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Changes in bioactive lipids, alkylacylglycerol and ceramide, occur in HIV-infected cells[1]

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The mass levels of bioactive lipids known to modulate signal transduction or to possess other biological activities were measured in HIV-infected CEM cells. The levels of diacylglycerol, an activator of protein kinase C, as well as of alkylacylglycerol were elevated. A more drastic increase was observed in the ceramide levels after HIV-infection, whereas sphingosine levels were hardly influenced. Interestingly, the magnitude of the changes was related to the infection time, being higher at 8 days after infection then at 4 days. The possible role of these lipids in the cytopathic effects of HIV-infection is discussed.
In addition, an improved methodology to quantitate simultaneously diacylglycerol and alkylacylglycerol in crude lipid extracts, based upon their phosphorylation by *E. coli* diacylglycerol kinase, is presented.

The depletion of T4-CD4$^+$ lymphocytes in AIDS and the CPE of HIV on different cultured cells is well documented (see 1). The biochemical mechanism(s) underlying this CPE is still under investigation. Whereas the syncytium formation was once believed to be the major determinant of CPE (2,3), several studies suggest that there is not a strict correlation between cell killing and syncytium formation (4-8). Possibly the envelope proteins (gp41, gp120 and gp160) play a role in cell killing (5,9). In this regard, HIV would resemble other retroviruses whose envelope proteins are harmful to the host cell (10-12). Most likely the insertion of envelope proteins in the cell membrane causes a perturbation

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Abbreviations . AAG : 1-alkyl-2-acyl-*sn*-glycerol; AIDS : acquired immunodeficiency syndrome; C/M : chloroform/methanol; CPE : cytopathic effect; DAG : *sn*-1,2-diacylglycerol; DTPA : diethylenetriaminopentaacetic acid; HIV : human immunodeficiency virus; HTLV-IIIb : human T-cell leukemia virus type III isolate b.

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of the lipid bilayer. As a consequence membrane-related processes might be affected (6,13,14). An important membrane-related event is the activation of PKC. This kinase is pivotal to the cell (see 15), but thusfar few papers have appeared on its role during HIV infection. Moreover, conflicting data have been presented. Phorbolesters enhance the CPE in Molt-4 cells by stimulating the viral replication (16). This appears to be related to the induction by phorbolesters, probably through the activation of PKC, of the nuclear transcription factor kB which binds to the viral enhancer (17). In ERIC cells however, addition of phorbolesters or didecanoylglycerol causes the opposite effect, a reduction in the cell injury (6). The reason for this discrepancy is not clear. On the other hand, different PKC inhibitors like staurosporin (18), hypericin (19,20), and H-7 (21) possess antiretroviral activity, suggesting again PKC-dependent processes during HIV infection.

The initial aim of this study was to investigate whether HIV infection perturbs the functioning of PKC, by changing the mass levels of its physiological activator, *sn*-1,2-diacylglycerol. It turned out that the changes observed in ceramide and 1-alkyl-2-acyl-*sn*-glycerol, which can be measured in a similar manner as DAG (as described in the method section), were more prominent than those in DAG. AAG (22-24) and ceramide (see 25) are known to possess bioactivity. Recently evidence has been provided that ceramide can act as a second messenger. Inducers of monocytic differentiation of HL-60 cells like vitamin D_3, γ-interferon and tumor necrosis factor α cause sphingomyelin breakdown (26-28). Moreover addition of cell-permeable ceramide analogues to these cells results in differentiation (27,28). The possible role of DAG, AAG and ceramide in CPE is discussed.

MATERIALS AND METHODS

Materials. Choline glycerophospholipids (bovine heart) were obtained from Avanti Polar Lipids, Inc., Pelham, AL. Phospholipase C (*Bacillus cereus*, type XIII, 2350 U/mg protein, suspension in 3.2 M $(NH_4)_2SO_4$ pH 6.0) and Lipidex 5000 (hydroxyalkoxypropyl-dextran, type IX, 50 % substituted with alkylchain length of C_{15}-C_{18}) were purchased from Sigma, St. Louis, MO. Methylamine (33 % (w/v) in absolute ethanol) was from Fluka AG, Buchs, Switzerland. Ceramide, obtained by alkaline hydrolysis of cerebrosides, was a gift of Dr. W.R. Bishop. Reagents for the DAG-kinase assay were obtained as described in ref. 29.

Preparation of 1-alkyl-2-acyl-*sn*-glycerol. Diradylglycerols, obtained by treating beef heart choline glycerophospholipids with phospholipase C (29), were separated by chromatography on a Lipidex-5000 column (30). Column fractions were monitored by TLC on Silica 60G plates in heptane/diethyl ether/acetic acid (25/75/1 - v/v), and those containing pure AAG (R_f=0.55) were pooled, dried under N_2, dissolved in chloroform, standardised by measuring their ester content (29) and stored at -20 °C.

Cell culture. CEM cells (ATCC-CCL 119) and HTLV-IIIb were propagated as described before (31).

Lipid extraction. At the indicated time after infection, 10^7 CEM cells were transferred to plastic tubes, pelleted by centrifugation, transferred in cold phosphate buffered saline into screw cap glass tubes and pelleted again. The supernatants were removed and the cells extracted with 3 ml C/M (1/2 - v/v). Denatured proteins were removed by centrifugation and reextracted. The organic extracts were combined and adjusted to 6 ml with C/M (1/2 - v/v). An aliquot (4.5 ml) was removed for the analysis of sphingosine (32), and the remaining extract was brought to 3 ml with C/M (1/2 - v/v), and phase-separated under neutral conditions (33). The chloroform phase was analysed for phospholipids (33) and DAG, AAG and ceramide as described below.

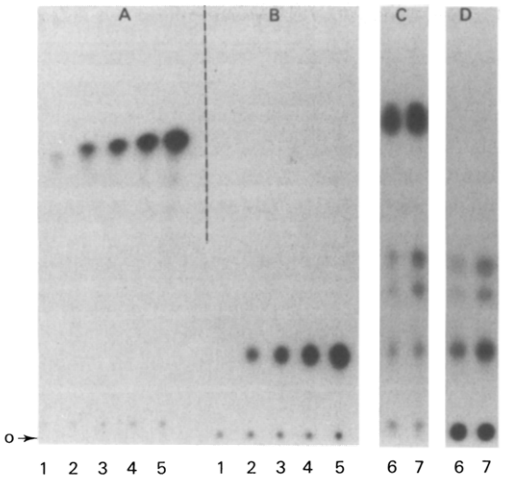

Figure 1. Thin layer chromatography of diacyl- and alkylacylglycerolphosphate before and after alkaline hydrolysis.

Increasing amounts of AAG (0, 100, 200, 400 and 800 pmoles; lane 1 to 5) or crude lipid extracts from CEM cells (control 4 days=lane 6; infected 4 days=lane 7) were phosphorylated and analyzed by TLC as described in the method section without hydrolysis (A,C) or after alkaline hydrolysis (B,D). Notice in lanes 6 and 7 the presence of ceramide-phosphate which is not affected by the alkaline hydrolysis. Some lysophosphatidate is also seen under the conditions without hydrolysis (lane 6 - 7 in C). This represents phosphorylated 1-alkenyl-2-acylglycerol, which is cleaved however during the acidic extractions to 2-acylglycerol-3-phosphate, and phosphorylated monoacylglycerol. Both are converted to glycerol-3-phosphate by the alkaline hydrolysis (Van Veldhoven, P.P. and Bell, R.M., unpublished data). The amount of lysophosphatidate found after hydrolysis represents therefore the cellular level of AAG.
(O:origin; R_f values of phosphatidate, non-hydroxy N-fatty acid ceramide-phosphate, hydroxy N-fatty acid ceramide-phosphate, lysophosphatidate and glycerol-3-phosphate are respectively 0.50, 0.26, 0.22, 0.15 and 0.01).

Quantitation of diacylglycerol, alkylacylglycerol and ceramide. The dried lipids were dissolved in 40 μl 3.75 % (w/v) octylglucoside - 12.5 mM 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol - 1 mM DTPA pH 6.6 by vortexing, followed by bath-sonication for 1 min. To the detergent mixture was then added 50 μl of reaction mixture (100 mM Hepes buffer[3] pH 7.0 - 100 mM LiCl - 25 mM $MgCl_2$ - 2 mM EGTA - 2 mM dithiotreitol - 5 μg *E. coli* strain N4830/pJW10 membrane protein). After 10 min reactions were started by adding 10 μl of 10 mM [γ-^{32}P]-ATP (specific activity ± 100000 cpm/nmol) in 20 mM imidazole - 1 mM DTPA pH 6.6. Reactions were allowed to proceed at 25 °C for 30 min and stopped by adding 0.7 ml of 1 % (w/v) $HClO_4$ (w/v) and 3 ml of C/M (1/2 - v/v). Tubes were mixed thoroughly and phase separated with 1 ml of chloroform and 1 ml of 1 % (w/v) $HClO_4$. Lower phases were washed twice with 2 ml of 1 % (w/v) $HClO_4$/methanol (7/1 - v/v) and analysed by chromatography on Silica 60G plates (0.25 mm) in chloroform/pyridine/formic acid (60/30/7 - v/v).

To differentiate between phosphorylated DAG and AAG, which run similarly in this solvent system, identical volumes of the kinase assay extracts (0.8 ml) were analyzed with and without an alkaline hydrolysis (see Figure 1). For the hydrolysis, the extract was transferred to a screw cap tube, dried under N_2, and dissolved in 0.5 ml methylamine reagent (made up by adding 30 % by volume of water to 33 % monomethylamine in ethanol). Tubes were capped and kept at 70°C for 90 min in a heating block. These conditions, which are somewhat more harsher than those described in ref. 35, were needed to achieve complete transacylation of AAG-phosphate. This is consistent with the slower deacylation of the esterbond in etherlinked phospholipids (36). After cooling, the content

[3] Since the optimal pH for the phosphorylation of DAG (or AAG) and ceramide are respectively 6.6 (34) and 7.4 (32), assays were run at pH 7.0, instead of pH 6.6 as originally described (29).

of the tubes were dried under N_2, dissolved in C/M (7/3 - v/v) and chromatographed. Labelled lipids were located by autoradiography and scraped into scintillation vials containing 4 ml scintillation fluid. Recovery of standards was calculated from the specific activity of the ATP used (33) and the volumes of the lower phases. In agreement with others (37,38), AAG is phosphorylated by DAG kinase. As observed with DAG (29), phosphorylation of ceramide and AAG is quantitative (Van Veldhoven P.P. and Bell R.M., unpublished data). Our modification of the original DAG assay (29) to quantify also AAG, eliminates the need for additional extraction steps (37) or the use of *Rhizopus* lipase (38) to differentiate between both diradylglycerol species. During the hydrolysis, DAG-phosphate and AAG-phosphate are converted to respectively glycerol-3-phosphate and alkylglycerol-3-phosphate, while ceramide-phosphate is not affected (see also Fig. 1). The amount of cellular AAG was calculated from the label associated with lysophosphatidate after hydrolysis, while the amount of DAG was equal to the amount of phosphatidate found without hydrolysis minus the amount of alkali-stable lysophosphatidate.

RESULTS AND DISCUSSION

To investigate the possible influence of HIV infection on bioactive lipids, their levels were measured in CEM cells 4 days after infection with the HTLV-IIIb virus strain. Day 4 was chosen as representative for the onset of the infection. Cells were just beginning to show evidence of syncitia formation but cell viability was high (> 90 %). Since the measured lipids partition in membranes, and to correct for possible losses during extraction, their mass levels are all expressed as molar percentages of phospholipids. As shown in Figure 2A, the phospholipid levels were not influenced by the infection, and a small in-

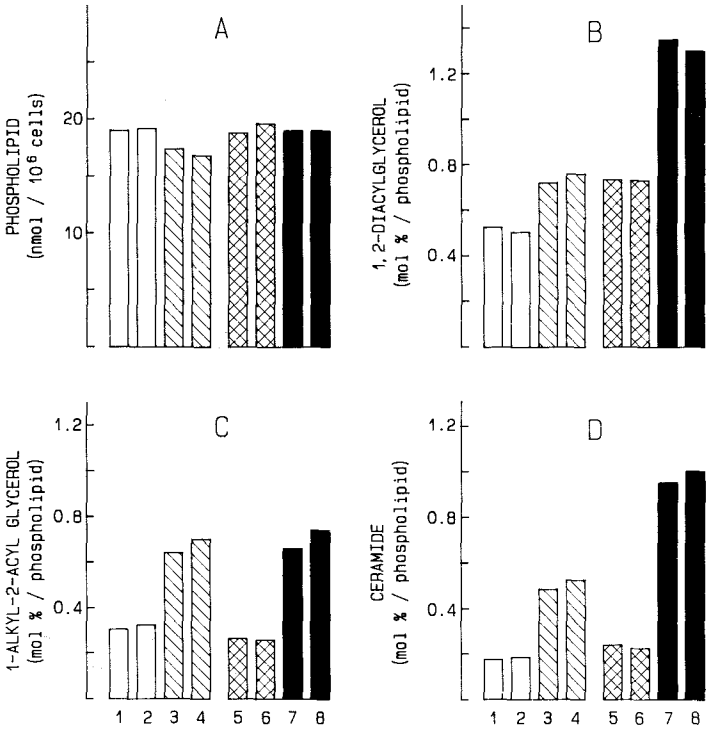

Figure 2. Mass levels of bioactive lipids in control and HIV-infected CEM cells.
Control (n° 1,2,5,6) and HIV-infected CEM cultures (n° 3,4,7,8) were analyzed at 4 (n° 1-4) and 8 days (n° 5-8) after infection with HTLV-IIIb virus (multiplicity of infection : 0.05) and analysed for phospholipids (A), diacylglycerol (B), alkylacylglycerol (C) and ceramide (D).

crease in DAG levels was noticed (Fig. 2B). The changes in AAG and ceramide were more pronounced, being respectively 2.1- and 2.8-fold higher (Fig. 2C-D).

In a separate experiment, cultures were analysed 8 days after infection. At this time point syncitial involvement was extensive and cell viability had fallen to about 65 %. Qualitatively, the same picture emerged as seen after 4 days of infection, but the changes were now even more striking. The DAG, AAG and ceramide levels in infected cells were respectively 1.8-, 2.6- and 4.1-fold higher compared to the non-infected cultures (Fig. 2B-D).

Except for DAG, the differences observed in the lipid levels in the non-infected cells on day 4 and 8 are small. The changes in DAG, levels of which are influenced by various factors in cultured monolayers (33), are probably not relevant.

The physiological importance or consequences of our findings in infected cells are not clear yet. The moderate increase in DAG levels is in contrast with the data of Lynn *et al.* (6), who found that the net synthesis, as well as the mass, of DAG in infected ERIC cells was lowered. Since mass levels were measured our data do not exclude the possibility that small pools of specific DAG, like those derived from phosphoinositides, are decreased as described in human lymphocytes (14).

The relevance of the increases in AAG remains to be established. It is becoming clear that AAG possess biological activity (22-24) and, like their diacyl-analogues, can fulfill a mediator rol although their action mechanism(s) is not known. AAG are generated in different cell types in responses to physiological stimuli like vasopressin, angiotensin II, ATP, and epinephrine (39) and interleukin-3 (40). Most likely, these compounds stimulate a phospholipase C and/or phospholipase D acting on phosphatidylcholine (see 41). In various cellular systems, the 1-position of this phospholipid is enriched in alkyl-groups (42,43), and can, after cleavage by these phospholipases, give rise to respectively AAG and AAG-phosphate. The latter one can subsequently be hydrolysed to AAG.

Finally, we like to discuss the changes in ceramide levels after HIV-infection. At this moment neither the exact role of this neutral lipid is known, nor its origin. A first possibility is that this ceramide serves as a precursor for glycosphingolipids like the Le^y-antigen (a higher order glycosphingolipid), which is selectively expressed in infected cells (44), or cerebrosides. In various host cells, an increased synthesis of glucosyl- and lactosylceramide, accompanied by an increased sphingomyelin turnover, is seen after viral infections (45-49). The precise role of these cerebrosides in virus maturation is not known, but impairing their synthesis drastically lowers the virus reproduction (45,46,50). Since glycosphingolipids constitute only a minor amount of the total cellular lipids it is unlikely, however, that such massive changes in ceramide levels, which last during the infection, would be required for these processes.

Another, more plausible possibility is that the elevations in ceramide are related to the syncytium formation seen upon infection of these cells. Indeed sphingomyelin hydrolysis towards ceramide seems to be involved in membrane fusion processes (51-54). Indirect evidence for sphingomyelin turnover during HIV infection is provided by the mag-

nitude of the observed changes[4] and sphingosine measurements. Free sphingosine was found in CEM cells in the same order of magnitude as in other cultured cells (32,56). Levels, expressed as mol % of phospholipids, were 0.033 and 0.037 in the control cells (at 8 days after infection), which is about 7-fold lower than the ceramide levels. Despite the 4.1-fold rise in ceramide in infected cells at that time (see Fig. 2D), sphingosine levels were only 1.6-fold higher (0.052 and 0.063 mol % sphingosine/phospholipids). So the ceramide increases do not seem to be related to the increased abundance of sphingosine; the deacylation of ceramide is probably responsible for the rise in sphingosine levels.

Whether the increases in ceramide are only related to the syncytium formation or play also a role in the virus induced cytopathology remains to be studied. Also the trigger of sphingomyelin turnover is not known, but it might be caused by the insertion of viral envelope proteins, which have indeed been shown to be involved in CPE (5,9), into the plasma membrane. If this membrane would be the main site of sphingomyelin hydrolysis, the concomittant physical changes in the bilayer must be very dramatic[4]. In sphingomyelinase-treated erythrocytes morphological changes are already apparent when 10 % of the sphingomyelin is hydrolysed (57,58). More extensive cleavage enhances the osmotic fragility of the erythrocytes (57). Lysis of HIV-infected cells, preceded by swelling of the plasma membrane, is characteristic for CPE (59). It is not unlikely that the decreased membrane potential and impaired Ca^{2+} fluxes (6,13) and impaired phospholipase C and PKC activity (14), observed in HIV-infected cells, are secondary effects of the sphingomyelin breakdown in the plasma membrane.

Further studies on the origin and fate of AAG and ceramide will shed more light on their role. Moreover, if these lipids play a role in the pathology of HIV as we propose, this might suggest new approaches (for example acting on phospholipase C or sphingomyelinase) to drug development.

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[4] If one assumes that the increase in ceramide (taken as 0.75 moles per 100 moles of phospholipids) is due to hydrolysis of sphingomyelin present in the plasma membrane, about 60 % of this phospholipid would be degraded. Such calculation is based on the facts that in most cell types sphingomyelin is concentrated in the plasma membrane (about 25 % of the total cellular content), where it accounts for 25 % of the plasma membrane phospholipids (which themselves represent about 5 % of the total cellular phospholipids) (see 55).

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