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Stimulation of Virus Production and Induction of Self-Syncytium Formation in Human T-Cell Leukemia Virus Type I- and Type II-Infected T Cells by 12-*O*-Tetradecanoylphorbol-13-Acetate

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Treatment of human T-cell leukemia virus type I (HTLV-I)- and HTLV-II-infected T-cell lines with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulated virus release. However, this stimulation was mainly detected at 42 to 48 h of treatment, whereas later virus release declined rapidly. During the first 48 h, TPA had no effect on cell growth, but later, the number of viable cells was profoundly lower in the TPA-treated than in the untreated cultures. This shift in virus release and cell number resulted from self-fusion of a large proportion of the virus-producing cells, which seemed to consequently enter into a dying process. This fusion, which resulted in syncytium formation, was strongly inhibited by anti-HTLV-I *env* monoclonal antibodies. Furthermore, no self-fusion was detected in three different uninfected T-cell lines similarly treated with TPA. On the other hand, stimulation of virus production by 3-methylcolanthrene (3-MC) treatment failed to induce self-fusion in the infected cells. Moreover, no syncytium was detected when these 3-MC-treated infected cells were cocultured with any of the TPA-treated uninfected cells. The effects of TPA on virus production and syncytium formation were both abolished by three different protein kinase C inhibitors. Taken together, these data suggest that the self-fusion observed in these experiments required both enhanced virus production and protein kinase C-phosphorylated viral or/and virally induced cellular component(s).

Human T-cell leukemia virus type I (HTLV-I) has been etiologically implicated with adult T-cell leukemia (2, 4, 6, 43) and certain neurological disorders (6, 43). A related human retrovirus, HTLV-II, has been associated with a T-cell variant of hairy cell leukemia (2, 4, 6, 43). However, the pathogenic mechanism of these viruses is unclear.

Both viruses possess a transcription transactivating gene, called *tax*, which activates viral RNA transcription from a promoter located at the viral 5′ long terminal repeat. Notably, *tax* can also transactivate the expression of various cellular genes and is therefore widely believed to be involved in the pathogenesis of these viruses (4, 6, 43). It should be emphasized, however, that the vast majority of HTLV-I- and HTLV-II-infected individuals never develop any of the diseases related to these viruses, and those who do so manifest the clinical symptoms after a latency of many years (2, 4, 6, 43). Moreover, virtually no viral gene expression can be detected in the infected cells of the virus-carrying individuals (2, 4, 6, 42). It is therefore conceivable that the inapparent HTLV infections reflect this lack of virus expression and that initiation of an HTLV-related pathologic process in such carriers requires an activation of the latent virus to provide the needed *tax* gene product.

Numerous studies from our and other laboratories (reviewed in reference 4) have shown that environmental carcinogens and tumor promoters stimulate the expression of various animal retroviruses and activate their latent proviruses. In experiments aimed to estimate whether such exogenous factors might be involved in HTLV activation in asymptomatic carriers, we have found that a chemical carcinogen such as 3-methylcholanthrene (3-MC) remarkably stimulates virus expression in HTLV-I (10)- and HTLV-II (unpublished data)-infected T-cell lines. Other studies have demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can activate the expression of reporter genes directed by the HTLV-I long terminal repeat in transient transfection assays (11, 12, 18, 31, 34). However, inconsistent data have been reported regarding the effect of this and other tumor promoters on HTLV expression and replication in infected cells (7, 15, 16, 22, 24, 40). Therefore, the present study was undertaken to further clarify this issue.

For this purpose, we used the HTLV-I-infected SLB-I and the HTLV-II-infected MoT T-cell lines (1). These cells were cultured in Iscove's medium containing 10% fetal calf serum (Biological Industries Ltd., Kibbutz Beith Haemmek, Israel) at an initial density of 10^5 cells per ml and exposed to 10^{-7} M TPA (Sigma Chemical Co., St. Louis, Mo.). TPA stocks were prepared in dimethylsulfoxide (Sigma) and diluted in the culture medium, keeping the dimethyl sulfoxide concentration in all cultures (including the TPA-untreated controls) at 0.1%. At various time intervals thereafter, aliquots of 5 ml were taken from each culture and cleared of cells by centrifugation at 3,000 × *g* for 10 min. Virus particles were pelleted from the supernatants by centrifugation through 1 ml of a 17% sucrose cushion for 1 h at 100,000 × *g* and quantitated by assaying their reverse transcriptase activity as described elsewhere (5). Figure

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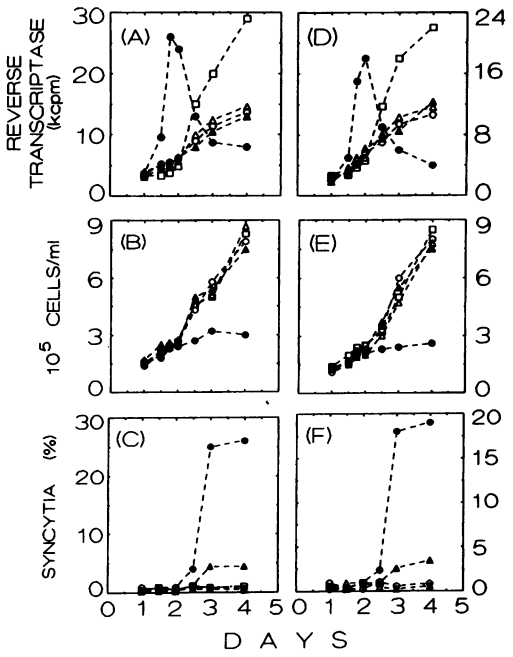

FIG. 1. Effects of TPA, H-7, and 3-MC on virus release, cell growth, and syncytium formation. SLB-I (A through C) and MoT (D through F) cells (initial density of 10^5 cells per ml) were incubated in the presence of TPA (●), H-7 (△), TPA plus H-7 (▲), or 3-MC (□); untreated cells (○) served as a control. At the indicated time intervals, 5-ml aliquots were taken from each culture for measurement of viral reverse transcriptase activity in the medium (A and D), for counting of viable cells (B and E), and for estimation of syncytium formation (C and F). Data represent the averages of three parallel cultures for each point.

1 illustrates representative data for several repeated experiments with both SLB-I and MoT cells. TPA profoundly stimulated virus release from both cells (Fig. 1A and D). This stimulation started to be detectable after 36 h of treatment and peaked at 42 to 48 h of treatment. Since TPA may, at certain doses, exert a mitogenic effect that increases the rate of cell growth (35) and since retrovirus replication is usually enhanced upon stimulating the growth rate of the host cells (37, 40), it was necessary to clarify whether this increased release of HTLV-I and HTLV-II resulted from a direct effect of TPA on virus production or was just an indirect consequence of a mitogenic effect of TPA on the host cells. Therefore, in each experiment, we monitored cell growth by counting viable cells, using the trypan blue exclusion method. The results depicted in Fig. 1B and E demonstrate that the TPA dose used in these experiments had no appreciable effect on cell growth during the first 48 h, suggesting that the increased virus release reflected a direct effect of TPA on virus replication. Of note, however, is that after this initial stimulation, virus release declined rapidly and reached, eventually, a level that was even lower than that of the untreated control cells. Furthermore, the number of viable cells also stopped increasing at this later stage of the TPA treatment.

A clue for the explanation of this time-dependent converse response to TPA was provided by the many unusually large rounded single cells which appeared in the TPA-treated SLB-I (Fig. 2A) and MoT (not shown) cultures at 72 h. Hematoxylin-eosin staining revealed that these large cellular structures were multinucleated syncytia. Figure 2B shows the syncytia formed in SLB-I cells, and similar multinucleated structures were seen also in the MoT cells (not shown). No such syncytia were noticed in the untreated control SLB-I (Fig. 2C) or MoT (not shown) cells. Scanning electron microscopy (SEM) analysis, performed as previously described (3), provided a closer look into the cell fusion process involved in the formation of these syncytia. Figures 2D through F may be interpreted as illustrating SLB-I cells at various stages of this process. Similar stages were seen in MoT cells as well (not shown). Figure 2D seems to show the initial cell aggregation occurring at the early stage of this process, Fig. 2E demonstrates single small cells in a process of fusion with an apparently intermediate syncytial structure, and Fig. 2F presents an ultimate giant syncytium (note scale bars in the photographs for estimating the relative sizes of the various cellular structures shown). At latter times of treatment (days 4 to 6), many of the syncytia appeared to be heavily vacuolated and disintegrating (not shown), suggesting that after cell fusion, these syncytia entered into a dying process. It should be noted that although such multinucleated giant cells comprised, in the experiments shown in Fig. 1, only 26% (Fig. 1C) and 19% (Fig. 1F) of the total cell number of the TPA-treated cultures, the percentage of cells involved in this fusion was certainly much higher, since each syncytium resulted from fusion of 5 to 20 cells. Furthermore, we did not include in these figures structures with two nuclei, since we could not distinguish by this method between mitotic cell structures and those that probably resulted from cell fusion and should have been also taken into account when we considered the percentage of cells participating in syncytium formation. Therefore, this syncytium-forming process, which became apparent on day 3, could likely account for the reduced virus release and the lower number of viable cells noted in the TPA-treated cultures at this late stage of the treatment.

Induction of syncytium formation has been known for many years to be a typical feature of various animal retroviruses (3, 8, 21, 26, 30, 33, 39) and has been demonstrated more recently also with the human retroviruses human immunodeficiency virus type 1 (14, 38) and HTLV-I (17, 19, 20, 27). However, in contrast to our present observations, in most of the previously reported cases of syncytium formation by HTLV-I, this process has been shown to involve fusion between the virus-producing cells and specifically selected target cells rather than self-fusion of the virus-producing cells themselves. A possible argument is that perhaps some of the infected cells used in our experiments were not expressing the viral genome and could therefore serve as target cells for fusion with the infected cells. However, this possibility was ruled out by indirect immunofluorescent staining, using an anti-HTLV *gag* monoclonal antibody (Genzyme Corporation, Boston, Mass.) as the first antibody as instructed by the supplier. This staining repeatedly showed that all of the cells expressed viral antigens regardless of whether they were treated with TPA (data not shown).

Of interest is our previous study demonstrating that retrovirally mediated cell fusion starts with bridging between neighboring cells via virus particles that bind concomitantly to more than one cell and that these bridged cells are subsequently unified into a multinucleated cellular structure by fusion of their membrane with the envelope of the bridging virus particles (3). Furthermore, functional analyses of the HTLV-I envelope proteins by Nagy et al. (28) and Weiss et al. (41) have revealed that the gp46 component is responsible for the virus attachment to specific receptors on the cell surface (although these receptors have not been identified yet), whereas the gp21 component is responsible for the fusion of the viral envelope with the cell membrane. These envelope components seem to be involved also in HTLV-induced syncytium formation (28,

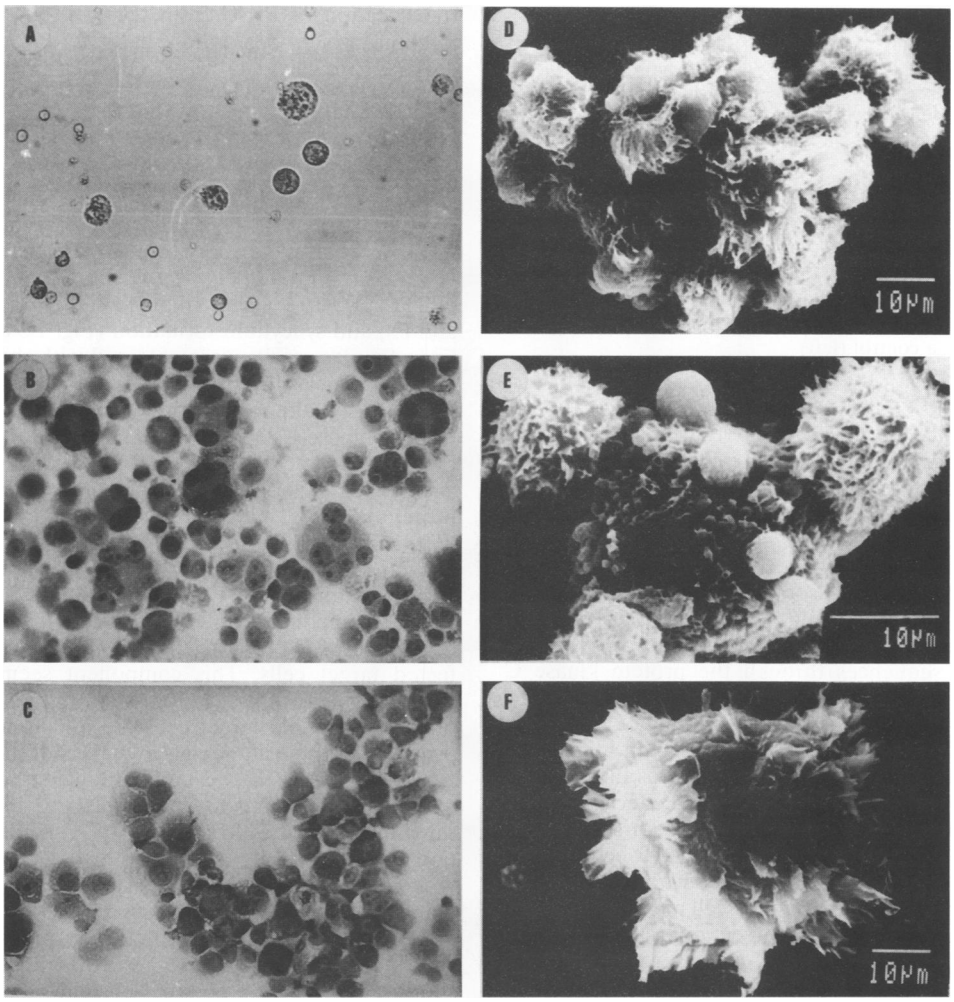

FIG. 2. Demonstration by light microscopy and SEM of the syncytium-forming process in TPA-treated SLB-I cells. (A) Unstained cells inspected by light microscopy on day 3 of TPA treatment. (B) Hematoxylin-eosin staining of fixed cells on day 3 of TPA treatment. (C) Hematoxylin-eosin staining of TPA-untreated control cells. (D) SEM presentation of initial cell aggregation. (E) SEM presentation of fusion of single cells to an intermediate syncytial structure. (F) SEM presentation of an ultimate giant syncytium.

41). On this ground, our present data could have been interpreted as suggesting that the increased virus production by the TPA-treated cells, seen at 42 to 48 h of treatment, facilitated such bridging between these cells and thereby mediated their subsequent self-fusion into the syncytia detected on the next day. This interpretation seemed to be supported by our next finding that addition of an anti-HTLV-I *env* monoclonal antibody (1:100 final dilution of the original stock; Genzyme) together with TPA abolished syncytium formation in SLB-I cells almost completely, whereas nonspecific antibodies had no effect in this respect (not shown). Moreover, TPA treatment of three different uninfected T-cell lines, Jurkat, Hut-78, and H-9, failed to induce syncytium formation. However, to further substantiate this conclusion, it was important to prove that stimulation of virus production by other agents would also induce self-fusion in these cells. Given our previous studies which revealed that subtoxic doses of 3-MC could stimulate virus production in both SLB-I (10) and MoT (unpublished data) cells, we examined whether such 3-MC doses could indeed induce self-fusion in these cells. As depicted in Fig. 1A and D, this carcinogen evoked a considerable stimulation of virus release in both cell lines. In addition, we compared the effects of TPA and 3-MC on the level of the viral *env* proteins within SLB-I cells. These proteins were quantitated in cell extracts by Western blot (immunoblot) analysis, using the above-mentioned anti-HTLV-I *env* monoclonal antibody in conjunction with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibodies as described elsewhere (10). Figure 3 shows that the level of these proteins was considerably increased after treatment with either of these agents, and the extent of this increase was comparable for both agents. Nevertheless, unlike TPA, 3-MC failed to induce syncytium formation in either SLB-I (Fig. 1C) or MoT (Fig. 1F) cells. It could be argued that TPA upregulated both the production of the virus and the expression of its receptor and thereby enhanced the bridging between the cells through the budding viral particles, whereas 3-MC enhanced only the production of the virus. To examine this possibility, 3-MC-treated SLB-I cells were cocultivated with TPA-treated uninfected Jurkat or Hut-78 cells at high (5×10^6 cells of each cell line per ml) and low (10^6 cells of each cell line per ml) densities, but no syncytium formation could be detected within 5 days. Taken together, these data strongly suggest that an active HTLV-I or HTLV-II production and a certain TPA-

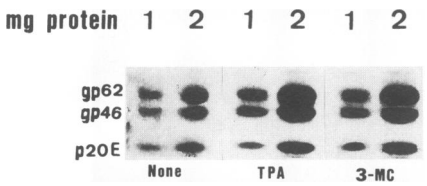

FIG. 3. Effects of TPA and 3-MC on the level of viral Env proteins in SLB-I cells. Cell were treated with TPA for 2 days (middle) or with 3-MC for 4 days (right), and then the indicated amounts of their extracts were analyzed by Western blot analysis for HTLV-I Env proteins with a specific monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibodies. Untreated cells served as a control (left).

affected viral or/and cellular factor are both needed for the self T-cell fusion. Furthermore, if a cellular factor is involved, its existence or function is evidently somehow dependent on the presence of the virus in the same cell. This factor might be induced or activated by the viral *tax* gene product. Alternatively, it might be activated by specific cleavage by the viral protease or by interaction with some other viral protein(s).

TPA has been shown to induce HTLV-I long terminal repeat-directed transcription by activating specific TPA-responsive enhancer sequences through the protein kinase C (PK-C)-associated signal transduction pathway (12, 34, 36). PK-C activation has been noted also to influence syncytium formation by HIV-I, eliciting an inhibitory effect in some cases (9) and a stimulatory effect in others (25). It was therefore of interest to assess the role of PK-C in the TPA effects found in our experiments. For this purpose, we examined first the effect of TPA on PK-C activity in SLB-I and MoT cells. PK-C activation by TPA is known to be associated with translocation of this enzyme from the cytoplasm to the cell membrane and to be followed by its downregulation (see reference 23 for a review). Therefore, the TPA-treated cells were fractionated into cytosolic and membrane fractions, which were analyzed for PK-C activity as described elsewhere (42). The results obtained with SLB-I cells (Fig. 4) show a rapid TPA-induced translocation of PK-C from the cytosolic to the membrane fraction, which was followed by a downregulation of the enzyme. Similar data were obtained with MoT cells (not shown). These results indicate that PK-C was indeed activated by TPA, under our experimental conditions, in both cell lines.

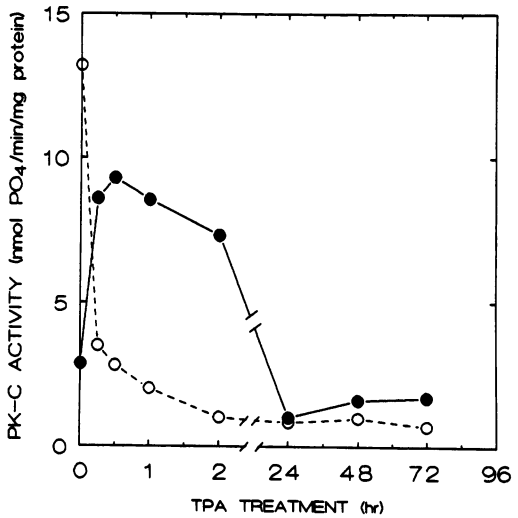

FIG. 4. TPA-induced subcellular redistribution of PK-C in SLB-I cells. Cells were treated with TPA and at the indicated time intervals were fractionated into cytosolic (○) and membrane (●) fractions, which were analyzed for PK-C activity.

To determine whether PK-C activation was needed for the TPA effect on virus production and syncytium formation, the cells were treated with TPA in the presence of three different PK-C inhibitors, 1-(5-isoquinolynyl)-2-methylpiperazine (H-7), staurosporine, and chelerythrin (23). The results obtained with H-7 are presented in Fig. 1. This inhibitor abolished the effects of TPA on virus release (Fig. 1A and D), cell growth (Fig. 1B and E), and syncytium formation (Fig. 1C and F) in both cell lines. Similar results were obtained with the other two inhibitors (not shown). It should be emphasized that these drugs can inhibit also, although with a lower efficiency, some other protein kinases (23). However, since the only protein kinase that TPA can activate is PK-C, it is rather unlikely that these agents would abolish its effects through inhibition of other protein kinases.

It is interesting in this context that in addition to the above-noted participation of the viral *env* components in syncytium formation, Fukudome et al. (13) have identified a cellular component, called C33 antigen, which is required for the fusion of HTLV-I-producing cells with appropriate uninfected target cells. This component seems to exist in the virus-producing cells at a glycosylation state different from that of the susceptible target cells (13). Therefore, these authors postulate that the altered glycosylation of this antigen prevents self-fusion between the virus-producing cells themselves. It would be interesting to determine whether TPA overcomes this inhibition of self-fusion by PK-C-mediated phosphorylation of the altered C33 antigen or of some other cellular or viral components. In preliminary experiments with cells incubated for 4 to 6 h with radioactive PO_4, we found a substantial difference in the cellular protein phosphorylation patterns of TPA-treated versus untreated SLB-I and MoT cells (not shown), but we have no solid evidence as yet that the TPA-induced self-fusion of these cells can be attributed to any of the differentially phosphorylated proteins detected in the TPA-treated cells.

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