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Propagation of a transmissible cytotoxic activity on cultures of human peripheral blood lymphocytes

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SUMMARY

Positive results were attained when human peripheral blood lymphocytes (PBLs) were investigated for their ability to propagate a transmissible cytotoxic activity (TCA) isolated on VERO cell cultures from a sample of cerebrospinal fluid (CSF) drawn from a woman with ischemic brain injury. In consideration of this finding it can be assumed that "in vivo" blood lymphocytes contributed to give rise to the TCA detected "in vitro" in the CSF inoculum.

KEY WORDS: Human preripheral blood lymphocytes, Transmissible cytotoxicity, Misfolded proteins

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Recently, from the cerebrospinal fluid (CSF) of a patient with brain ischemia we isolated a transmissible cytotoxic activity (TCA) on VERO simian kidney cell cultures (Portolani *et al.*, 2005). The cytotoxicity showed particular properties such as resistance to proteolysis and the ability to cause apoptosis of permissive cells. In addition, two protease-resistant proteins of molecular mass of ~39 and ~27 kDa were seen to be present in the cytotoxic preparations obtained by subsequent passages of the CSF toxicity on fresh cell cultures. Repeated electron microscopic examinations both of cytotoxic cell cultures and respective supernatants did not disclose viral particles which might explain the infectious nature of this cytotoxicity. In consideration of the fact that protein molecules with conformational changes are cytotoxic, protease resistant (Bucciantini *et al.*, 2004) and capable of self amplification (Prusiner, 1982), we suggested that one or two misfolded proteins formed *in vivo* following the ischemic injury of brain tissue might be present in the CSF sample inoculated in cell cultures. Investigations performed to identify human cell cultures able to propagate the cytotoxicity under study showed that the astroglioma cell line D54-MG (Bigner *et al.*, 1981) was an efficient cell substrate for the cytotoxicity propagation. Negative results obtained when a series of human lymphoid cell lines (U937, Molt-3, SupT-1, HSB-2, JJhan) were tested seemed, instead, to exclude a susceptibility of the lymphocytes. Contrary to this prior assumption, this paper reports on the successful propagation of the TCA on fresh cultures of peripheral blood lymphocytes (PBLs).

Two batches of blood (200 ml each) from healthy subjects were obtained from the Transfusional Center of the University Hospital of Modena and

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Reggio Emilia. PBLs were isolated from whole blood by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Some of the cells were immediately cultured to be tested for the propagation of TCA. Aliquots of the remaining cells, suspended in RPMI medium with 40% FBS and 10% DMSO, were kept in liquid nitrogen to be used in subsequent experiments in the case of a positive result. TCA propagation by PBLs was investigated in different experimental cell culture conditions. Cell cultures were maintained both in growth medium consisting of RPMI supplemented with 10% foetal bovine serum (FBS) and in RPMI growth medium supplemented with 20 U/ml of IL-2 (Merck, Whitehouse Station, NJ, USA). In some experiments, cells were stimulated with 20 µg/ml phytohemoagglutinin (PHA) (Merck, Whitehouse Station, NJ, USA) for 48 hours and then cultured in the presence or absence of 20 U/ml of IL-2. Each type of cell culture, containing at the seeding 10^7 lymphocytes in 10 ml of RPMI growth medium was inoculated after 48 h-cultivation and removal of 8 ml of medium with cytotoxic and control supernatant (SPN) from D54-MG cultures (D54-MG/SPN). Following 1 h-absorption of the inoculum, 10 ml of the RPMI medium were added to the cell cultures according the different culture conditions. Lymphocyte cultures treated with cytotoxic preparations showed acidification of the culture medium and larger and more numerous cell aggregates than the corresponding control cultures (Figure 1 A, B). The propagation and then the titration of the TCA produced by

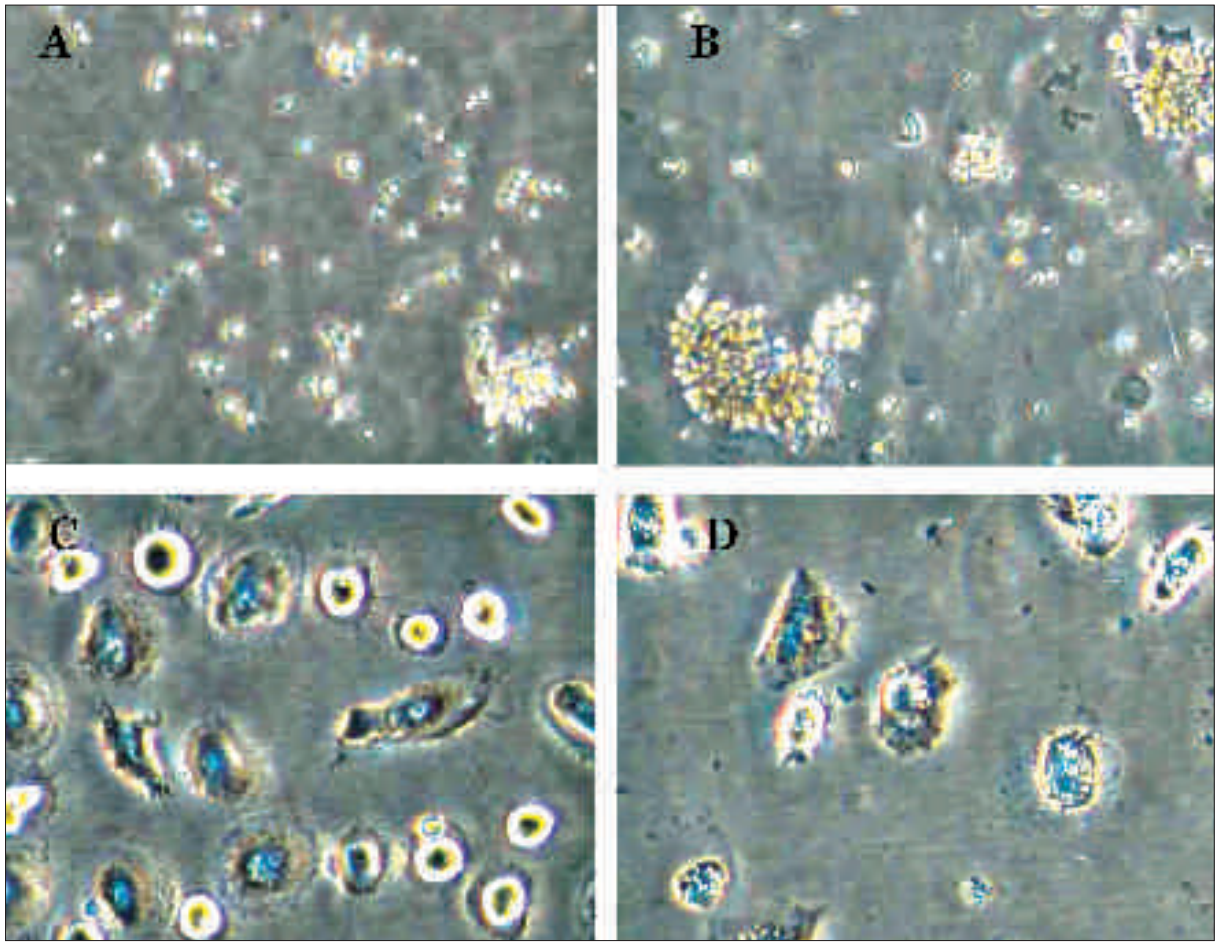

FIGURE 1 - ***A***, ***B*** *Aggregates of lymphocytes cultured in growth medium alone six days after the inoculation of mock and cytotoxic D54-MG/SPN respectively (10 x).* ***C***, ***D*** *Monolayers of macrophagic cells from cultures grown in medium supplemented with IL-2, 40 days after the inoculation of mock and cytotoxic D54-MG/SPN respectively (20 x).*

PBLs was assessed according to Portolani *et al.*, 2005 on the basis of the appearance of a cytotoxic effect (CTE) induced on D54-MG cell cultures both by SPNs and cell lysates from lymphocyte cultures. In particular, the samples under study were titrated as follows. After substitution of growth medium (DMEM 10% FBS) with maintenance medium (DMEM 2% FBS), 24 h-growth D54-MG cells on 96 well plates were inoculated with 20 µl of tenfold serial dilutions of each sample, in triplicate. D54-MG cell wells were subjected to microscopic observation for the appearance of CTE. The TCA titre, determined according to Reed and Muench (Lennette, 1964), was expressed as Total Cytotoxic Dose 50/20 µl (TCTD50/20 µl). Six days after inoculation, SPNs and lymphocyte lysates had TCA titres of $10^{3.25}$ and 10^{5} TCTD50/20 µl respectively when cultured in the growth medium without IL-2 and PHA. Similar titres ($10^{3.25}$-$10^{3.5}$ and $10^{5.5}$-$10^{5.74}$ TCTD50/20 µl) were found in SPN and cell lysates respectively, in all other culture conditions. The fact that the quantity of TCA from lymphocyte culture is larger than that of the inoculum (a total of 2.5 x $10^{4.74}$ TCTD50) is indicative of an ability of PBLs to TCA propagation. This conclusion was confirmed by analogous TCA titres obtained when SPNs from cytotoxic lymphocyte cultures (Lymph/SPNs) were used instead of cytotoxic D54-MG/SPNs to seed PBL cultures.

Macrophages proved to be able to propagate the TCA under study. Cell monolayers formed by cells adhering to the culture flask surface arising from lymphocytes cultured in the absence of PHA and IL-2 and treated with cytotoxic D54-MG/SPNs, showed morphologically altered cells which detached from the flask surface. Figure 1 (C, D) shows macrophage cultures at the 40^{th} day of the treatment with control and cytotoxic D54-MG SPNs. Figure 1D shows a thinning out of the cell monolayer due to the cell detachment. The cytotoxic titres of the SPNs harvested from cell cultures at 4, 8, 12 and 40 days of seeding, were $10^{5.25}$, $10^{4.5}$, $10^{4.25}$ and $10^{3.5}$ TCTD50/20 µl respectively.

DNA laddering assays showed that the production of TCA by PBLs causes cell death by apoptosis (Figure 2A, lane 3). Lymphocytes cultured in growth medium supplemented with IL-2 were processed to prepare genomic DNA, which was fractioned by electrophoresis using a 2% agarose gel and stained by ethidium bromide (Butler *et al.*, 1997). In the same electrophoretic pattern, the DNA fragmentation induced by proteinase K (pK) (Eurobio, France)-pre-treated cytotoxic Lymph/SPN, shows that the TCA produced by PBLs is protease resistant (lane 5).

Lymphocytes cultured in the growth medium alone were studied by electron microscopy. Lymphocytes were fixed with 2.5% glutaraldehyde in Tyrode's buffer pH 7.4 for 2 h at 4°C, post-fixed in osmium tetroxide in the same buffer, dehydrated through graded concentrations of ethanol and finally embedded in epoxide resin (Durcupan ACM, FLUKA, Buchs, Switzerland). Semi-thin sections stained with toluidine blue were used for high-resolution light microscopy and for the selection of areas for thin sectioning. For electron microscopy, thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss M109 electron microscope. Morphological alterations typical of apoptosis, consisting of condensed mitochondria, apoptotic bodies and cytoplasmic vacuolization (Figure 2 B-C-D-E), were detected in lymphocytes from cell samples treated both with cytotoxic and control preparations. When the rate of cell apoptosis was analysed by a digital image analyser (NIKON ACT-1 for DXM 1200F), the percentage of apoptotic lymphocytes from cytotoxic cell cultures proved to be as much as 30% higher than apoptotic lymphocytes from control cell cultures.

In order to identify the population of lymphocytes involved in the propagation of the TCA in addition to macrophages, electroimmunocytochemical technique (immunogold) was used. Assays were carried out employing monoclonal antibodies to B and T lymphocyte antigens Anti-CD20 and Anti-CD45R0 respectively (Dako, Copenhagen, Denmark) and a secondary antibody conjugated to colloidal gold particles (EY Laboratories, Inc., San Mateo, CA). Sections of 80 nm of mock and cytotoxic samples of lymphocytes embedded in epoxide resin were mounted on nickel grids for immunogold reactions performed according to Tong *et al.* (2004). After immunotests were carried out, sections were stained with 1% uranyl acetate and lead citrate and examined under a Zeiss EM109 electron microscope (Carl Zeiss, Inc., Thornwood, NY). A semi-quantitative analysis (Mayhew *et al.*, 2004) was then carried out, and the digital image analyser was used to compare the number of apoptotic T

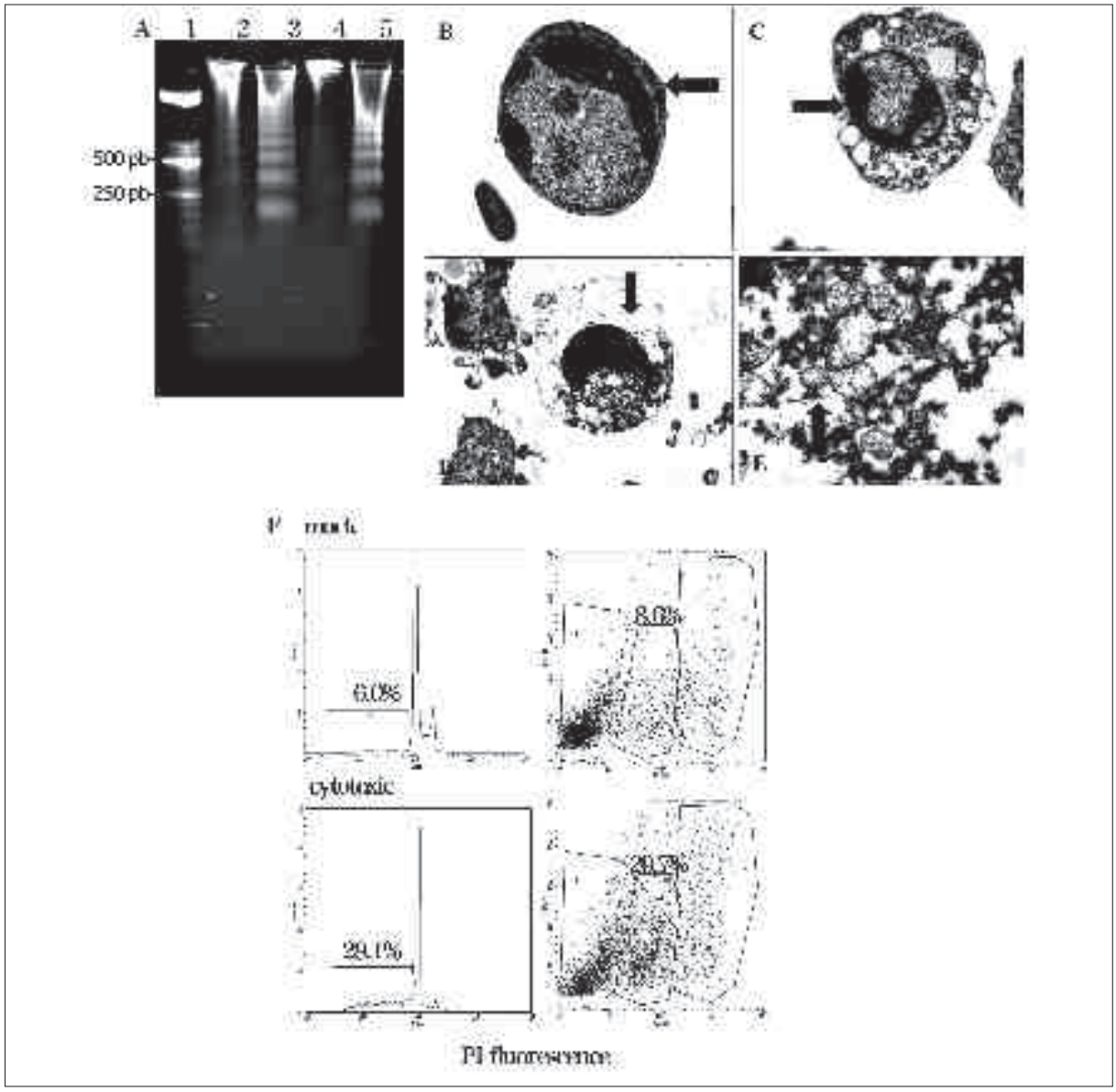

FIGURE 2 - ***A****: Fragmentation of the DNA of PBLs from cell cultures inoculated with mock (lane 2) and cytotoxic (lane 3) Lymph/SPN and fragmentation of the DNA of PBLs from cell cultures inoculated with Lymph/SPN pre-treated with 50 µgr/ml pK mock (lane 4) and cytotoxic (lane 5). DNA MW marker (50-base pair ladder marker) was loaded onto lane 1.* ***B****,* ***C****,* ***D****,* ***E****: Semithin sections of PBLs grown in the growth medium alone, gathered six days after treatment with cytotoxic Lymph/SPN.* ***B****: Initial step of an apoptotic lymphocyte with condensed chromatin close to the nuclear membrane and intact cytoplasm (14500x).* ***C****: A more advanced step of apoptosis with more condensed chromatin, empty cytoplasm and many vacuolizations (9500x).* ***D****: Final step of apoptosis with chromatin displaying the typical half-moon shape, cytoplasm disrupted because of plasmic membrane break; mitochondria and other cell debris can be seen among cells (8500x).* ***E****: Details of mitochondria can be seen: they are still present but completely decondensed (17000x).* ***F****: Cytofluorimetric analysis of PBLs six days after the inoculation of mock and cytotoxic Lymph/SPN. Cells were from cultures supplemented with IL-2. Left column: DNA content assay. The DNA content in PBLs was assessed using propidium iodide (PI) staining. Numbers represent the percentage of cells with <2c DNA content, and thus considered apoptotic. Right column: plasma membrane integrity test. The test was performed using PI in isotonic solution as a viability stain. R1: intact, viable cells; R2: apoptotic cells; R3: necrotic cells. Numbers represent the percentage of cells in R2. The percentage of apoptotic cells obtained with the two methods is quite consistent.*

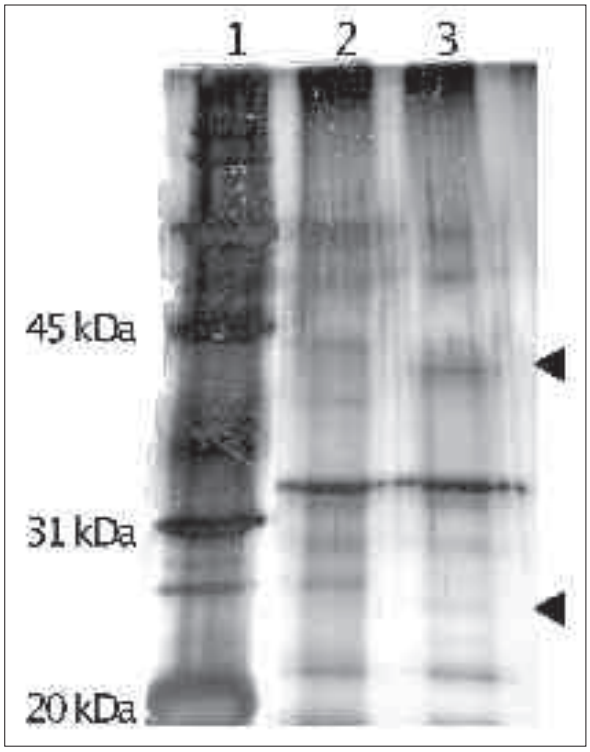

FIGURE 3 - *Gel (12%) electrophoresis showing ~39 and ~27 kDa protease- resistant bands in protein samples from lysates of lymphocytes cultured in growth medium supplemented with IL-2. Mock (lane 2) and cytotoxic (lane 3) lymphocyte lysate treated with 20 µg/ml proteinase K for 1 h at 37°C. Lane 1: proteins of molecular mass standards (Bio Rad) are indicated in kDa; protein bands were visualized by silver staining.*

and B lymphocytes present in mock and cytotoxic sections. The results showed that the percentages of apoptotic T and B lymphocytes from cytotoxic cell cultures were as much as 30% and 20% higher than apoptotic T and B lymphocytes from control cell cultures respectively.

Flow cytometric analysis (Salvioli *et al.*, 2000) of PBLs treated with cytotoxic Lymph/SPNs in the different experimental conditions of cell growth showed that in each situation the percentage of lymphocyte death by apoptosis varied, but was never more than 30% higher than that of lymphocytes from control cell cultures. Cells with damaged plasma membrane consistent with necrotic cell death were also found, as shown in Figure 2F. This mortality was probably due to the use of lymphocytes from liquid nitrogen and also to the PHA (Lorenz *et al.*, 1997) if present in the growth medium.

In conclusion, in addition to the TCA obtained on VERO cells following the inoculation of the CSF from the patient with brain ischemia (Portolani *et al.*, 2005), the TCA passaged on blood lymphocytes was protease resistant, able to induce apoptosis of permissive cells, and as shown in figure 3, able to stimulate in these cells the production of two protease-resistant proteins of ~39 and ~27 kDa. The sharing of these characteristics shows that the PBLs are capable of expanding the cytotoxic activity under study. In consideration of this, it may be hypothesized that "in vivo" blood lymphocytes cooperated to give rise to the TCA detected in the CSF "in vitro".

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