The Cytopathic Effect of HIV Is Associated with Apoptosis

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Large amounts of histones, H1, H2A, H2B, H3, and H4, were observed in total extracts of T4 lymphocytes and derived cell lines infected with the human immunodeficiency virus (HIV) type 1 or type 2. These histones were simply detectable by analysis of crude cellular extracts by polyacrylamide gel electrophoresis in SDS and staining the proteins with Coomassie blue or by immunoblot assays using specific polyclonal antibodies. The histones were found to be localized in the nucleoplasm, bound to low molecular weight (LMW) DNA in the form of nucleosomes. The mechanism responsible for the accumulation of nucleosomes during HIV infection was found to be due to fragmentation of cellular DNA, a mechanism referred to as apoptosis or programmed cell death in which a nuclear endonuclease becomes activated and cleaves DNA at internucleosomal regions. Accordingly, the LMW DNA accumulated in the course of infection was found to have a characteristic pattern of nucleosomal ladder and its accumulation was reduced in the presence of zinc, a known inhibitor of the endonuclease. Routinely in acute HIV infections, the accumulation of nucleosomes was observed at least 24 hr before lysis of infected cells. In a particular HIV-1 infection, in which the first signals of the cytopathic effect (vacuolization of cells and appearance of syncytia) was observed at Days 6-7 whereas maximal virus production occurred at Days 10-17, the accumulation of nucleosomes was at its maximal level already on Day 6 postinfection. In the nucleoplasm of chronically infected cells producing virus but not manifesting a cytopathic effect, no LMW DNA or histones were detectable. These observations indicate that the cytopathic effect of HIV infection is associated with apoptosis. The detection of histones and oligonucleosomal DNA fragments in the nucleoplasm can be used as a convenient marker for chromatin fragmentation during this process.

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INTRODUCTION

The human immunodeficiency virus (HIV) infects lymphocytes, monocytes, and macrophages by binding to its principal receptor, the CD4 molecule (Marsh and Dalgleish, 1988). It has been demonstrated that HIV recognition of CD4 is mediated by the external envelope glycoprotein of the virus (McDougal et al., 1986). Consequently the entry of the virus particle in the cell might occur by viral envelope and cell membrane fusion (Lifson et al., 1986; Sodroski et al., 1986; Stein et al., 1987; McClure et al., 1988). Once the HIV core is found in the cytoplasm of the infected cells, viral RNA becomes transcribed by the reverse transcriptase. The proviral DNA, which can exist in a linear or circularized form, is then integrated into the host chromosomal DNA (for a recent review see Cann and Karn, 1989). In addition, large amounts of unintegrated viral DNA become accumulated in infected cells (Shaw et al., 1984; Muesing et al., 1985). In general, HIV infection of cell cultures might generate an acute or/and chronic infection. In both cases virus is produced and becomes released by budding at the cellular membrane. An acute infection is characterized by a typical cytopathic effect manifested by vacuolization of cells and formation of syncytia and consequently by cell lysis. On the other hand chronically infected cells do not show this typical cytopathic effect despite their constant capacity to produce infectious HIV particles. This difference between acute and chronic infection might be in part due to a lower amount of virus particles produced and down-regulation of CD4 receptors in chronically infected cells (Fauci, 1988; Stevenson et al., 1988; Besansky et al., 1991, personal observations). The cytopathic effect of HIV has recently been suggested to involve a mechanism whereby virus infection primes a series of processes leading to apoptosis or programmed cell death (Ameisen and Capron, 1991).

Apoptosis and necrosis are the two stereotyped mechanisms by which nucleated eukaryotic cells die (Duvall and Wyllie, 1986). Necrosis is considered as a pathological reaction occurring in response to major perturbations in the cellular environment such as a lytic viral infection. On the other hand, apoptosis is considered as a physiological process taking part in homeostatic regulation, when death is part of reactions involved in normal tissue turnover (Duvall and Wyllie, 1986).
1986; McConkey et al., 1989; Rothenberg, 1990). The mechanism(s) leading to apoptosis is not yet very clear and it might be different from one system to another. In most cases, apoptosis follows an increase in cytosolic Ca²⁺ concentration, requires protein synthesis, and is manifested by endogenous nuclease activation leading to fragmentation of DNA into oligonucleosome-length DNA fragments (reviewed in McConkey et al., 1990). Here we provide evidence to demonstrate that LMW DNA in the form of nucleosomes become accumulated in the nucleoplasm of HIV-infected cells during an acute infection. Our results favor the hypothesis that HIV-infected cells die because infection triggers physiological events which lead to fragmentation of cellular DNA. These events occur during the course of infection at a time when de novo protein synthesis is comparable to that of uninfected cells.

MATERIALS AND METHODS

Viruses and cells

The HIV-1 Lai isolate previously referred to as HIV-1 Bru (Wain-Hobson et al., 1991) was used in this study. This virus and the HIV-2 Rod have been extensively studied in our laboratory (Laurent et al., 1989, 1990; Rey et al., 1989a). CEM cells were cultured in suspension medium RPMI-1640 (GIBCO-BRL) containing 10% (v/v) fetal calf serum and 2 kg/ml Polybrene (Sigma). CEM clone 13 cells are derived from the human lymphoid cell line CEM (ATCC-CCL 119) and express the T4 antigen to a high level. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from heparinized venous blood by density centrifugation on Ficoll metrizoate (lymphoprep; Nycomed, Oslo), as previously described (Salmon et al., 1988). The lymphocytes were stimulated for 3 days with 0.2% (w/v) phytohemaglutinin fraction P (Difco Laboratories, Detroit, MI) in RPMI 1640 medium containing 10% (v/v) fetal calf serum and 2 µg/ml Polybrene (Sigma). The CD4 positive enriched PBMC were prepared as previously described (Salmon et al., 1988). The lymphocytes were stimulated for 3 days with 0.2% (w/v) phytohemaglutinin fraction P (Difco Laboratories, Detroit, MI) in RPMI 1640 medium containing 10% (v/v) fetal calf serum. Cells were then cultured in RPMI 1640 medium containing 10% (v/v) T-cell growth factor (Biotech). After infection with HIV, lymphocytes were cultured in the presence of 10% (v/v) T cell growth factor and 2 µg/ml Polybrene. For metabolic labeling of proteins, infected cells were incubated for 6 hr at 37° in minimum essential medium without L-methionine and serum but supplemented with 200 µCi/ml [³⁵S]-methionine. For metabolic labeling of histones, cells were incubated (18 hr) in minimum essential medium without L-lysine and serum but supplemented with 70 µCi/ml of [³H]lysine. [³⁵S]-methionine (specific activity > 1000 Ci/mmol) and [³H]lysine (sp act 75–100 Ci/mmol) were purchased from Amersham (Amersham, UK).

Preparation of extracts

Total cell extracts (cytoplasm plus nucleoplasm) were prepared in lysis buffer containing 10 mM Tris-HCl, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.2 mM PMSF, 100 units/ml aprotinin (Iniprol), and 1% Triton X-100. This suspension was then centrifuged at 12,000 g to pellet aggregated material and cellular chromatin and recover the supernatant comprising cytoplasm and nucleoplasm. For the recovery of the cytoplasm separately from the nucleoplasm, cells were disrupted in the cytoplasm extraction buffer containing 10 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 5 mM MgCl₂, 0.2 mM PMSF, 100 units/ml aprotinin, and 0.5% Triton X-100. After centrifugation at 1000 g, the supernatant contained the cytoplasm whereas the intact nuclei were pelleted. The nuclei were then extracted in the lysis buffer and centrifuged at 12,000 g to separate the nucleoplasm from the high molecular weight chromatin.

Antibodies

Rabbit antiserum specific for isolated histones H1, H2A, H2B, H3, and H4 were those previously described (Mullor et al., 1991). They were obtained by immunizing rabbits with each histones purified from calf thymus and complexed to RNA as described by Stollar and Ward (1970). Murine polyclonal antibodies raised against commercial preparations of human H2A and H2B (Worthington) were prepared using poly(A)·poly(U) as adjuvant (Hovanessian et al., 1988). Mice were injected intraperitoneally at 12 days interval with a suspension of 200 µg of H2A or H2B mixed with 200 µg of poly(A)·poly(U). Six days before the fifth immunization, mice were injected intraperitoneally with a suspension of sarcoma 180/TG cells to prepare hyperimmune fluid. Aaicid fluids were then recovered and tested by immunoblot assay. By this technique, we were able to generate powerful antibodies although in addition to H2A or H2B they recognized also other histone subtypes. This was probably due to the fact that the commercial preparations of H2A or H2B were not pure.

Analysis of histones

Crude extracts were diluted onefold in electrophoresis sample buffer (250 mM Tris–HCl, pH 6.8, 2% [w/v] SDS, 20% glycerol, and 1% β-mercaptoethanol) and boiled 5 min before analysis by polyacrylamide gel (15%) electrophoresis in SDS. Histones were clearly detectable by staining the gel with Coomassie blue. Two-dimensional gel isoelectric focusing was performed as previously described (Laurent et al., 1989). For this latter technique, cell extracts diluted in electro-
Analysis of nucleosomal DNA fragments

The nucleoplasma (prepared as described above) contained the low molecular weight (LMW) DNA whereas the pellet of the nuclear extracts contained the high molecular weight (HMW) chromatin. Nucleoplasma was incubated with 20 μg/ml RNase for 1 hr at room temperature, then 100 μg/ml proteinase K for 2 hr at 37°C, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with ethanol. The DNA pellet was resuspended in electrophoresis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and analyzed by electrophoresis on 1.4% agarose gels containing 0.5 μg/ml ethidium bromide at 60 V for 2.5 hr. Unintegrated HIV-DNA was also analyzed by slot-blot. The radiolabeled HIV-DNA probe corresponding to the entire HIV-1 genome was from p-BRU2, a molecular clone of HIV-1Lai (K. Peden et al., manuscript in preparation).

Results

Detection of histones in total cell extracts of HIV-infected cells

All the experiments presented here were carried out with a stock of HIV 1 (Lai) prepared on CEM cells. At a dose of HIV-1 50–100 TCID50, the cytopathic effect (c.p.e.) can be clearly observed 2 to 3 days postinfection (p.i.) by vacuolization of cells and appearance of syncyta. Fig. 1A shows a typical experiment presenting the synthesis of HIV proteins at different days p.i. By immunofluorescence studies (data not shown), it is possible to demonstrate that the infection is gradual, in that less than 15% of cells are positive for HIV proteins (gag p18, p25) at 48 hr p.i. but reaching more than 90% at 4 days p.i. Most of the cells become lysed on the fifth day p.i. In agreement with the immunofluorescence studies, more and more HIV proteins are synthesized during the kinetics of infection (Fig. 1A).

Analysis of total cellular extracts, containing cytoplasmic and nucleoplasmic proteins, by SDS–polyacrylamide gel electrophoresis indicated the presence and accumulation of histones during the course of infection. These histones were clearly recognized by their electrophoretic mobility in polyacrylamide gels stained with Coomassie blue (Fig. 1B). The presence of different histone subtypes, H1, H2A, H2B, H3, and H4, were confirmed by immunoblot analysis using specific antibodies (Fig. 1C). These results indicated the presence of considerable amounts of H2A, H2B, H3, and H4 in infected cell extracts in contrast with corresponding extracts from uninfected cells. The level of H1 was high in control cell extracts but there seemed to be a slight increase in infected cell extracts (Fig. 1C, H1). The presence of H1 in cell extracts was probably due to the fact that the lysis buffer contained 0.4 M NaCl. Normally, H1 stabilizes the nucleosome structure and is located in the region of the exit and entry points of the DNA (Thoma et al., 1979) and as a contrast to the other histone subtypes found trapped in the core particles of nucleosomes, it becomes released at high ionic concentrations (Kornberg et al., 1989).

Accumulation of histones was routinely detectable in CEM cells infected with HIV-1 or HIV-2. The kinetics of accumulation were always correlated with the kinetics of infection. The maximum accumulation occurred at least 24 hr before lysis of cells at a time when more than 90% of cells were viable by their capacity to exclude Trypan blue (data not shown). During this period, the cells were actively involved in the synthesis of viral proteins (Fig. 1A). It should be emphasized that histones were not found to be associated with virus particles nor were they detectable in the culture medium. These observations suggest that the presence...
FIG. 1. Detection of histones in total cell extracts during the course of HIV infection. (A) CEM cells infected with HIV-1 were labeled with [35S]methionine for 6 hr at 1, 2, 3, and 4 days postinfection (lanes 1 to 4). Cell extracts were then immunoprecipitated using HIV-1 seropositive patient serum (Materials and Methods). The fluorograph shows the different HIV-1 proteins: the external (gp120) and transmembrane (gp41) envelope glycoproteins, the gag precursor (p55) and its partially cleaved product (p40), and the major core protein (p25). (B) Polyacrylamide gel (15%) electrophoretic analysis of total extracts (cytoplasm + nucleoplasm) from uninfected (Control: Days 2 and 4) or HIV-1-infected (HIV-1, Days 2, 3, and 4) CEM cells. The gel was stained with Coomassie blue. On the left is the position of molecular weight markers and on the right is the position of histone subtypes. (C) Immunoblot assay for identification of different histone subtypes using rabbit antibodies specific for H1, H2A, H2B, H3, and H4 (Materials and Methods). Lanes minus or plus correspond to extracts (Day 4 p.i. in (B)) from uninfected (−) or infected (+) cells. For the different experiments shown in (A, B and C), each sample corresponded to material from 1 x 10^8 cells.

of histones in total cell extracts was not a consequence of cell lysis but it was probably due to an abnormal event primed by virus infection.

Synthesis of histones is not enhanced during the course of HIV infection

In the experiment shown in Fig. 1B, the amount of histones at Day 4 p.i. could be estimated to be about 10 μg/1 x 10^6 cells. As a first step to find an explanation for this dramatic increase of histone accumulation, we studied their synthesis during the kinetics of infection by metabolic labeling of cells with [3H]lysine or [3H]arginine for 2 to 6 hr at every day p.i. (data not shown). The results clearly demonstrated that there was no significant difference in the level of newly synthesized histones between infected and uninfected CEM cells. However, a small proportion of histones seemed to be modified in infected cells since their isoelectric points shifted from basic to acidic pH values. Figure 2 shows a two-dimensional gel isoelectric focusing analysis of [3H]lysine-labeled extracts at Days 1 and 4 p.i. In parallel, cold extracts were also analyzed by the same method and the position of histones was revealed by immunoblot analysis using specific antihistone antibodies (Fig. 2, W.B). In [3H]-labeled extracts from infected cells, two new spots were revealed at pl values at pH 8.0–8.2 and corresponding to proteins of 16–18 kDa. These proteins were identified as being H2A and H2B by the immunoblot assay. It should be noted that the modified histones should represent less than 10% of the amount observed in the cell extracts since they were not revealed by Coomassie blue staining but were detectable weakly by silver nitrate staining (data not shown). The major proportion of histones because of their basic pl were excluded from the gel dur

FIG. 2. Modified H2A and H2B revealed by two-dimensional gel isoelectric focusing in extracts from HIV-infected cells. Cold extracts (W.B. for immunoblot assay) or [3H]Lys-labeled extracts ([H][Lys]; metabolic labeling for 18 hr) from CEM cultures 1 and 4 days p.i. were analyzed by two-dimensional gel isoelectric focusing (Materials and Methods). The immunoblot assay was carried out with mixed rabbit polyclonal antibodies against H2A and H2B. The figure shows a section of each radiograph. The pH gradient obtained by isoelectric focusing (1st dimension) was between 8.0 and 8.5. In the second dimension, the proteins were resolved by SDS–PAGE. It should be noted that all samples were first suspended in SDS before isoelectric focusing (Materials and Methods).
**Histones become accumulated in the nucleoplasm complexed to LMW DNA**

Routinely we prepared extracts in the lysis buffer containing 1% Triton X-100, 0.4 M NaCl, and 1 mM EDTA. Under these experimental conditions, we recovered the cytoplasm together with the nucleoplasm separately from the aggregated chromatin. In order to find out the subcellular localization of the increased histones, cells at 2, 3, and 4 days p.i. were first disrupted in a low salt buffer containing magnesium to separate the cytoplasm from the nuclei which were then lysed in the lysis buffer. In this particular experiment lysis of infected cells occurred 5 to 6 days p.i. Samples corresponding to the different preparations were analyzed by SDS–polyacrylamide gel electrophoresis and the proteins were revealed by Coomassie blue staining (Fig. 3). It is apparent from this experiment that histones observed in total extracts from HIV-infected cells are localized in the nucleoplasm and that very little is found in the cytoplasm. The increase in the level of histones (H2A, H2B, H3, and H4) in the nucleoplasm on Day 4 is highly significant since its amount was almost equivalent to that found in the cellular chromatin. At Days 2 to 4, most of HI was recovered in the nucleoplasm because of the high ionic concentration in the lysis buffer.

The nucleoplasm of HIV-1 infected cells at 4 days p.i. was passed through a Sephadex G-50 column and the elution was monitored by measuring absorbance at 260 nm. Most of the material in the nucleoplasm was eluted just after the void volume as a sharp peak (Fig. 4a). This fraction was found to contain LMW DNA of size less than 23,000 bp (see Fig. 5, lane 3), the unintegrated extrachromosomal DNA of HIV (Fig. 4b) and each of the histone subtypes, H1, H2A, H2B, H3 and H4.

**Fig. 3.** The accumulated histones are localized in the nucleoplasm of infected cells. CEM cells were infected with HIV-1, and at 2, 3, and 4 days p.i. (lanes 2, 3, and 4) cells were disrupted to prepare extracts corresponding to the cytoplasm, nucleoplasm (Nuclear-SN) and high molecular weight DNA or chromatin (Nuclear-pellet). The nuclear pellet (chromatin) was directly suspended in the SDS–electrophoresis sample buffer whereas cytoplasmic and nucleoplasmic extracts were diluted once in twofold concentrated SDS–electrophoresis buffer. Aliquots corresponding to material from 1 × 10⁷ cells were analyzed by polyacrylamide gel (15%) electrophoresis. The figure shows the Coomassie blue-stained gel.

**Fig. 4.** Histones in the nucleoplasm are all associated together in a high molecular weight complex. The nucleoplasm from HIV-1-infected cells (10⁷) at Day 4 p.i. was loaded on a Sephadex G-50 column (0.8 × 30 cm) equilibrated in the lysis buffer. Elution was also in the same buffer. Fractions (1.0 ml) were collected immediately after the void volume (10 ml). (a) The absorbance value at 260 nm of each fraction (abscissa). The peak of bovine plasma albumin (indicated by the arrow 66 kDa) was in fraction 8. (b) Slot blot analysis to reveal HIV DNA in the different fractions; an autoradiogram is shown. (c) Immunoblot analysis of the different fractions using rabbit antibodies specific for H2A. (d) Immunoblot analysis of fractions 1 to 3 using rabbit antibodies specific for H1, H2A, H3, and H4. In sections c and d the autoradiograms show only the lower portion of gels. In (b, c and d), the samples 1–3 correspond to the peak in section a (i.e., fractions 2–4).
Accumulation of nucleosomes in the nucleoplasm of HIV-infected cells. The profile of LMW DNA extracted from the nucleoplasm (A) and the profile of total proteins (B) from uninfected (lanes 4 and 6) or HIV-infected CEM cells (lanes 1–3, 5, and 7). Lanes 1–3: HIV-1-infected cells at 2, 3, and 4 days p.i., respectively. Lanes 5 and 7: HIV-1 and HIV-2-infected cells respectively, at Day 4 p.i. Material corresponding to $2 \times 10^6$ cells (A) and $1 \times 10^6$ cells (B) were analyzed. The DNA markers are: 100 basepair ladder on the right and HaeIII digest of $\phi X 174$ on the left.

Accumulation of nucleosomes in the nucleoplasm due to fragmentation of cellular chromatin

Figure 5A shows the profile of the LMW DNA found in the nucleoplasm of CEM cells infected with HIV-1 (lane 5) or HIV-2 (lane 7). In both cases there was a typical ladder of DNA fragments, corresponding to oligonucleosomes made up of multimers of 146-bp DNA fragments. In contrast, nucleoplasm of uninfected cells contained very little LMW DNA (Fig. 5, lanes 4 and 6). The amount of LMW DNA in the nucleoplasm of HIV-infected cells 4 days p.i. could be estimated to be 5 to 10 $\mu$g/1 $\times 10^6$ cells. In a kinetics experiment in HIV-1-infected cells, the amount of LMW DNA and the degree of fragmentation were significantly increased during the course of infection (Fig. 5, lanes 1 to 3 corresponding to Days 2, 3, and 4 p.i.). Previously, such fragmentation of DNA has been correlated to an apoptosis process, i.e., programmed cell death due to the activation of a Ca$^{2+}$-dependent cellular endonuclease which cleaves the linker DNA that connects adjacent nucleosomes (reviewed by McConkey et al., 1990). Comparison of the DNA pattern in the nucleoplasm (Fig. 5A) with that of histones observed in total extracts (Fig. 5B) from the same experimental cultures indicated that detection of extrachromosomal histones could be used as a convenient marker to monitor apoptosis in HIV-infected cells.

It has been reported that incubation of apoptotic cells with ZnSO$_4$ reduces fragmentation of DNA probably due to inhibition of the nuclear endonuclease (Cohen et al., 1984; Bell et al., 1990). Accordingly, the addition of 1 mM ZnSO$_4$ in HIV-1-infected cultures on Day 3 p.i. for 24 hr resulted in a significant reduction in the level of histones accumulated in the nucleoplasm and also a reduction in the amount and the degree of DNA fragmentation (Fig. 6). These results confirm that accumulation of histones is due to fragmentation of cellular DNA.

Accumulation of nucleosomes occur before maximal production of virus in HIV-infected cells

Recently it has been demonstrated that mycoplasmas can enhance the ability of HIV to induce a cytopathic effect in vitro T cell cultures (Montagnier et al., 1990; Lo et al., 1991). We therefore investigated the effect of an inhibitor, mycoplasma removal agent (MRA) on the HIV-1 infection in CEM cells. Under our experimental conditions, addition of MRA in HIV-infected cultures routinely resulted in a significant delay
(3 to 4 days) in the development of syncytia. Furthermore, the cytopathic effect was transient and fewer cells were killed although large quantities of infectious virus were produced. Few days after the peak of virus production, cells were found to be chronically infected.

Figure 7 shows a typical experiment in which the infection was carried out under similar conditions as the experiment described in Fig. 1 except in the presence of 0.5 μg/ml MRA. At different days postinfection, total cell extracts were analyzed by immunoblot assay for the presence of HIV proteins as a marker to monitor the kinetics of infection. The same extracts were also assayed by immunoblot analysis using anti-H2B antibodies as a measure of accumulation of nucleosomes. Six days p.i., HIV proteins (gp120, gp41, p55, p40, and p25) were clearly detectable and the maximal levels were reached on Days 10 to 13 p.i. (Fig. 7A). Interestingly, this kinetic of accumulation of HIV-proteins was in parallel with the degree of accumulation of unintegrated HIV DNA (Fig. 7C). In contrast, the cytopathic effect, estimated by the percentage of syncytia formation in the culture was 18% on day 6 p.i. reaching a maximal degree (65%) on Day 6 (Fig. 7D). These cell extracts when analyzed for accumulation of histones indicated that their level (in this case H2B) was increased 4-5 days p.i. and reached a maximum on Day 6 (Fig. 7B). Thus, accumulation of nucleosomes and its maximal peak occurred several days before the maximal accumulation of unintegrated HIV DNA. In view of these observations, it is possible to suggest that accumulation of histones in HIV-infected cells is mostly due to fragmentation of cellular DNA which is correlated with the appearance of the first signs of the cytopathic effect.

Fragmentation of DNA does not occur in chronically infected cells

Chronically infected cells might be isolated following an acute infection with HIV. In our laboratory, such chronically infected CEM cells were obtained following infection with a freshly isolated HIV-1 (NRK) from an AIDS patient (M. McChesney and Y. Riviere, unpublished). The infected cells which survived the transient cytopathic effect of HIV were cultured for 8 weeks to generate the chronically infected cell line. These chronically infected cells produced infectious virus continuously, they did not express cell surface CD4 receptors, and did not manifest the characteristic cytopathic effect of HIV, i.e., formation of syncytia. Extracts corresponding to the cytoplasm and the nucleoplasm were analyzed for the presence of HIV proteins and histones. Furthermore, DNA extracts in the nucleoplasm were analyzed by electrophoresis in a 1.4% agarose gel for the detection of LMW DNA. Figure 8 shows that

![Figure 7](https://example.com/figure7.jpg)

**Fig. 7.** Accumulation of nucleosomes is correlated with the cytopathic effect of HIV. CEM cells were infected with HIV-1 (as in Fig. 1) in the presence of 0.5 μg/ml MRA (Flow Lab.). Different days p.i., total cell extracts (cytoplasm plus nucleoplasm) were analyzed by immunoblotting using HIV-1 positive serum (A) or rabbit anti-H2B antibodies (B). Autoradiographs are shown in (A) and (B), in (B) only a section of the gel is shown. (C) DNA was extracted from the nucleoplasm and analyzed by slot-blot hybridization with a labeled probe corresponding to the entire HIV-1 genome; an autoradiogram is shown. (D) Cell cultures were examined under microscope and the number of syncytia were estimated.
Fig. 9. The accumulation of nucleosomes during HIV infection of CD4 lymphocytes. CD4 lymphocytes from a normal donor (Materials and Methods) stimulated with PHA were infected or not with HIV-1 in the presence of TCGF (IL-2). Different days p.i. total extracts were analyzed by immunoblotting using HIV-1 positive serum and murine anti-H2B antibodies. The bottom section of the autoradiographs are shown. The arrows indicate the position of p25 and of histones. The murine anti-H2B antibodies reacted with H2A and H2B since the commercial antigen preparations were not pure.

Accumulation of nucleosomes in HIV-1-infected CD4 lymphocytes

CD4 lymphocytes isolated from a normal donor were stimulated with PHA before infection with HIV-1 in the presence of TCGF (IL-2). Figure 9 shows that the major HIV core protein p25 was produced at its maximum 4 days p.i. During this kinetics of infections, H2B and H2A became detectable 3 days p.i. and clearly apparent at 4 days p.i. Intriguingly, on the 6th day p.i., the histones became detectable in uninfected, PHA stimulated cells. The 24-hr advance in infected cells might demonstrate the intrinsic effect of HIV infection. Overall, the level of histones increased in infected CD4 lymphocytes was much lower than that observed in infected CEM cells (shown in Fig. 1). This was probably due to IL-2 which was added to the CD4 lymphocyte cultures. Previously, it has been demonstrated that IL-2 can protect T lymphocytes from glucocorticoid-induced DNA fragmentation (Fernandez-Ruiz et al., 1989; Nieto et al., 1989). These results demonstrate that HIV infection of peripheral blood mononuclear cells leads to apoptosis, a mechanism which might also occur in the absence of infection due to mitogen treatment of these cells. Apoptosis therefore, is a physiological process triggered by different factors (virus, mitogens) which have in common the capacity to stimulate mechanisms leading to DNA fragmentation.

DISCUSSION

The fundamental unit of chromatin of all eukaryotic cells is the nucleosome which is constituted of approximately 200 bp DNA making two turns around an octamer composed of two each of histones H2A, H2B, H3, and H4. In the nucleosome the two turns of DNA are stabilized by one extranucleosomal molecule of histone H1 (Pederson et al., 1986). Adjacent nucleosomes are connected by the linker DNA which represents the DNase sensitive site. Accordingly, in the presence of a DNase, a ladder of DNA fragments is generated, with each fragment differing in length from the next by 200 bp. More extensive digestion will cause the release of histone H1 molecules and produce highly protected DNA segments of 146 bp surrounding the histone octamer, referred to as the nucleosome core particle (Thoma et al., 1979; Noll and Kornberg, 1977; Pederson et al., 1986). Under physiological conditions, fragmentation of cellular DNA is a consequence of programmed cell death or apoptosis (Duvall and Wylie, 1986; McConkey et al., 1990; Rothenberg, 1990). Here we have shown that in the course of infection with HIV, the different histone subtypes are dramatically increased and become accumulated in the nucleoplasm. The fact that these histones are found to be associated with LMW DNA suggested that they become accumulated as oligonucleosomes. Our results favor the hypothesis that HIV infection triggers an apoptosis mechanism which can be demonstrated either by the accumulation of histones in the nucleoplasm or by fragmentation of DNA into a characteristic pattern of nucleosomal ladder. Both of these events were reduced or inhibited when infected cells were cultured in the presence of Zn⁺⁺, an inhibitor of the nuclear endonuclease responsible for the DNA fragmentation. Recently, apoptosis has been suggested to be the cause of cell death in HIV infected cell cultures and in vivo in AIDS patients (Terai et al., 1991; Gougeon et al., 1991; Groux et al., 1991). Apoptosis seems to be a general mechanism associated with HIV-related infections since both types of HIV (Fig. 5) and as well as the simian immunodeficiency virus SIVmac...
(data not shown) are capable of priming this process in different T-cell lines and in normal CD4 lymphocytes.

An acute infection by HIV is characterized by a typical cytopathic effect manifested by the vacuolization of cells, formation of syncytia, and is followed by cell lysis. In CEM cells, the appearance of the first signs of apoptosis were associated with the appearance of the first signs of the cytopathic effect, i.e., formation of syncytia. Inhibition of mycoplasma growth during an acute infection (Fig. 7) resulted in a modified kinetics of infection in which maximal nucleosomal accumulation coincided with maximal syncytia formation and occurred several days before maximal production of HIV proteins. Further correlation between the cytopathic effect and apoptosis, was provided by CEM cells chronically infected with HIV-1 (Fig. 8). These cells produced continuously infectious virus without forming syncytia. Careful investigation of the nucleoplasm of chronically infected CEM cells revealed the absence of histones and LMW DNA. Similarly, the nucleoplasm of a monocytic cell line, U937, chronically infected with HIV-1 or HIV-2 contained very little histones and LMW DNA (data not shown). In chronically infected cells, formation of syncytia probably does not occur due to reduced CD4 expression on the cell surface. The interaction of the viral envelope with the CD4 receptor might then be required for triggering the apoptotic process.

In vitro appearance of the first signs of apoptosis is also correlated with the dose of HIV. For example, at a dose of HIV-1 50–100 TCID50, the infection gradually develops and at 4 days p.i. 90–95% of cells produce HIV proteins. Under these experimental conditions the nucleosomes in the nucleoplasm become clearly apparent at day 3 p.i. (Fig. 3). At a higher dose of HIV-1 (500–1000 TCID50), the infection is rapid in which 95% of cells become producers of HIV proteins 2 days p.i. whereas significant levels of nucleosomes become accumulated already by 24 hr p.i. (data not shown). Consistent with the observations on chronically infected cells, these results indicate that apoptosis is highly correlated with virus infection. The mechanism by which HIV infection triggers the apoptosis process to occur in vitro cell cultures remains to be clarified. Such a direct mechanism might be also functional in patients infected with HIV. However, in vivo this direct mechanism might probably be supplemented by other direct or indirect mechanisms since in AIDS patients the number of T4 lymphocytes becomes drastically reduced (Fauci, 1988) under conditions when less than 0.1% of cells are in fact infected (Harper et al., 1986; Schnittman et al., 1989). In vitro and in vivo, soluble gp120 might interact with CD4 receptors on uninfected cells leading to an abortive cell activation and thus triggering apoptosis (McConkey et al., 1990; Pinching and Nye, 1990). In vitro, it has been demonstrated that gp120 can interact with CD4 and modify cellular functions (Hoxie et al., 1986; Diamond et al., 1988; Gurley et al., 1989). In rodent neurons, gp120 has been shown to cause an increase in intracellular calcium and neuronal toxicity (Dryer et al., 1990), an effect which might be mediated by activation of the nuclear endonuclease. It should be emphasized that besides gp120, other viral proteins or cellular factor(s) produced during infection might interact with uninfected cells and trigger apoptosis.

McConkey et al. (1990) have proposed a model for regulating signals in the induction of apoptosis in immature thymocytes. According to this model, an increase in calcium or cAMP concentration stimulate endonuclease activation and apoptosis. However, when these signals occur with concomitant protein kinase C activation, then DNA fragmentation does not occur and cell proliferation may result. Therefore, in immature thymocytes apoptosis is the consequence of incomplete or unbalanced signalling. In the case of mature T lymphocytes, it has been reported that they resist apoptosis under normal conditions. However, an unbalanced signalling causes an increase in calcium concentration leading to apoptosis (Jenkins et al., 1987; McConkey et al., 1989). The results presented in Fig. 9 show that mitogen stimulation of peripheral blood CD4 cells might be sufficient for apoptosis. Interestingly, HIV infection of such mitogen stimulated cells resulted in a slight acceleration of the first signs of apoptosis, thus indicating the intrinsic effect of HIV infection. These results demonstrate that apoptosis is a physiological process triggered by various agents such as virus and mitogens, which have in common the capacity to stimulate mechanisms mediating an apoptotic state. An unbalanced signalling in PHA treated CD4 cells might be the cause of apoptosis. On the other hand, the mechanism by which HIV infection triggers apoptosis remains to be elucidated.

Nucleosomes accumulated in the nucleoplasm during an acute infection in vivo are probably released after cell lysis. These nucleosomes might then stimulate a pathological state manifested by abnormal polyclonal B cell proliferation and autoantibody formation. Consistent with this, considerable degree of polyclonal B cell activation has been reported in patients with AIDS (Clifford et al., 1983). In vitro, nucleosomal preparations have been shown to exert a mitogenic effect on normal murine splenic lymphoid cells (Bell et al., 1990). A recent survey that we have carried out on 150 HIV positive sera or plasma, indicated the presence of high affinity autoantibodies against DNA and different subtypes of histones (Muller and Hovanessian, in prepara-
tion). Previously, anti-histone H2B autoantibodies have also been reported in AIDS patients (Strickler et al., 1987). Furthermore, the development of anti-H2B antibodies has been correlated to the development of disease associated with infection by simian immunodeficiency viruses (Fultz et al., 1990; Morrow et al., 1991). The presence of antibodies specific for nucleosome constituents in HIV seropositive individuals suggest that nucleosomes become also accumulated during HIV infection in vivo following an apoptosis process.

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REFERENCES


