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Anti-hepatitis B virus activity of *N*-acetyl-L-cysteine (NAC): new aspects of a well-established drug

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Abstract

N-acetyl-L-cysteine (NAC) is commonly administered as an antidote against acetaminophen intoxication and is the preferred agent in the treatment of pulmonary diseases. It is furthermore commonly considered that it restrains human immunodeficiency virus (HIV) replication by scavenging reactive oxygen intermediates (ROI) and thus suppressing activation of nuclear factor κB (NFκB). We show here that NAC is in addition able to inhibit hepatitis B virus (HBV) replication, but by a mechanism independent of the intracellular level of reactive oxygen intermediates. Treatment of HBV-producing cell lines with NAC resulted in an at least 50-fold reduction of viral DNA in the tissue culture supernatant within 48 h. This decrease of viral DNA and thus of virions in the tissue culture supernatant is caused by a disturbance of the virus assembly, rather than by a reduction of viral transcripts. Our data strongly suggest a potential use of this well-established, non-toxic drug for the treatment of HBV infection. Since NAC, in contrast to interferon, exerts its anti-HBV activity at a posttranscriptional level, a combination of NAC with the established interferon therapy could also be considered.

Keywords: Hepatitis B virus (HBV); *N*-acetyl-L-cysteine (NAC); Interferon; Hepatitis B virus surface antigen

1. Introduction

Despite the development of vaccines, HBV infection remains a serious health problem, affecting more than 300 million people worldwide who are chronically infected by the virus. Chronic infection is correlated with HBV replication in the hepatocytes of the liver and the presence of virions in the bloodstream. The risk of developing a hepatocellular carcinoma (HCC) has been estimated to be more than 100-fold higher in chronic carriers of HBV than in non-infected persons (Beasley and Hwang, 1991). Although interferon-α has been used with promising results in the therapy of chronic infection in some patients treated for prolonged periods (Perrillo et al.,

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1990), overall response rates have been unsatisfactory. Thus the development of new, non-toxic and more efficient strategies for the treatment of HBV infections is a matter of urgent necessity.

NAC is commonly administered as an antidote against drug-induced hepatotoxicity for paracetamol poisoning, counteracting the effects of oxidative stress (Smilkstein et al., 1988). In this context, NAC exerts its chemoprotective activities by scavenging reactive oxygen intermediates (ROIs) such as hydrogen peroxide, super oxide anions and others. In various in vitro systems, oxygen radical scavengers, like dithiocarbamates (PDTC) and NAC, have been shown to block the stimulation of HIV-1 LTR by transactivator Tax from human T-cell leukaemia virus (HTLV-I) by inhibiting the activation of transcription factor NF-κB (Schreck et al., 1992). It has been suggested that NAC suppresses the replication of human immunodeficiency virus (HIV-1) by employing the same signal transduction pathway (Roederer et al., 1990).

This study was performed to find out whether NAC is also able to restrain the replication of HBV. HBV is a small enveloped DNA virus with a partially double-stranded genome of approximately 3.2 kb in size. After infection, the viral genome is converted into a covalently closed molecule and transcribed into RNA molecules of 3.5 kb, 2.4 kb, 2.1 kb and 0.8 kb in length (reviewed in Ganem and Varmus, 1987). The longest transcript serves as a template for the core antigen and the viral polymerase. Furthermore, the 3.5-kb RNA represents the viral pregenome and is packaged into nucleocapsids together with the viral polymerase. The transcription of this terminally redundant RNA is initiated at the enhancer II/core promoter (E_{II}/C_p) which is therefore also called the pregenomic promoter region. After reverse transcription of the pregenomic RNA, nucleocapsids are surrounded by HBV surface antigens (HBsAg) and released from the host cell. The expression of the surface proteins occurs from a 2.1-kb RNA which represents the middle-sized and the small HBsAg and from a 2.4-kb transcript that stands for the large surface antigen. Besides these transcripts there is a minor RNA 0.8 kb in length. This 0.8-kb message is derived from the X-gene open reading frame (ORF), which encodes a transcriptional transactivator (HBx transactivator) that stimulates the expression of a broad variety of viral and cellular genes.

We show here that *N*-acetyl-L-cysteine is a helpful tool to block the extracellular spread of HBV. In the case of HIV replication, NAC is suggested to exert its antiviral activity on a transcriptional level. In contrast to this, the data presented here show that the anti-HBV effect of NAC is not caused by a reduction of viral transcripts but by a disturbance of the virus assembly. Thus, NAC should be a helpful agent to support the immune-mediated endeavour of HBV-infected persons to eliminate the virus and should therefore be tested in an appropriate clinical trail.

2. Materials and methods

2.1. Tissue culture cells:

Tissue culture cells were maintained in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL), supplemented with 10% foetal calf serum (FCS), 50 units/ml of streptomycin and penicillin and 1 mM glutamin. For HepG2-4A5 cells (Weiss et al., 1996) and HepG2-2.2.15 cells (Sells et al., 1987) 250 μg/ml G418 (GIBCO) were added to the medium. Cell line HepG2-2.2.15 had been established by stable transfection of HepG2 cells (American Type Culture Collection (ATCC) No. HB8065) with a plasmid carrying multiple copies of the HBV genome under the control of the LTRs of Moloney murine leukaemia virus. HepG2-4A5 cells were established by stable transfection of HepG2 cells with a terminally redundant HBV plasmid (pSPT1.2xHBVneo), containing all the viral genes exclusively under the control of their autologous regulatory elements. Thus, HepG2-2.2.15 cells are capable of producing higher amounts of HBV particles than HepG2-4A5 cells, containing only one terminally redundant HBV genome in a chromosomal integrated state.

2.2. Detection of viral DNA in the medium

For dot-blot hybridisation, viruses from two independent experiments were precipitated from

50 ml culture medium with polyethylene glycol ($M_w = 8000$). Viral DNA was isolated by proteinase K digest, phenol/chloroform extraction and analysed by dot-blot hybridisation using a ^{32}P-labelled HBV DNA fragment. The amount of viral DNA was quantified by a Bio-Imaging analyser (Fujix; BAS 1000; MacBAS). To determine the number of virions released from the cells, serial dilution of 3.2 kb HBV-DNA (25–800 pg) were used as a positive control. Mean PSL values (photo stimulated luminescence) were calculated from two independent experiments.

For a semiquantitative polymerase chain reaction (PCR), viral DNA from 400 μl of tissue culture supernatant was extracted using a commercial kit (QIAamp®, DIAGEN GmbH) and amplified in an automatic DNA thermal cycler (denaturation: 30 s, 95°C; annealing: 30 s, 55°C; extension: 90 s, 72°C) using HBV primers PCR-HBV$_{510-529}$ (5′-CCTATGGGAGGGGGCC-TCAG-3′) and PCR-HBV$_{630-609}$ (5′-CCAATAC-CACATCATCCATATA-3′) corresponding to regions within the ORF of the HBV surface antigen. PCR reaction products were analysed by agarose gel electrophoresis and Southern blotting using a ^{32}P-labelled oligonucleotide PCR-HBV$_{601-580}$ (5′-GCCAAACAGTGGGGGAAA-GCCC-3′) corresponding to an internal part of the amplified DNA. As an external standard, 1.5 μg of a CAT reporterplasmid were added to the tissue culture supernatant, extracted together with the viral DNA and amplified in the same way as described above using appropriate primers.

One of the characteristic features of hepadnaviruses is that virions contain an only partially double-stranded genome with a single-stranded gap in the (+)-strand DNA. This gap can be completed in an in vitro assay system called endogenous polymerase reaction (EPR). In this assay system the enzymatic activity of the viral polymerase, which is packed together with the DNA genome into the nucleocapsid, is used to elongate the viral genome with dNTPs, one of which is radioactively labelled. EPR was carried out using an anti-HBcAg (HBV core antigen) antibody (DAKO, Hamburg) for immunoprecipitation of viral core particles, essentially as described previously (Radziwill et al., 1988) with the difference that all four dNTPs were used at the same concentration (10 μM).

2.3. CAT assays and CAT plasmids

The day before transfection, HepG2 cells were split into 6-cm tissue culture plates at a density of 10^6. Transfection was performed by the calcium phosphate coprecipitation method, applying a glycerol shock 6 h later (Graham and van der Eb, 1973). Cells were transfected with 6 μg pSPT1.2xHBV, containing a terminally redundant, 1.2-fold HBV genome (Weiss et al., 1996), or with an equimolar amount of cloning vector pSPT19 (Boehringer Mannheim) together with 7.5 μg of reporterplasmids (this ratio has been optimised in previous experiments). CAT reporterplasmid pHBV-E$_{II}$-CAT harbours the enhancer II and the core gene promoter of HBV subtype adr4 (*Bam*HI-*Bgl*II fragment) in front of the CAT gene. Reporterplasmid pHIV-LTR-CAT has been described elsewhere (Nabel and Baltimore, 1987) and contains the CAT gene under the control of the HIV-1 LTR (nucleotides −453–+80). Inhibitors (30 mM NAC) or stimulating agents (30 ng/ml tumour necrosis factor α (TNF-α); 100 ng/ml tetradecanoylphorbol acetate (TPA)) were added to the medium after the glycerol shock. 36 h after transfection, cells were lysed and the protein concentration of the cytoplasmic extract was determined. CAT activity was assayed with 100 μg protein as described (Gorman et al., 1982). Quantitative values were obtained by measuring the amount of acetylated and unreacted [^{14}C]chloramphenicol with an isotope scanner (Berthold), and mean CAT activities were calculated from three independent assays.

2.4. Chemicals

A 1-M stock solution of NAC was prepared in serum-free DMEM and neutralised by addition of NaOH. This stock solution was degased and stored for less than 1 week at +4°C. NAC, TPA and TNF-α were supplied by Sigma. Dideoxycytidine (ddCTP) was purchased from Pharmacia.

2.5. Cytotoxicity and cell proliferation assay

The effects of NAC on cell growth were determined by the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) into cellular DNA. For this purpose HepG2-4A5 cells were seeded into flat-bottomed 96-well plates at a density of $2 \cdot 10^4$ cells/well and incubated in the absence or in the presence of various amounts of NAC for 54 h. After this incubation period, tissue culture medium was replaced by DMEM containing BrdU as a labelling reagent, and the cells were incubated for another 2 h. BrdU incorporated into cellular DNA was assayed according to the manufacturers instructions (Boehringer Mannheim, cell proliferation assay) and the amount of labelled DNA was quantified by using a peroxidase-labelled antibody to BrdU.

2.6. Northern blot analysis

12 h after treating cells with NAC or TNF-α respectively, polyadenylated RNA was isolated and analysed by Northern blotting using a ^{32}P-labelled HBV DNA probe. As a loading control, membranes were rehybridised with a β-actin probe.

2.7. Southern blot analysis

HepG2-4A5 cells were incubated with NAC for 4 days. DNA was isolated from the cells of one 6-cm dish and intracellular extrachromosomal DNA was separated as described (Hirt, 1967). 20 μg of episomal DNA were analysed by agarose gel electrophoresis and subsequent Southern blotting using a ^{32}P-labelled HBV probe.

3. Results

3.1. Treatment of HBV-producing cell lines with NAC results in a dose-dependent reduction of virions in the tissue culture supernatant

To find out whether NAC is able to restrain the replication of HBV two stably transfected, HBV-producing cell lines HepG2-2.2.15 (Sells et al., 1987) and HepG2-4A5 (Weiss et al., 1996) were employed. Both cell lines harbour a single terminally redundant (HepG2-4A5) HBV genome or multiple copies (HepG2-2.2.15) of the HBV genome in a chromosomal integrated state and thus are capable of producing potentially infectious hepatitis B virions. HepG2-4A5 cells were treated with increasing amounts of NAC up to 30 mM and the amount of viral particles in the tissue culture supernatant was analysed by quantification of viral DNA in the tissue culture supernatant (Fig. 1(a)). By comparison with serial dilution of HBV-DNA it can be calculated that untreated HepG2-4A5 cells secrete about 10^5–10^6 virions/ml (by assuming 2.6 pg DNA/10^6 virions). This number decreased by about 2.5-, 10- and at least 50-fold after cells were treated with 3, 10 and 30 mM NAC respectively within 48 h. Similar results were observed when HepG2-2.2.15 cells were used instead of HepG2-4A5 cells (data not shown), excluding a cell type-specific effect. The dose-dependent reduction of viral DNA in the tissue culture supernatant was also confirmed by semiquantitative PCR (Fig. 1(b)) and EPR (Fig. 1(c)). As the EPR requires a replication-competent viral DNA-genome which is, together with the viral polymerase, encapsidated into core particles, the decrease of reaction products also demonstrates a clear reduction of viral particles in the supernatant of HepG2-4A5 cells. Taken together we could show by employing three independent methods that NAC treatment of HBV-producing cell lines results in a dose-dependant reduction of virions in the tissue culture supernatant, as measured by quantification of viral DNA.

3.2. Inhibition of HBV replication in HepG2-4A5 cells is not due to a loss of cell proliferation

To rule out the possibility that the observed inhibition of HBV replication by NAC is the consequence of a non-specific cytotoxic effect, cellular proliferation and viability were assayed by the incorporation of 5-bromo-2'-deoxyuridine into the chromosomal DNA (Boehringer Mannheim, cell proliferation kit). HepG2-4A5 cells were split into 96-well plates and treated with NAC in in-

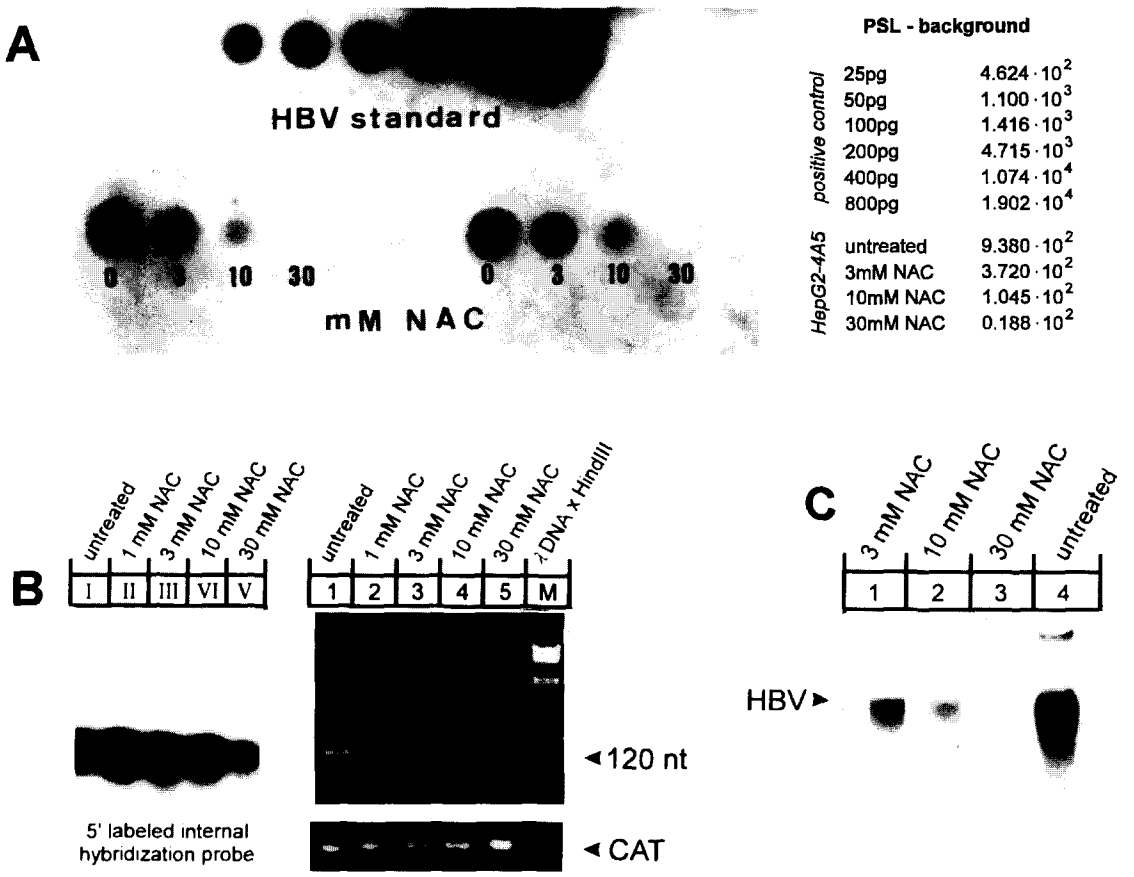

Fig. 1. Treatment of HBV-producing cell line HepG2-4A5 with NAC results in a dose-dependent reduction of viral DNA in the culture medium. HepG2-4A5 cells were seeded at a density of $1 \cdot 10^6$ cells per 6-cm dish. Cells were grown for 48 h in the presence or absence of NAC and the quantity of viral DNA in the tissue culture supernatant was analysed by dot-blot hybridisation (A), quantitative PCR (B) and endogenous polymerase reaction (EPR) (C). One of the characteristic features of HBV is its partially double-stranded DNA genome found in extracellular virions. The single-stranded gap can be completed by elongating the (+)-strand DNA in an in vitro assay system called EPR (Kaplan et al., 1973). Thus, this assay is an appropriate tool to detect and to quantify hepatitis B virions in the tissue culture supernatant in a very specific way.

creasing amounts ranging from 1 to 200 mM for 56 h. No cytotoxic effects up to 30 mM NAC were observed, whereas concentrations higher than 50 mM resulted in a complete loss of cell proliferation (Fig. 2). In addition, no morphological changes were visible up to 30 mM NAC. For further experiments, NAC concentrations no higher than 30 mM and incubation periods no longer than 48 h were used. Since no cytotoxic side effects occurred, neither morphologically nor by a change in cell proliferation, the plain reduction of viral particles (Fig. 1(a)–(c)) must be caused by an antiviral activity of NAC.

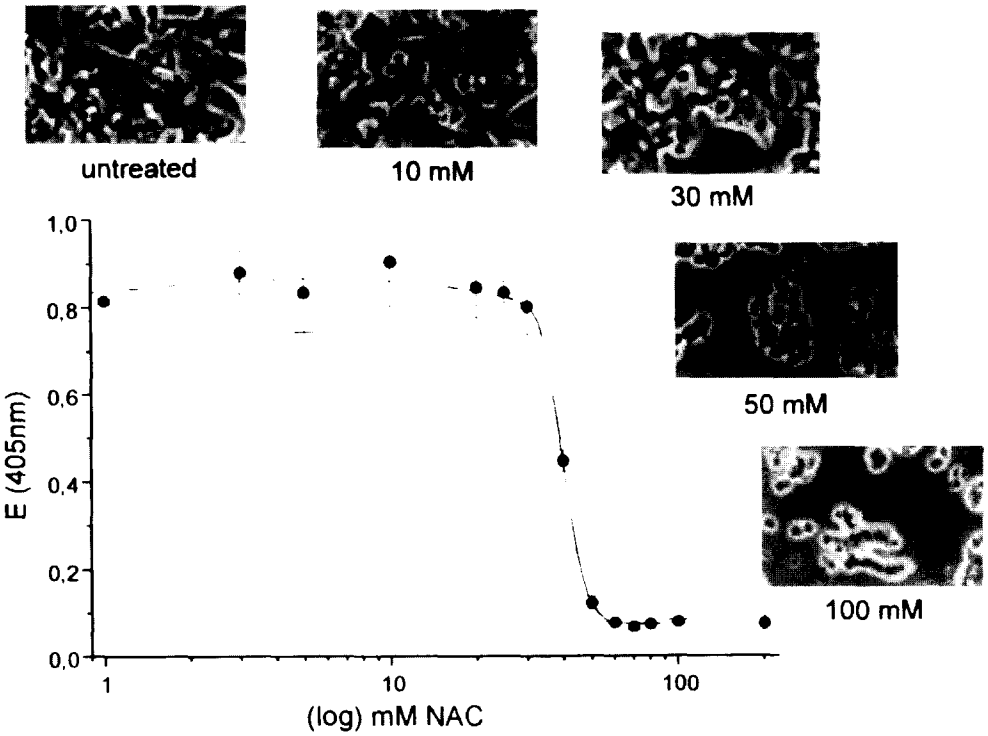

Fig. 2. NAC up to 30 mM has no effect on DNA synthesis and viability of HepG2-4A5 cells. HepG2-4A5 cells were split into 96-well plates and treated with NAC in increasing amounts ranging from 1 to 200 mM, for 56 h. Afterwards, cellular proliferation and viability were assayed by the incorporation of 5-bromo-2′-deoxyuridine into the chromosomal DNA (Boehringer Mannheim, cell proliferation kit). To document morphological changes, photographs of the cell monolayer are shown.

3.3. In contrast to the HIV-LTR, the HBV pregenomic promoter is not influenced by reactive oxygen intermediates

A group in our laboratory has recently shown that antioxidants selectively suppress transactivation of NF-κB-dependant CAT reporterplasmids by HBx, a transcriptional transactivator encoded in the HBV genome (Meyer et al., 1992). Furthermore, NAC is known to antagonise the phorbolester- and cytokine-induced stimulation of HIV-LTR directed transcription by inhibiting NF-κB activation via scavenging ROIs. It has been suggested that the same mechanism counteracts the effect of TNF-α- and TPA-induced stimulation of HIV-1 replication in latently infected monocytes and T-lymphocytes (Roederer et al., 1990; Kalebic et al., 1991). To find out whether NAC exerts its anti-HBV effect, similar to the situation in HIV, by repressing the activity of the pregenomic promoter, a CAT assay was performed. The HBV pregenomic promoter region (E_{II}/C_p) shows several similarities to retroviral LTR regions and, like these, controls transcription of the pregenomic RNA. Therefore, we used reporterplasmids containing these regulatory regions to study the effect of NAC on transactivation by HBx. Cotransfection of liver cells with reporterplasmid pHIV-LTR-CAT together with an HBx expressionplasmid is known to cause a distinct activation of the HIV-LTR. Thus, a combination of pHIV-LTR-CAT reporterplasmid and the HBx transactivator containing plasmid pSPT1.2xHBV (Weiss et al., 1996) was used. Cotransfection of HepG2 cells with these two plasmids caused an approximately 10-fold stimulation of the HIV-LTR (Fig. 3(a), lane 4 as compared with lane 1). This transeffect was partially sup-

A

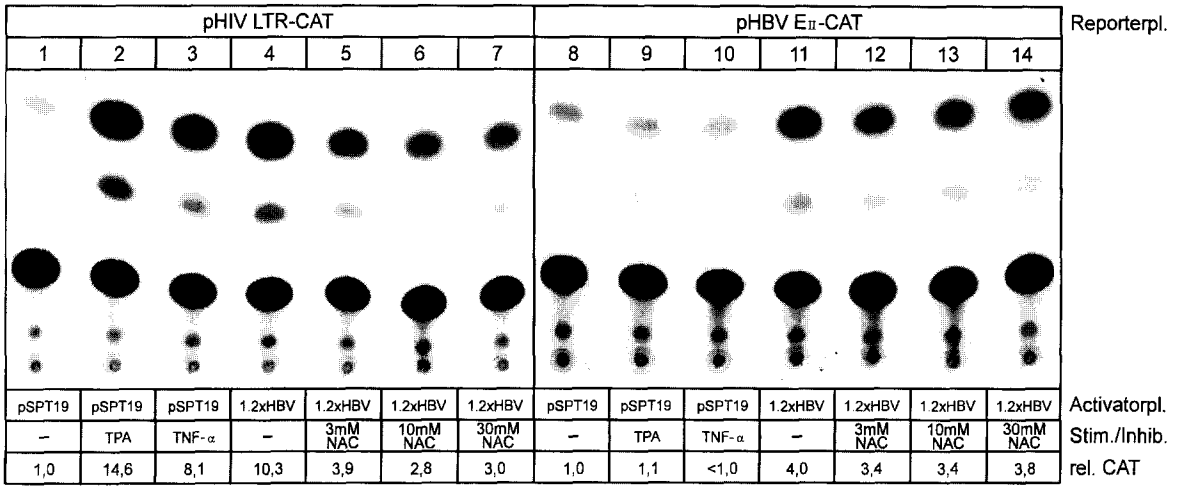

Fig. 3. Suppression of HBV replication by NAC is not caused by a transcriptional inhibition. (A) CAT assays were performed after cotransfection of HepG2 cells with reporterplasmids pHIV-LTR-CAT or pHBV-E_{II}-CAT respectively. In contrast to the HIV-LTR, the addition of ROI-inducing agents such as TNF-α (30 ng/ml) or TPA (100 ng/ml) showed no stimulating effect on the HBV pregenomic promoter region. Although the regulatory regions of both viruses could be activated by the HBx transactivator, only transcription initiated from the HIV-LTR region could be suppressed by NAC but not the HBV pregenomic promoter. These data were supported by Northern blot hybridisation of PolyA$^+$ RNA from HepG2-4A5 and HepG2-2.2.15 cells (B).

pressed by NAC (lanes 5–7), while stimulating the cells with TPA (lane 2) or TNF-α (lane 3) was able to cause the same effect as the HBx transactivator in a mock transfected control. TNF-α and the tumour promoter TPA, are both known to induce intracellular ROIs and to activate NF-κB (reviewed in Schreck and Baeuerle, 1991). This supports the idea that ROIs are involved in mediating the HBx transeffect on the HIV-LTR by activating NF-κB. In accordance with data published previously (Roederer et al., 1990), the data presented here also indicate that ROIs are involved in regulating the transcriptional activity of the HIV-LTR. When reporterplasmid pHIV-LTR-CAT was replaced by pHBV-E_{II}-CAT, containing the HBV-pregenomic enhancer/promoter region in front of the CAT gene, no stimulation was observed, either by TPA or by TNF-α (Fig. 3(a), lanes 9 and 10). Correspondingly, the HBx transeffect, although it was only about 4-fold, could not be suppressed by NAC (lanes 12–14). These data show that the HBV-E_{II}/C_P region is, in contrast to the HIV-LTR, not regulated by ROIs. Thus NAC cannot exert its antiviral activity by suppression of the HBV pregenomic promoter, which controls the transcription of the 3.5-kb pregenomic RNA. To confirm these data by an independent method, a Northern blot analysis of HBV-RNAs from HepG2-2.2.15 and HepG2-4A5

B

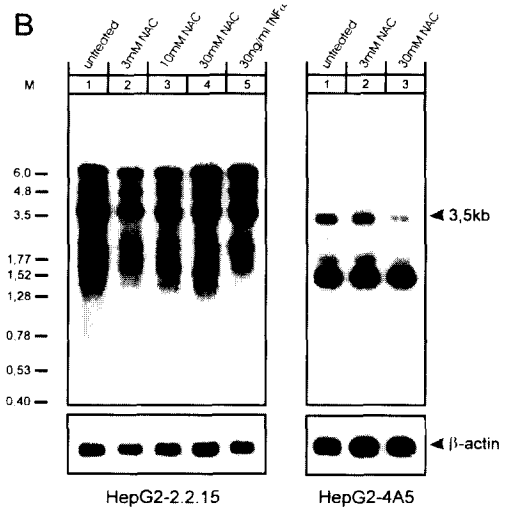

Fig. 3B

cells was performed (Fig. 3(b)). The amount of each viral transcript was not significantly affected by treating cells with NAC (Fig. 3(b), lanes 2–4 as compared with lane 1) and consequently also was not increased by TNF-α (lane 5) in HepG2-2.2.15 or HepG2-4A5 cells. Taken together, these data indicate that the decrease of viral DNA in the medium of HepG2-4A5 cells as caused by NAC (Fig. 1) must be due to a posttranscriptional effect, rather than to a decline of viral pregenome formation. Thus the decrease of viral DNA in the medium of HepG2-4A5 cells as caused by NAC (Fig. 1(a)–(c)), must be due to a posttranscriptional or even posttranslational effect.

3.4. Inhibition of HBV replication by NAC is exerted on a posttranscriptional level

To further distinguish between these two possibilities, HBV DNA-replication intermediates were extracted by the method of Hirt (Hirt, 1967) and quantified by Southern blotting. HBV replication intermediates are synthesised by reverse transcription of pregenomic RNA within viral core particles. Thus the quantity of DNA replication intermediates as measured by this assay reflects viral transcription and translation as well. Treatment of HepG2-4A5 cells with NAC revealed an elevation rather than a decrease of DNA replication intermediates (Fig. 4), demonstrating an intracellular increase of viral core particles. Thus, the anti-HBV activity of NAC must be exerted at a posttranslational step, leading to the conclusion that viral particles are either not properly formed or that they can no longer be secreted by the cells, resulting in an accumulation of core particles in the cytoplasma under the influence of NAC.

3.5. Addition of NAC renders HBsAg to be no longer detectable by a commercial enzyme immunoassay

For more than two decades it has been known that the structure of HBsAg is stabilised by extensive disulphide linkage (Vyas et al., 1972). Since NAC is known to rupture disulphide bridges, which may lead to a disturbance of virus assembly, we analysed the amount of viral antigens in

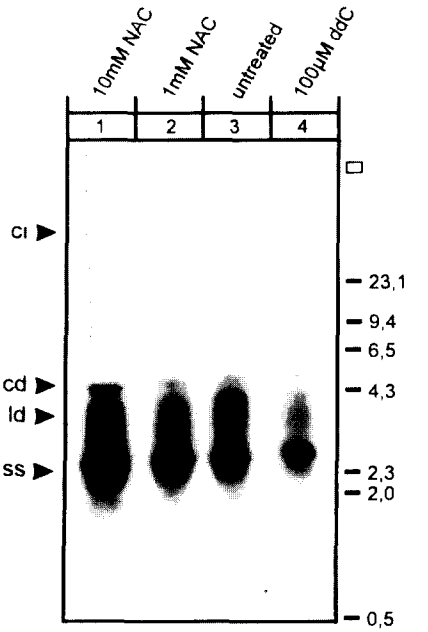

Fig. 4. Suppression of HBV replication by NAC is exerted at a very late step in HBV replication. Episomal viral DNA was prepared from HepG2-4A5 cells treated for 4 days with NAC and analysed by Southern blotting. As a control, the reverse transcriptase inhibitor ddCTP was used. The blot shows chromosomal integrated (ci), circular double-stranded (cd), linear double-stranded (ld) and single-stranded (ss) HBV DNA replication intermediates.

the tissue culture supernatant of HepG2-4A5 cells. As is to be expected from the reduction of virions (Fig. 1(a)–(c)), HBc/eAg in the medium is reduced in a dose-dependent manner as well (Fig. 5(a)). Under the same conditions, HBsAg secretion appeared to be much more affected (Fig. 5(b)). To assess the nature of this difference, tissue culture supernatant of untreated HepG2-2.2.15 cells was removed from the cell monolayer and afterwards treated with 50 mM NAC. While there was no difference in the HBV envelope antigen (HBeAg) level, HBsAg was no longer detectable by a commercial enzyme immunoassay (Fig. 5(c)). As the major antigenic loop of the HBsAg is stabilised by extensive disulphide linkage, a rupture of disulphide chemical bounds by NAC is the most likely explanation for this observation. These data support the idea that NAC exerts its anti-HBV activity at a posttranslational level and

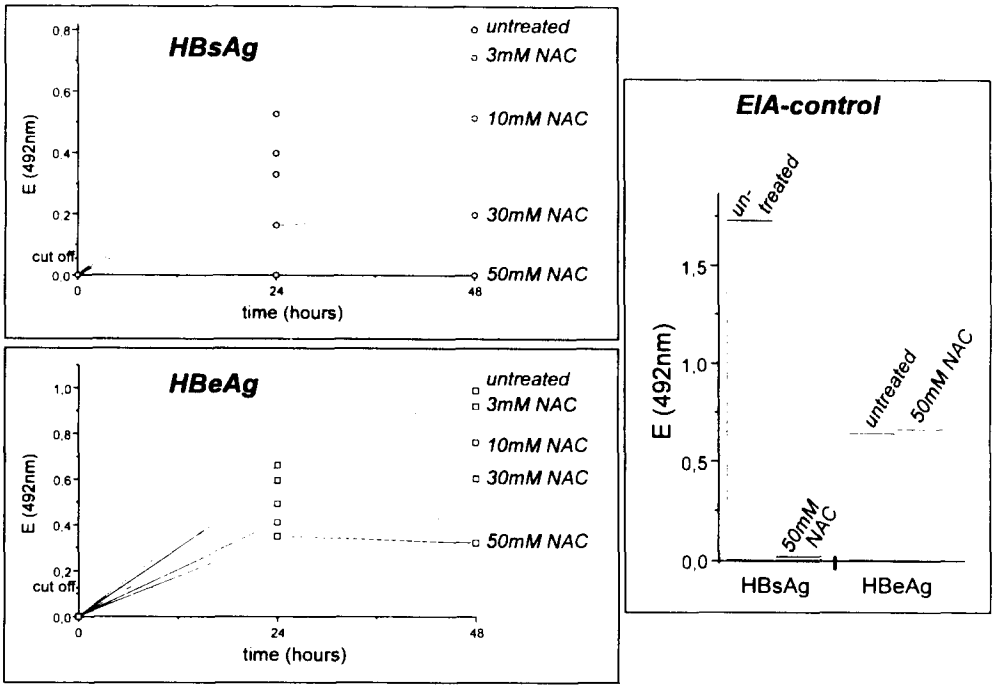

Fig. 5. Disturbance of disulphide chemical bonds is the most likely explanation for the anti-HBV activity of NAC. HepG2-4A5 cells were treated with increasing amounts of NAC (3–50 mM) and the amount of HBc/eAg (A) and HBsAg (B) was measured by enzyme-linked immunosorbent assay (ELISA) 24 and 48 h later (Auszyme II and HBe (rDNA) from Abbott Laboratories, respectively). As a control, 50 mM NAC was added to HBsAg and HBc/eAg positive tissue culture supernatant from untreated HepG2-2.2.15 cells (C).

thus are in accordance with the results observed by analysing the amount of episomal viral DNA (Fig. 4). Further studies will have to show which disulphide bridge(s) are affected by NAC and how the assembly of HBV is influenced by thiol compounds.

4. Discussion

Although further work will be necessary to unravel the details of the anti-HBV effect of NAC, the data presented here clearly demonstrate a dose-dependent antiviral activity of NAC. Furthermore, they show that the mechanism of this antiviral effect is different for HIV and for HBV. The outstanding amount of cysteine residues and the stabilisation of HBsAg by disulphide bridges is known to be essential for antigenic activity of this protein (Vyas et al., 1972). In addition, it is known that HBsAg forms disulphide-linked dimers, acting as an intermediate in HBsAg assembly. These dimers have recently been shown to be converted into high-molecular-weight disulphide-linked oligomers (Huovila et al., 1992). Thus, the correct formation of disulphide bridges is a very crucial step in HBV assembly. Since NAC is known to rupture disulphide bridges, it is suggestive that the anti-HBV effect of NAC—which has been demonstrated by employing different methods (Fig. 1)—is, as well as the disorder of the HBsAg antigenicity (Fig. 5), based on a disturbance of HBV assembly.

Immune-mediated destruction of infected hepatocytes is often not powerful enough to rapidly and completely clear an HBV infection in the presence of circulating virus, resulting in chronic infection. Thus any agent that can block the

extracellular spread of HBV and that can be used in a long-term therapy might assist the immune system in eliminating the virus. NAC meets both conditions: (i) the amount of mature virions is clearly reduced after NAC treatment, and (ii) NAC is a widely used drug in the treatment of chronic bronchitis with no toxic side effects and therefore can be administered in long-term treatment, helping to prevent the spread of the virus. Loading dosages of up to 140 mg/kg body weight followed by doses of 70 mg/kg, can be given every 4 h over a period of at least 3 days without severe side effects (Miller and Rumack, 1983; Gavish and Breslow, 1991). Calculated from these data, NAC serum concentrations of about 1 mM are easily attainable. Whether such high amounts of NAC are necessary in a long-term treatment in order to suppress HBV replication in an HBV-infected patient cannot be answered from the data observed in our in vitro system. NAC is known to bind to protein very rapidly after administration. Within 1 h after administration 50% of NAC is already bound to protein (Olsson et al., 1988). This shows the extraordinarily high affinity of this agent to proteins, a mechanism which strongly supports the anti-HBV effect of NAC as postulated from our data. Therefore, even lower dosages of NAC should be helpful in a long-term treatment of HBV infection. Furthermore, as the targets of NAC in the multiplication cycle of HBV are different from those influenced by interferon-α, a combination of NAC with the established interferon therapy can also be considered. Such a combination has already been used in the treatment of hepatitis C virus infection (Beloqui et al., 1993). Considering the data presented here, the negligible side effects of NAC treatment and the unfavourable prognosis after developing an HBV-associated hepatocellular carcinoma, the question of how well NAC can restrain HBV production in the HBV-infected patient should be investigated in an appropriate clinical trial.

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