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Neutralization by human serum samples of a transmissible agent isolated from the cerebrospinal fluid of neurological patients

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SUMMARY

A transmissible cytotoxic agent thought to be associated with one or more misfolded protein(s) was found in several cerebrospinal fluid (CSF) samples from neurological patients. Since some experiments carried out to identify this unusual infectious factor showed the block of its propagation by rabbit gammaglobulins (IgGs), the search for such an activity by human IgGs was programmed. Neutralizing assays carried out using human sera as IgGs source showed a blocking property displayed by: twenty serum samples from as many patients with a diagnosis of acute infection, two of ten sera from healthy subjects and four serum samples from patients with lupus erythematous (SLE). When neutralizing sera were tested on cell cultures in immunofluorescence assays for the serum ability to label specific protein(s), similar fluorescent pictures resulted in treated and control cells. On the other hand, the SLE serum samples disclosed a granulosity of the nuclear material of cytotoxic cells in accordance with the DNA apoptotic laddering reported in previous papers. Oxidative disorders, as suggested by the immunoblotting analysis of the antioxidant enzymes Mn-superoxide dismutase (SOD2) and heme-oxygenase 1 (HO-1), point to an alteration of the oxidative pathway among the causes of the DNA damage induced by the cytotoxic transmissible agent under study.

KEY WORDS: Cytotoxic transmissible agent, Misfolded proteins, Human serum neutralizing activity, Antioxidant enzymes, Mn-superoxide dismutase, Heme-oxygenase 1

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INTRODUCTION

Of the numerous samples of cerebrospinal fluid (CSF) sent to the Laboratory for Diagnosis of Viral Diseases of the University of Modena and Reggio Emilia for a search for cytopathic viruses, seven specimens drawn from patients with various neurological disorders showed they were carriers of an unconventional unknown cytotoxic agent serially transferable to fresh cell cultures of VERO cells (Portolani *et al.,* 2005; Beretti *et al.,* 2006). In addition to VERO cells, when glial cells from the human astrocytoma cell line D54-MG (Portolani *et al.,* 2005) and human primary lymphocytes (Portolani *et al.,* 2008) were tested with crude preparations obtained from VERO cytotoxic cell cultures they showed susceptibility to the cytotoxic factor. Human primary fibroblasts (HFs) were considered unable to propagate the cytotoxic activity as no morphological change was observed on cell monolayers at the time of cerebrospinal fluid inoculation. Later, when the challenge of human fibroblast cell cultures with cytotoxic preparations obtained from cytotoxic cell cultures of VERO was repeated, the appearance of a moderate acidity of the cell culture medium suggested testing this material on VERO,

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D54-MG and human blood cells. The rescue of transmissible cytotoxicity in all the cases showed that the propagation of the cytotoxic agent also occurs in human fibroblasts (unpublished data). The wrong initial conclusion may be explained because morphological alterations appear on the human fibroblast monolayers rather late compared to what occurs on VERO cell cultures.
Results of chemical-physical studies (Portolani *et al.* 2005) implemented to characterize the agent responsible for the transmissible cytotoxicity suggested a possible association with one or two proteins of molecular weight about 27-39 kDa. The hypothesis was considered that these proteins, showing resistance to proteolysis, tendency to aggregation and ability to induce apoptosis, are misfolded proteins formed in the brain tissues of the hosts studied, in response to agents remained unknown.
Antioligomeric antibodies are able to recognize oligomeric species common to amyloidogenic polypeptides as Abeta42, prions, polyglobuline, etc. (Kayed *et al.,* 2003; Shorter *et al.,* 2004).
In order to find a support for the proteomic origin of the propagable cytotoxicity under study, experiments were performed according to the following protocol. Ten micrograms of a commercial rabbit antioligomer antybody suitable for use in neutralizing assays (Invitrogen rabbit, polyclonal, anti-oligomer antibody A11) in 0.1 mL of PBS solution was incubated for 1 hour at room temperature with 0.1 mL of a cytotoxic preparation obtained from cell cultures "infected" with the cytotoxic factor, then the mixture was inoculated on D54-MG cell cultures. Control mixtures formed by cytotoxic preparations challenged with both 10 µg of rabbit antibodies to mouse IgGs (Santa Cruz Biotechnoloy) and PBS solution were similarly prepared and then inoculated into cell cultures. The unforeseen result of these experiments was the absence of cytotoxicity in the cell cultures inoculated with the mixtures containing rabbit IgGs apart from their specificity (unpublished data). Taking a cue from this result, the existence of a similar neutralizing activity by human IgGs was investigated. To this aim, a series of human serum specimens used as source of human IgGs were subjected to neutralizing assays. Subsequently, the reactivity of identified neutralizing serum samples to cell specific proteins was tested on mock and cytotoxic cell in immunofluorescence tests. The defensive response to possible oxidative damage was also investigated in cytotoxic cell cultures by studying several antioxidant enzymes.

MATERIALS AND METHODS

Cells

D54-MG glial cells and human fibroblasts (HFs) were cultured in 5% CO_2 atmosphere at 37°C. The media employed for cell growth, supplemented with 10% heath inactivated foetal bovine serum (FBS) were: DMEM F12 medium for D54-MG cells and MEM Eagle medium for HFs. At the cell monolayer confluence, the growth medium was replaced with maintenance medium containing 5% FBS.

Cytotoxicic and mock preparations employed in this study

The cytotoxic agent originally present in the cerebrospinal fluid sample collected from a patient with brain ischemic disorders was propagated on both D54-MG and HF cell cultures using cytotoxic and mock initial preparations obtained from VERO cell cultures as reported in Portolani *et al.*, 2005. Both types of inocula were incubated on D54-MG and HF cell monolayers for 1 hour at 37°C, then each type of cell culture was supplemented with a suitable maintenance medium. Microscopic observation disclosed that the cytotoxic inoculum had induced alterations exceeding about 75% of the cell monolayer, both the damaged and the control cell cultures were frozen and thawed three times. After removal of cell debris by a 1500 rpm centrifugation for 10 minutes, cytotoxic and mock supernatants were aliquoted and maintained at -20°C until use.

Human serum and CSF samples

Human serum samples were from: ten blood donors, twenty patients with a diagnosis of suspected bacterial or viral infection and four patients with lupus erythematous (SLE). The cerebrospinal fluid of twelve multiple sclerosis (MS) patients was also examined.

Neutralyzing assays

A 0.5 mL volume of cytotoxic cell preparations obtained from D54-MG cell cultures were incu-

bated both with 0.5 mL of a 1:5 dilution of each serum sample and with 0.5 mL of PBS. After 1 hour incubation at room temperature, two cell tubes of D54-MG cells were inoculated with 0.2 mL of each type of mixture. After 1 hour absorption at 37°C, maintenance medium was added to each cell tube. The cell cultures were subjected to microscopic observation until the appearance of the maximum cytotoxicity in the cell tubes inoculated with the mixture formed by cytotoxic preparation and PBS. The neutralizing activity of a serum sample was suggested by the concomitant absence of cell monolayer changes, and confirmed by the absence of cytotoxic activity of cell extracts prepared from these undamaged cell cultures. The same protocol was followed when CSF samples were examined.

Immunofluorescence assays

D54-MG and HF cells grown on glass coverslips were treated with cytotoxic and mock inocula, as previously reported. Three and six days respectively after treatment, the cells were rinsed once with PBS, fixed with methanol-acetone 3:1 for 30 minutes, then rinsed again with PBS. The fixed cells were incubated with 1:5 dilution of each serum sample for 30 minutes at 37°C, rinsed with PBS, then incubated with 1:50 dilution of FITC-conjugated rabbit antihuman IgG (Sigma) for 30 minutes at 37°C. After PBS washings, the coverslips were stained with Evans blue for 5 minutes.

Western blotting assays

D54-MG cell cultures inoculated with cytotoxic and mock cell preparations were lysed in 10 mM Tris base pH 7.4, 0.5% sodium deoxycholate, 0.5% NP-40 at appropriated times post inoculation. After sonication and centrifugation at 10.000xg for 10 minutes, the content of proteins extracted from the cell lysates was quantified with Bradford method (Bradford, 1976). Eight micrograms of proteins, arising both from mock and cytotoxic cell cultures, were separated by 12% acrylamide SDS-PAGE and transferred onto nitrocellulose membrane. Following blocking with milk solution (5% milk powder in 10 mM Tris base pH 7.4, 0.1% Tween 20), the membranes were incubated for 1 hour at room temperature with each of the selected antibodies (Santa Cruz Biotechnology) diluted in the blocking solution: Cu/Zn-superoxide dismutase 1 (SOD1) (Santa Cruz Biotechnology) 1:500, Mn-superoxide dismutase 2 (SOD2) (Calbiochem) 1:3000, and heme-oxygenase 1 (HO-1) (Santa Cruz Biotechnology) 1:500. After washings with blocking solution, immunoreactive bands were visualized after 1 hour incubation at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) 1:5000 and enhanced stimulation chemiluminescence. Band intensities, expressed in arbitrary units (AU), were quantified by densitometric scanning using the Image I Data Analyzer software.

RESULTS

On the basis of initial experiments showing a neutralizing property of the cytotoxic factor under study by commercial preparations of rabbit IgGs, the search for a similar activity by human IgGs contained in human serum samples was planned. Results of neutralizing assays to which the serum

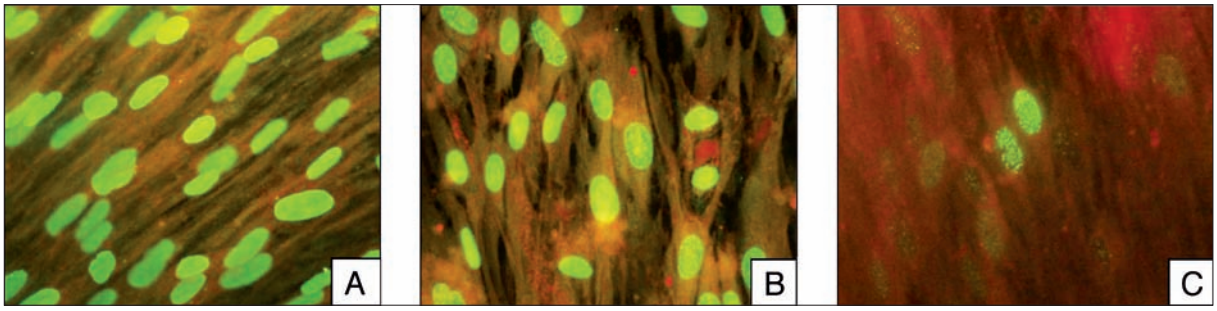

Figure 1 - *Immunofluorescence tests (40x magnification). Lupus erythematous (SLE) serum sample tested on control (A) and cytotoxic human fibroblast (HF) cell cultures (B). Cytotoxic HF cell cultures tested with another SLE serum sample (C).*

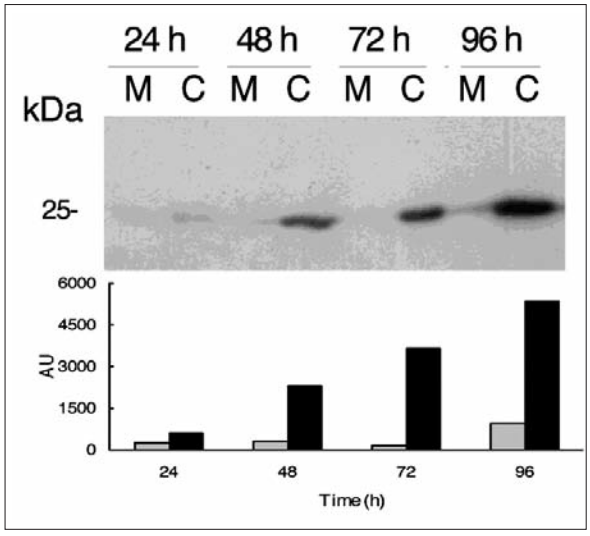

Figure 2 - *Analysis of SOD2 expression. Western blot analysis of SOD2 in mock (M) and cytotoxic (C) D54-MG cells, harvested at 24, 48, 72 and 96 hours after inoculation. (AU = Arbitrary Units).*

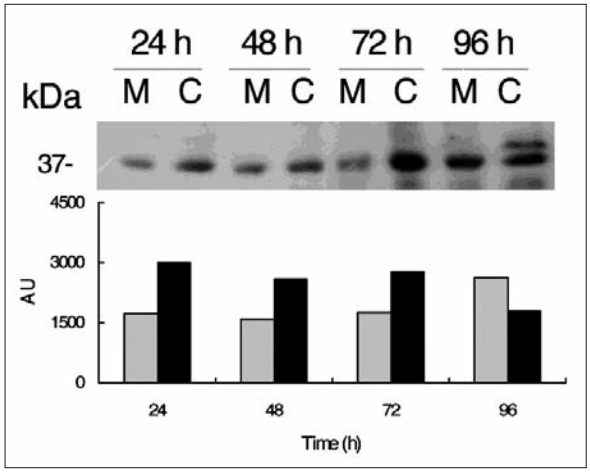

Figure 3 - *Analysis of HO-1 expression. Western blot analysis of HO-1 in mock (M) and cytotoxic (C) D54-MG cells harvested at 24, 48, 72 and 96 hours after inoculation. (AU = Arbitrary Units).*

specimens were subjected varied according to the origin of the samples examined. The activity of cytotoxic inocula was neutralized by: two of the ten serum samples from blood donors, all twenty samples from patients with suspected bacterial or viral infection and the four specimens collected from patients with lupus erythematous. When tested in immunofluorescence assays, the neutralizing serum specimens from patients with lupus reacted, as expected, with the nuclear proteins of D54-MG glial cells and of human fibroblasts inoculated both with mock and cytotoxic cell preparations (Figure 1). All the other neutralizing samples reacted giving origin to aspecific cell pictures (data not shown).

The production of the antioxidant enzymes SOD1, SOD2 and HO-I was investigated by Western blotting analysis. Results concerning SOD2 and HO-1 are illustrated in figure 2 and figure 3, respectively. Both the enzymes increased in cytotoxic cell cultures beginning 24 hours after the cytotoxic treatment. In particular, the SOD2 level increased progressively and clearly throughout the experiment (96 hours), whereas the HO-1 content increased as a whole to a lesser extent and even at 96 hours of cytotoxic treatment, it dropped below the enzymatic level of the control cells. No increase in SOD1 was observed (data not shown).

DISCUSSION

The influence of human IgGs on an unusual cytotoxic agent detected in the CSF of a patient with brain ischemic disorders was investigated. The study was suggested by the observation (unpublished) that commercial preparations of rabbit IgGs were able to block the propagation of this agent.

Results of neutralizing assays performed with human serum samples used as IgGs source showed that among the collected specimens some were able to protect cell cultures from the action of cytotoxic preparations obtained from extracts of cells infected by the agent under study. In particular, the neutralizing property was detected in all twenty sera of patients suspected to have bacterial or viral infection and in the four sera of patients suffering for lupus erythematous, whereas it was displayed by only two of the ten serum specimens collected from blood donors. Cerebrospinal fluid samples drawn from patients with multiple sclerosis lacked this property.

The neutralizing sera processed by immunofluorescence assays to find a correlation between protective activity and reactivity to components of the cell substratum gave the following results. The two serum specimens from patients included into the group of blood donors, together with

all the specimens collected from subjects investigated for bacterial or viral infection, produced various immunofluorescent pictures, each of which without intrinsic differences between mock and cytotoxic cells. As expected, serum specimens from patients with lupus reacted both with nuclear proteins of control and cytotoxic cells. However, in contrast with the uniform colouring of the nucleus of mock cells, an evident granulosity of the nuclear material appeared in cytotoxic cells (Figure 1). The latter immunofluorescent picture, which reflects the DNA apoptotic laddering reported in previous papers (Portolani *et al.,* 2005; Beretti *et al.,* 2006) stimulated a study on molecular alterations which might have contributed to the appearance of this event. Among the causes of programmed death, an oxidative derangement was investigated by Western blotting analysis of several antioxidant enzymes. Of the three antioxidant enzymes investigated, namely SOD1, SOD2 and HO-1, the production both of SOD2 and HO-1 increased beginning 24 hours after the treatment of the cell cultures with cytotoxic preparation. In particular, SOD2 (Figure 2) increased clearly and progressively throughout the experiment (96 hours) whereas the increase in HO-1 (Figure 3), compared with that of SOD2, was less substantial, and even at 96 hours of cytotoxic treatment the enzymatic level was below that of the control cells. This finding may be due to the digestion of HO-1 by proteases activated following of the metabolic processes resulting in cell death (Boya and Kroemer, 2006). A possible reason for the absence of an analogous degradation of SOD2 may be linked to the resistance of the enzyme to proteinase K, as shown by our unpublished experiments. Taken together, the increases in the antioxidant enzymes SOD2 and HO-1 suggest oxidative stress is one of the causes of the DNA apoptotic damage associated with the propagation of the cytotoxic agent under study (Hassan, 1988; Choi and Alam, 1998).

From the results of the immunofluorescence assay to which the neutralizing serum samples were subjected, it appears that the neutralizing antibody component of the sera examined is not able to point to the supposed protein(s) associated with the cytotoxic agent because of the similarity of mock and cytotoxic fluorescent pictures and their variability. This disappointing result could be due to a neutralizing antibody titre of the sera (not higher of 1 to 5 dilution) at the sensitivity limit of the immunofluorescence assay. Another reason could lie in the fact that the factor neutralizing the cytotoxicity is not an antibody which makes the immunofluorescence test, and any other immune assay unsuited to the detection of its target. The neutralizing activity was considered to have an antibody nature because of the personal observation that commercial preparations of rabbit IgGs disclose this property. However, the presence in these materials of other serum components not removed during the antibody purification process cannot be ruled out. The fact that the neutralizing activity is common to all the serum samples drawn from patients in whom the response to an infectious agent was underway at the time of serum collection accords with a possible association of the neutralizing activity with some serum factor formed during the host's response to an antigen. This may also apply to the neutralizing sera of the patients with lupus erythematous, a disease based on immune mechanisms. In addition, the absence of a neutralizing activity by the antibody component of the cerebrospinal samples from patients with multiple sclerosis is in line with this supposition.

In conclusion, the aim of the present research was to obtain additional information on the characteristics of an infectious cytotoxic agent isolated in our laboratory from the cerebrospinal fluid of several neurological patients. The results show that the transmissibility of this agent may be blocked by human sera drawn, in particular, from patients suspected to have an acute infection, and patients with lupus. The initial assumption of a possible association of the serum blocking effect with an antigen-antibody reaction appears to be unlikely since immunofluorescence tests failed to reveal a specific localization of the cytotoxic agent within the treated cells compared to controls. Immunofluorescence tests performed with sera from patients with lupus erythematous revealed nuclear fragmentation in cytotoxic cells. The mechanism of DNA damage most probably involves oxidative disorders, as shown by the increase in oxidative enzymes. Notwithstanding the possible non-antibody nature of the neutralizing activity, it cannot be excluded that human neutralizing sera may play a role in the identification of the cytotoxic agent under study.

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