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Cerebrospinal fluid samples from patients with various neurological disorders have a transmissible cytotoxic activity with similar properties

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SUMMARY

When inoculated into cell cultures to search for cytopathic viruses, six out of 384 cerebrospinal fluid (CSF) samples from patients with different neurological disorders proved to have a transmissible cytotoxic activity (TCA) not correlated to a conventional infectious agents. Properties shown by a TCA previously detected in the CSF sample of a patient with brain ischemia (Portolani *et al*., 2005) were shared by each of the newly isolated TCAs. We conclude that independently of the neurological clinical picture shown by the patient, the TCA detected in the CSF samples under study could have the same origin.

KEY WORDS: Cerebrospinal fluid cytotoxicity, misfolded proteins, apoptosis.

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INTRODUCTION

In a previous paper, we reported the results of a study concerning a transmissible cytotoxic activity (TCA) isolated on VERO cell cultures from the cerebrospinal fluid (CSF) of a patient with brain ischemia (Portolani *et al*., 2005). A traditional agent responsible for this cytotoxicity was excluded on the basis of negative electron microscopy and a lack of immunoreactivity both with the patient's serum and with human γ-globulin preparations. The hypothesis was made that transmissible cytotoxicity might have to do with the misfolding of one or more proteins. This supposition was suggested by the results of chemico-physical investigations on cytotoxic materials obtained from serial passages of the CSF cytotoxicity on fresh cell cultures. These materials were resistant to proteolysis and to DNase I and RNase A, had a tendency to aggregations and cytotoxicity, which are all properties shared with conformationally altered proteins (Prusiner, 1982; Bucciantini *et al.*, 2004). In a later study, we also demonstrated that this TCA may be propagated by peripheral blood lymphocytes (PBLs) (Portolani *et al.*, manuscript subjected for publication).

In the years following the finding of this cytotoxic sample of CSF, six out of 384 CSF specimens, when inoculated into cell cultures (VERO and human fibroblast cells) for virus isolation displayed a cytotoxicity for the VERO cells alone, which proved to be transferable and not associated with a conventional infectious cause. Since

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all the TCAs detected in CSF samples were from patients with different neurological disorders (Table 1), we decided to verify whether some properties shown by the TCA detected in the first sample of CSF (TCA1) were shared or not by the newly isolated TCAs (TCA2-7). The choice of properties to be investigated fell on the ability of the TCA to:

a) propagate on D54-MG cells and PBLs, in addition to VERO cells;
b) induce NO by RR4 cells;
c) induce apoptosis of permissive cells;

TABLE 1 - *TCA (1-7): cytochemical characteristics of the CSF samples, clinical diagnosis of the neurological disorders, sex and age of the patients.*

TCA N°	*CSF Cytochemical analysis*					*Clinical diagnosis*	*Patient* sex*	*Age*
	Glucose mg/dl	*Proteins mg/dl*	*Lactate mg/dl*	*Clorures mEq/L*	*Cells N° (prevalence)*			
1	58	158	n.a.	n.a.	200 (Lymphocytes)	Brain ischemia	F	69
2	56	27	9.10	124	2	Transverse myelitis	F	14
3	59	46	n.a.	n.a.	100 (Neutrophils)	Meningoencephalitis	F	49
4	56	36	10.7	119	1	Multiple sclerosis	M	44
5	56	82	n.a.	n.a.	100 (Neutrophils)	Meningoencephalitis	M	52
6	54	30	n.a.	n.a.	2	Transverse myelitis	M	33
7	62	38	n.a.	120	1	Encephalitis	F	72

*Some patients from whom the cytotoxic CSF were obtained were admitted to the Neurological Division of the University Hospital of Modena and Reggio Emilia, while others were admitted to Neurological Divisions of Hospitals in the province of Modena.

TABLE 2 - *Properties shared by the seven TCAs isolated from as many CSF samples.*

[a]Propagation on VERO, D54-MG, PBLs. The titres obtained on each type of cells ranged between $10^{3.5}$-10^{5} TCT50/20µl.

[b]Induction of NO by RR4 cells. The NO values obtained ranged between 15-25 µM.
Induction of death by apoptosis of permissive cells documented by cell DNA laddering.
Resistance to proteolysis documented by resistance to proteinase K (pK) (Fig. 1A).
Overexpression of two proteins of ~39 kDa and ~27 kDa molecular weight in the host cells (Fig. 1B).

[a]EMEM, DMEM and RPMI supplemented with 10% foetal bovine serum were used to growth VERO, D54-MG cells and PBLs respectively. Cytotoxic preparations formed by VERO cell lysates obtained by serial passages of the toxicity of each CSF sample through VERO fresh cell cultures were inoculated both into one-day cultures of D54-MG cells and into two day-PBL cultures. Six days after seeding, D54-MG and PBLs lysates were prepared by freezing and thawing of the respective type of cell cultures three times. The propagation of the TCA was verified by titration of the cytotoxic activity of each type of cell lysate. To this end, one to ten dilutions of the cell lysate under study were tested for their ability tp induce a cytotoxic effect on D54-MG cell cultures. TCA titres were then determined by the evaluation of cytotoxic and non-cytotoxic cell cultures according to Reed and Muench (Lennette, 1964).
[b]RPMI supplemented with 10% foetal bovine serum was used to grow RR4 cells. One to five dilutions of supernatants from VERO, D54-MG and PBL cytotoxic cell lysates were inoculated into RR4 cell cultures at the time of cell plating. Five days later, the NO produced by RR4 cells was evaluated by the Griess reaction which consists in optical density measurement at 570 nm (Ding et al., 1998) of the stable oxidation of the product NO_2^- present in the cell culture medium. A standard curve, prepared using sodium nitrite (Merck) in a range between 3 and 200 µM, determined the µM NO induced by the sample under study. The µM NO released by RR4 cells treated with lipopolysaccharidic toxin from *Escherichia coli* (serotype 0128: B12, Sigma) was used to validate the assay. Negative controls included in each assay consisted of the same number of RR4 cells inoculated with one to five dilutions of supernatants from VERO, D54-MG and PBL mock cell lysates.

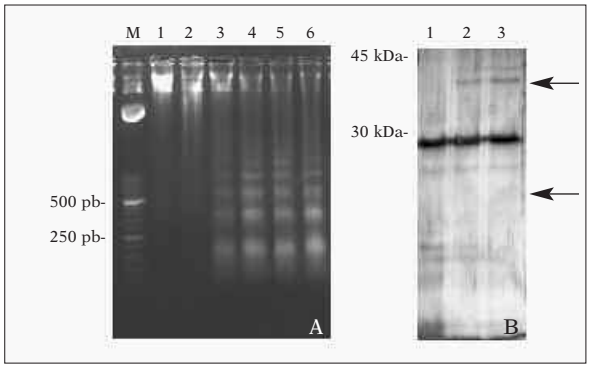

FIGURE 1 - ***A****: DNA fragmentation induced in PBLs by TCA 2 (lanes 3, 4) and TCA 7 (lanes 5, 6). DNA extracted by PBLs (Sikora et al., 1993) inoculated with cytotoxic lysates was untreated (lanes 3, 5) and treated (lanes 4, 6) with 50 µg/ml pK (Eurobio, France) for 1 hour at 37°C (Prusiner, 1982). Lanes 1 and 2: DNA from PBLs inoculated with mock cell lysates, untreated (lane 1) or treated (lane 2) with pK. Genomic DNA was fractionated by electrophoresis using a 2% agarose gel and stained by ethidium bromide. The DNA MW marker (50-base pair ladder marker) was loaded onto lane M.* ***B****: Polyacrylamide gel (12%) electrophoresis (Laemmli, 1970) showing pK-resistant bands in protein samples from PBL cell lysates: mock (lane 1), TCA1 (lane 2), TCA 7 (lane 3) cell lysates. Molecular masses of marker proteins are indicated in kDa to the left. Protein bands were visualized by Silver staining.*

d) resist proteolysis;
e) overexpress two proteins of ~39 kDa and ~27 kDa in the host cell.

Each of these properties was investigated according to specific procedures reported in detail in Portolani *et al.*, 2005. Results of these studies showed that all the properties taken into consideration are common to all the TCA isolates (Table 2). On this basis we conclude that independently of the neurological clinical picture shown by the patients, the TCA detected in the CSF samples under study could have the same origin.

A variety of pathological conditions, for example, ischemia or viral infections may provoke endoplasmatic reticulum (ER) stress in the cells of injured tissue (Kaufman, 1999). ER is a factory for folding and maturation of newly synthesized transmembrane and secretory proteins so an ER perturbation compromises the ER folding capacity, resulting in the accumulation of unfolded proteins (Shen *et al.*, 2004). The CSF samples with TCA were from patients with ischemia (TCA1) and from disorders possibly of infectious origin (TCA2-7) so we can postulate the appearance of ER stress in the course of the pathogenetic process of each of the clinical neurological pictures. The same properties shared by the TCA isolates could be explained by the presence in the corresponding samples of CSF, of the same misfolded proteins formed in the cells of the brain area involved in the pathogenetic process, because of the ER stress.

The infrequent finding of CSF samples with TCA compared to the numerous CSF specimens from neurological disorders sent to laboratories for the diagnosis of viral infections needs an explanation. Two possible reasons may be proposed. In a few individuals, the system controlling protein folding is more susceptible to noxious stimuli than in the rest of the population so, only in these people will the higher production or the slower degradation of proteins with altered conformation, or both these events, allow the presence in the CSF of the critical amount of proteins necessary for the appearance of CSF cytotoxicity. Alternatively, for the cytotoxicity associated with the misfolded proteins contained in the CSF samples to be microscopically detectable on the inoculated cell cultures, a certain number of concomitant events is necessary (for example, a critical amount of misfolded proteins at the time of seeding, a particular metabolic state of the permissive cells, the absence in the CSF of molecules able to interact with the proteins). In support of the latter assumption, we can cite the great difficulty in detecting human herpes simplex in the CSF of immunocompetent patients with herpetic encephalitis by cell culture isolation (Mattison *et al.*, 1991).

In conclusion, seven CSF samples drawn from as many patients with neurological disorders both of vascular (TCA1) and possibly of infectious origin (TCA2-7) displayed a toxicity for simian and human cells including human PBLs. This toxicity is transferable despite the impossibility of bringing to light a conventional agent to account for this. On the basis of results obtained in previous studies (Portolani *et al.*, 2005), a correlation was hypothesized between the TCA of the CSF deriving from a case of brain ischemia (TCA 1) and the presence in the CSF of misfolded proteins. Implicitly, this hypothesis suggests that such proteins, like agents with nucleic acid, may

be isolated and propagated on cell cultures. This suggestion sounds like a paradox so we cannot rule out the possibility that a virus is responsible for the TCA associated with the CSF cytotoxic samples. If this supposition is true, it should be an unknown virus with peculiar properties including the structure and dimensions of the virion so that its detection both outside and inside the host cell is made very difficult.

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