassan M, Selimovic D, Ghozlan H, et al. (2007) Induction of high-molecular-weight (HMW) tumor necrosis factor (TNF) alpha by hepatitis C virus (HCV) non-structural protein 3 (NS3) in liver cells is AP-1 and NF-kappa B-dependent activation. *Cell Signal*. 19:301–11.
32. Kanda T, Yokosuka O, Nagao K, Saisho H. (2006) State of hepatitis C viral replication enhances activation of NF-kB- and AP-1-signaling induced by hepatitis B virus X. *Cancer Lett*. 234:143–8.
33. Muto Y, Nouri-Aria KT, Meager A, Alexander GJ, Eddleston AL, Williams R.(1988) Enhanced tumor necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet* 2:72–4.
34. de la Mata M, Meager A, Ralando N, Daniels HM, Nouri-Aria KT, Goka AK. (1990) Tumor necrosis factor production in fulminant hepatic failure: relation to etiology and superimposed microbial infection. *Clin. Exp. Immunol*. 82:479–84.
35. Andus T, Bauer J, Gerok W. (1991) Effects of cytokines on the liver. *Hepatology* 13:364–75.
36. Mellor AM, Munn DH. (1999) Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? *Immunol. Today* 20:469–73.
37. Vancurova I, Wu R, Miskolci V, Sun S. (2002) Increased p50/p50 NF-kappa B activation in human papillomavirus type 6- or type 11- induced laryngeal papilloma tissue. *J. Virol*. 76:1533–6.
38. Prusty BK, Husain SA, Das BC. (2005) Constitutive activation of nuclear factor-kB: preferential homodimerization of p50 subunits in cervical carcinoma. *Front. Biosci*. 10:1510–9.
39. Mishra A, Bharti AC, Varghese P, Saluja D, Das BC. (2006) Differential expression and activation of NF-kappa B family proteins during oral carcinogenesis: Role of high risk human papillomavirus infection. *Int. J. Cancer* 119:2840–50.
40. Ponta H, Kennedy N, Scroch P, Hynes NE, Groner B. (1985) The hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. *Proc. Natl. Acad. Sci. USA*. 1020–4.
41. Gopalkrishna V, Murthy NS, Sharma JK, et al. (1995) Increased human papillomavirus infection with increasing number of pregnancies in Indian women. *J. Infect. Dis*. 171:254–5.
42. Karpa R, Laub O. (1990) Corticosteroids stimulate hepatitis B virus DNA, mRNA and protein production in a stable expression system. *Hepatol*. 11:34–6.
43. Piyathilake CJ, Henao OL, Macaluso M, et al. (2004) Folate is associated with the natural history of high-risk human papillomaviruses. *Cancer Res*. 64:8788–93.
44. Dhur A, Galan P, Hercberg S. (1991) Folate status and the immune system. *Prog. Food Nutr. Sci*. 15: 43–60.
45. Jukes TH. (1983) Folic acid deficiency. *Nature* 301: 192.
46. Mukherjee M, Joshi S, Bagadi S, Dalvi M, Rao A, Shetty KR. (2002) A low prevalence of the C677T mutation in the methylenetetrahydrofolate reductase gene in Asian Indians. *Clin. Genet*. 61: 155–59.
47. Radha Rama Devi A, Govindaiah V, Ramakrishna G. (2004) Prevalence of methylene tetrahydrofolate reductase polymorphism in south Indian population. *Curr. Sci*. 86:440–43.
48. Hohler T, Reuss E, Evers N, et al. (2002) Differential genetic determination of immune responsiveness to hepatitis B surface antigen and to hepatitis A virus: a vaccination study in twins. *Lancet* 360:991–95.
49. Thio CL, Carrington M, Marti D, et al. (1999) Class II HLA alleles and hepatitis B virus persistence in African Americans. *J. Infect. Dis*. 179:1004–6.