Protection of cells against HIV infection by the dialyzable leukocyte extract prior to cell culture duplication

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RESEARCH

ABSTRACT

The search for new therapeutic agents to treat the acquired immunodeficiency syndrome (AIDS) continues, since therapeutic anti-retroviral combinations already employed to treat AIDS patients do not eradicate the human immunodeficiency virus (HIV) infection. Our group recently demonstrated the inhibitory effect of the Dialyzable Leukocyte Extract (DLE) on HIV replication in cells, by using an *in vitro* assay system in the human MT4 cell line. We have also reported a long-term inhibition of viral replication when cells were treated with this leukocyte derivative, prior to viral challenge. In the present trial, our results showed that the DLE-mediated inhibition of HIV in cultured MT4 cells did not depends on cellular duplication, and its inhibitory effect on viral replication is achieved by cellular exposure to DLE for at least 24 h. Inhibition is absent when cells are incubated for shorter periods of time, suggesting that the inhibitory mechanism triggered by DLE is more strongly related to the modification of cellular factors than to its direct action on the viral particle. Modification of viral factors during the virus replication cycle is also considered.

Keywords: Dialyzable Leukocyte Extract, Transfer Factor, HIV, MT4, anti-HIV activity, Sendai virus, AIDS

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RESUMEN

El extracto dializable de leucocitos protege a las células de la infección por el VIH antes de la duplicación del cultivo celular. La combinación de retrovirales como estrategia terapéutica para tratar a los enfermos con síndrome de inmunodeficiencia adquirida (SIDA), no erradica la infección por el virus de la inmunodeficiencia humana (VIH), de ahí que continúe la búsqueda de otros agentes terapéuticos para el tratamiento de esta enfermedad. Utilizando un sistema de estudio *in vitro* de la línea celular humana MT4, nuestro grupo demostró el efecto inhibidor del extracto dializable de leucocitos (EDL) en la replicación del VIH. En publicaciones previas reportamos que la replicación del virus se inhibía cuando se trataban las células con este derivado leucocitario por tiempo prolongado antes del reto viral. Los resultados del presente ensayo demostraron que los cultivos de células MT4 no necesitan duplicarse para que ocurra la inhibición del VIH mediada por el EDL, y el efecto inhibidor de la replicación viral se puede constatar siempre que se pretraten las células por un período igual o mayor que 24 horas. Esta inhibición no puede observarse cuando el tiempo de pretratamiento es corto, lo que sugiere que el mecanismo de inhibición desencadenado por el EDL parece estar más relacionado con la modificación de factores celulares, que con la acción directa sobre la partícula viral, sin descartar la posibilidad de modificación de factores virales durante el desarrollo del ciclo viral.

Palabras clave: extracto dializable de leucocitos, factor de transferencia, VIH, MT4, actividad anti-VIH, virus Sendai, SIDA

*I*ntroduction

Immunomodulatory drugs can restore or increase the capacity to develop cytokine and endogenous factors-mediated responses. They act by inducing maturation, differentiation or proliferation of cellular subsets that are essential for the defense mechanisms of the body, increasing host resistance to infections caused by bacteria, parasites and viruses [1, 2].

The Dialyzable Leukocyte Extract (DLE) is a preparation obtained from healthy human leukocytes, showing diverse effects on the immune system. It is commonly employed to treat diseases caused by viruses, parasites, fungi, mycobacteria, and in cancer, among others [3]. Formerly, Lawrence [4] reported that leukocyte extracts administered to patients that are unable to develop delayed-type hypersensitivity against a given antigen, allowed them to react in this manner. This type of activity was named Transfer factor (TF), assuming that it contained molecules that were able to transfer cellular immunity from one individual to another. DLE and TF are used indistinctly in the literature, although some authors prefer to use TF to define DLE components which can mediate antigen-specific T cell responses [5].

In addition to transferring cell-mediated immunity, DLE also mediates effects on immune system functions, further influencing that type of response; these include: cytokine release, acceleration of CD2 receptor regeneration on T cells and the activation of monocyte-macrophages and chemotaxis [6-8].

In the 1970's, in spite of the scarce information available on DLE composition, the treatment with DLE of Wiskott-Aldrich patients was started, with satisfactory results in one-half of the patients. Since then, this product has been used to treat several diseases of

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different origin, with beneficial results, including mucous-cutaneous candidiasis, herpes simplex, herpes zoster, chronic infections, sepsis and hepatitis B. Several researchers have used DLE to treat human immunodeficiency virus (HIV) patients, either asymptomatic or at the acquired immunodeficiency syndrome (AIDS) phase, resulting in a partial immune reconstitution [10], a lower incidence of opportunistic infections [11], and clinical improvement [12]. In Cuba, a follow-up clinical trial was carried out using a Cuban DLE preparation in asymptomatic HIV patients [13]. Eighteen percent of the untreated individuals showed disease progression, with only 7% for DLE-treated patients [14].

These results indicate that the DLE preparation delays disease progression. However, the molecular basis supporting this effect remained unknown. We have previously demonstrated an inhibitory effect on HIV-infected MT4 cells pre-treated with DLE, in an *in vitro* infection model [15, 16]. The mechanism by which DLE inhibits HIV replication *in vitro* remains unknown. In this trial, we studied the time needed for the incubation of MT4 cells with DLE, to decrease the production levels of HIV particles. Since this effect was not demonstrated in short pre-treatment periods, we studied the need of duplicating the cell population in the culture.

Materials and methods

Dialyzable Leukocyte Extract

Certified whole blood from healthy human donors was employed to generate leukocyte concentrates by centrifugation, which were stored at 4 ºC for at least 24 h. Hemolysis was carried out by adding a sterile, cold NH_4Cl solution to leukocyte concentrates (to a final 83% concentration). Cellular suspensions were continuously centrifuged at 3 000 rpm and 1L/3.5 min flow-rate, at 4 ºC. The pellet was suspended in cold 1 X phosphate-buffered saline (PBS), supplemented with 17% v/v gammaglobulin-free human serum (agamma) and 50 µg/mL neomycin. A second hemolysis was carried out as previously described, for 10 min. Cells were suspended in a MEM medium supplemented with agamma serum at 1.8 mg of proteins/mL and 50 mg/mL neomycin, at 4 ºC, and shaken in an orbital shaker at 15 rpm for 15 to 20 min, at 4 ºC. The final cellular suspension was adjusted to 1.2×10^7 cells/mL and further incubated at 37 ºC with agitation. After two hours, Sendai virus was inoculated at a final 150 HAU/mL concentration and further incubated for 18 h. The culture was subsequently centrifuged and the cells washed with PBS and stored at -20 ºC. Cells were subjected to ten cycles of freezing and thawing, followed by three cycles of ultrasound rupture of 15 s each, at 60 s intervals with an outlet potency of 100W/min, and continuous regularity. Cells were further vacuum-dialyzed in PBS at 4 ºC for 24 h, and the supernatant was filtered through a 0.2 mm membrane at a pressure of less than 2 atm. One unit (1 U) of DLE was estimated as the amount of the dialyzed product obtained from 5×10^8 leukocytes.

Antiviral activity of DLE against HIV

The MT4 cell line was cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), at 37 ºC in a 5% CO_2 atmosphere. Cells were treated for 24 h, 72 h or 7 days with 0.15 or 0.3 U/mL doses of DLE. After that time, the culture medium was changed for all cultures (after three days, the culture medium was changed and ensuring the presence of DLE). Then, DLE-treated or untreated cells were infected with the HIV 1 Bru strain at 0.05 or 0.1 multiplicity of infection (m.o.i.), for one hour. Cells were further incubated for 7 days, with or without DLE, accordingly. Two control conditions were included: cell line control (cells without DLE treatment) and viral control (cells without DLE treatment and challenged with the virus). Culture supernatants were collected for p24 antigen determinations.

p24 antigen determinations

The presence of the p24 viral antigen in the supernatants of the culture was determined by using an ELISA method (DAVIHAgp24, Cuba), according to the instructions of the manufacturers. All determinations were done in triplicate. Results were expressed as percent of inhibition, calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{Ag p24 virus control culture} - \text{Ag p24 DLE-treated culture}}{\text{Ag p24 virus control culture}} \times 100$$

Growth curve

MT4 cells were seeded at 1.4×10^5 or 2.5×10^5, in culture plates containing the RPMI-1640 medium supplemented with 10% FBS, and incubated at 37 ºC at a 5% CO_2 atmosphere. Cultures were done in triplicate. The amount of total cells was determined through the Newbauer chamber counts at 7, 12 and 24 h, and every 24 h for 7 days. Viability was estimated by the Trypan blue dye exclusion method. Doubling times were established as the time in which cultured cells duplicate during the exponential phase of growth.

Results

Effect of DLE on HIV infection in MT4 cells

The MT4 cell line was used for *in vitro* studies, because it is highly susceptible to HIV infection [17]. The non-toxic DLE concentrations for this cell line were established by the Trypan blue dye exclusion method, and cultures were initiated with viability higher than 95% MT4 cells were treated 24, 72h or 7 days with DLE, it was kept during incubation after viral challenge. Both doses of DLE induced a 90% inhibition of viral replication at 0.05 m.o.i., seven days after incubation with DLE (Figure 1). This effect was also observed when cultures were subjected to the highest m.o.i. (0.1), with viral inhibition of 80% or higher (Figure 1B). Two DLE concentrations were evaluated for each experimental variant, and the inhibitory effect was detected even at the lowest DLE concentration.

Based on these results, lower DLE incubation periods were evaluated prior to viral challenge. Again, viral inhibition values above 90% were obtained for both viral doses after a 72 h incubation period (Figure 1 A and B).

When cells were pre-incubated with DLE for 24 h, p24 protein levels corresponding to 80% inhibition or higher were measured in the supernatants of cultures challenged either with 0.05 or 0.1 m.o.i. (Figures 1A

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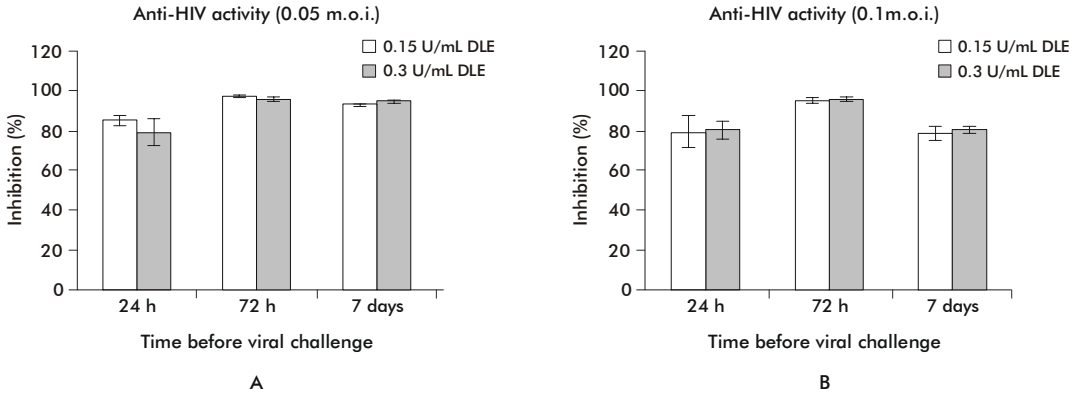

Figure 1. Effect of DLE treatment on HIV replication. MT4 cell concentration was adjusted to 2.5 x 105 cells/mL and cells were treated for 24 or 72 h, or 7 days, with 0.15 or 3.0 U/mL of DLE. They were then washed and infected at 0.05 (A) or 0.1 m.o.i. (B) with HIV-1, Bru strain. After viral challenge, virus was eliminated. The medium was replaced with a fresh medium with or without DLE and supernatants were collected after 7 days to determine p24 antigen levels. Results were expressed as percentage of inhibition, compared to the control of DLE-untreated cells and carried out in triplicate. Error bars represent the standard deviation.

and 1B, respectively). This effect was also detected for both DLE concentrations.

Determination of the doubling time for the MT4 cell population

A growth curve was established to determine the doubling time of MT4 cells, starting from 1.4 x 10^5 or 2.5 x 10^5 cells/mL. An exponential growth curve as typical for continuous cultured cell lines was obtained. The cellular doubling time was estimated as 35 h, being determined in the phase of exponential growth (Figure 2).

Discussion

The incidence of HIV infection continuously increases throughout the world, in spite of intense search for more than 25 years for interventionist therapeutic strategies. More than two dozen anti-retroviral therapies have been developed to fight HIV infection. They effectively decreased AIDS-related morbidity and mortality in developed countries [18], but they neither eradicated HIV infection [19, 20], nor its spread.

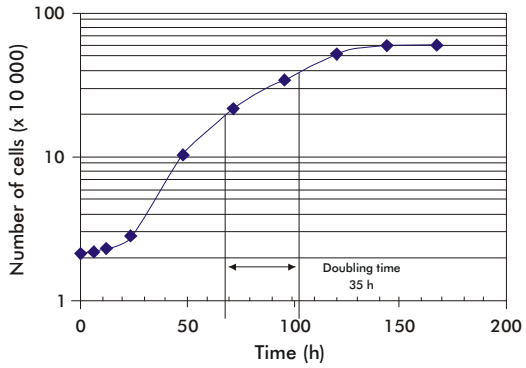

Figure 2. Growth curve of the MT4 cell line in culture. Cells were seeded in culture plates at 2.5 x 10^5 cells/mL in RPMI-1640 medium supplemented with 10% FBS, and incubated at 37 °C in a 5% CO2 atmosphere. Total concentration of cells was determined by counting them in a Newbauer chamber at 7, 12 and 24 h, and every 24 h. Plotting in a logarithmic-linear chart was used to estimate doubling time. Results are representative of five independent experiments.

About fourteen thousand new infections have been found each day [18]. The nature of the interaction between HIV and the immune system is complex, and the relevance of the different immune responses for infection control is only partially understood. The complete restoration of the immune system and virus eradication seem to be improbable. It requires increased specific and unspecific host immune responses, emphasizing on the research of immunotherapies focused on preserving and sustaining anti-HIV immunity. Moreover, although the cost of anti-retroviral therapy has considerably decreased globally, 5 000 000 HIV/AIDS patients needing treatment cannot afford it [21].

Therefore, the search for new drugs to inhibit viral replication or to restore the immune system in HIV patients continues. Newly discovered natural or chemically-synthesized substances are being evaluated as therapeutic drug candidates with antiviral activity, using *in vitro* systems of cultured cell lines susceptible to HIV infection, which include the CEM, U1 and MT4 cell lines [22, 24], many researches indicate the use of MT4 cells on *in vitro* studies related with HIV-1 infection. Ross et al considered MT4 as one of the most susceptible lymphoblastoid CD4+ cells [25]. Our results confirm the anti-HIV activity of DLE. In this study, HIV replication inhibition values reached 80-90% when MT4 cells were treated with the lowest dose of the extract and challenged with twice the viral dose (0.1 m.o.i.).

HIV replication was inhibited after a pre-treatment with DLE for seven days, and even for a time as short as 24 h. An inhibitory effect was reported for a similar extract on *in vitro* HIV replication in lymphocyte culture, in 1987 [26]. That effect was obtained by treating lymphocytes one hour after infection, inhibiting RT activity.

Although it has not been tested under those conditions, it is unlikely that a similar effect could be obtained with our system, since DLE does not influence HIV expression for a short period of time as established for reference drug evaluations in MT4 cells [15]. Up to date, there are no reports confirming those results. In fact, the use of different DLE preparations and *in vitro*

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models of HIV replication hampers further comparison. This results indicate that anti-HIV activity triggered by DLE is a maintained effect, since cellular contact with DLE lasts for as short a period as 24 h, prior to viral challenge for inhibition.

Current therapeutic approaches include the improvement of immune response with the synergistic administration of the antiretroviral therapy commonly used to control HIV-to-AIDS progression [27]. DLE simultaneously shows anti-HIV activity, modulates different types of immune effectors (*e.g.*, cytokines and transcription factors) and restores leukocyte subsets in treated patients. All these properties make DLE a potential drug to be used in a therapeutic combination with antiretrovirals. Other types of immunomodulators showing high inhibitory capacity, such as chemokines, would be difficult to use, because of the increased risks of unspecific inflammation and immune cell activation processes [28].

Cells tend to proliferate in a controlled fashion according to the needs of the organism. The regulation of the cell cycle depends on the cell type: some cells divide rapidly, while others lose that capacity. In our study, we established the growth curve for the MT4 cell line and determined the duplication time by semilog plotting [29, 30]. We found that the inhibition of HIV replication by DLE does not depend on cell division.

Moreover, MT4 cell culture density was greater after 24 hours than after 3 hours. The inhibition of viral replication does not detect when cell cultures are treated with DLE for 3 hours. Hence, the anti-HIV activity would require more cells in the culture to produce larger amounts of one or several factors, which would modify endogenous cellular effectors that would directly or indirectly interfere with the viral life cycle. Nevertheless, these factors do not seem to be required at very high concentrations, since cells have not yet been duplicated after 24 h in culture time in wich, an inhibitor effect of viral replication can observe. Understanding the mechanism of action of DLE will undoubtedly contribute to the design of antiviral drugs.

If the pre-treatment period is reduced to 3 hours, it is not possible to demonstrate the inhibitory effect of DLE on the replication of HIV. This seems to be more related to the modification of cell factors than to the particle itself, although the possibility of the modification of viral factors cannot be discarded.

In a previous study, our group demonstrated that DLE inhibits the production of some soluble factors involved in the pathophysiology of HIV, such as TNF-α [16] and the NF-κB and Sp1 transcription factors [31]. We are currently investigating other factors that could be mediating the anti-HIV activity of DLE.

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