MICROBIOLOGY AND IMMUNOLOGY

Infectivity of 18-20 kD Proteins Isolated from the Brain of Victims of Amyotrophic Leukospongiosis

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UDC 578.89

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 116, № 10, pp. 409-412, October, 1993
Original article submitted December 22, 1992

Key Words: *amyotrophic leukospongiosis; prion; low-molecular protease-resistant protein*

Amyotrophic leukospongiosis (AL) is a slow neurodegenerative disease of humans; its agent is referred, along with the agents inducing kuru, Creutzfeld-Jakob disease, Gerstmann-Straussler syndrome, and scrapie in animals, to the nonclassical (unconventional) viruses, or prions [2,4]. Opinions concerning their nature vary. Some scientists, who detected a correlation between infectivity and the presence of a protease-resistant protein PrP 27-30, consider it to be an infectious agent (the "prion" hypothesis) [15]. This uncommon protein, when purified, can aggregate and form specific rod-shaped scrapie-associated filaments [9,13,14]. We previously characterized filamentous structures in brain homogenate of AL patients and demonstrated their infectivity [6]. Still it is not quite clear what is the minimal infective unit of AL agent. The present research was undertaken to further disclose the structure of the AL agent.

MATERIALS AND METHODS

Brain tissue (cerebral cortex) from two patients who had died of amyotrophic leukospongiosis was the source of infectious material. The agent was isolated and concentrated by a previously described method [10] modified by us. Brain specimens (5 g) were thoroughly homogenized and a 10% suspension was prepared in phosphate saline buffer (PSB), pH 7.4, containing 10% sodium lauryl sarcosinate (Sigma). The resultant homogenate was twice centrifuged: at 22,000 g for 30 min at 4°C and at 215,000 g for 2 h at 4°C. The sediment was diluted with 1% lauryl sarcosyl and 10% NaCl solution prepared in PSB, pH 7.4, and then centrifuged again at 215,000 g for 2.5 h at 4°C. The sediment was then dissolved in the same solution and enzymatic digestion with proteinase K (Sigma), 20 μg/ml, was carried out for 2 or 24 h. The resultant material was placed in Eppendorfs and centrifuged at 6000 g for 15 min at 4°C. The supernatant was collected after the last centrifugation and denoted as fraction S (1). Later it was dialyzed against PSB, pH 7.4, for 24 h at room temperature and exposed to ultracentrifugation at 215,000 g for 2.5 h at 4°C. The resultant sediment fraction S (2), was diluted with PSB, pH 7.4 and stored at -20°C.

Protein electrophoresis was carried out in 12.5% PAGE according to a previously described method [12]. Before the material was imbedded in gel it was treated with RNAse A (Sigma) (10 μg/ml for 30 min at 20°C). Phoresis was carried out for 16-18 h at current strength 10 A/gel. The protein

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0007-4888/93/0010-1271$12.50

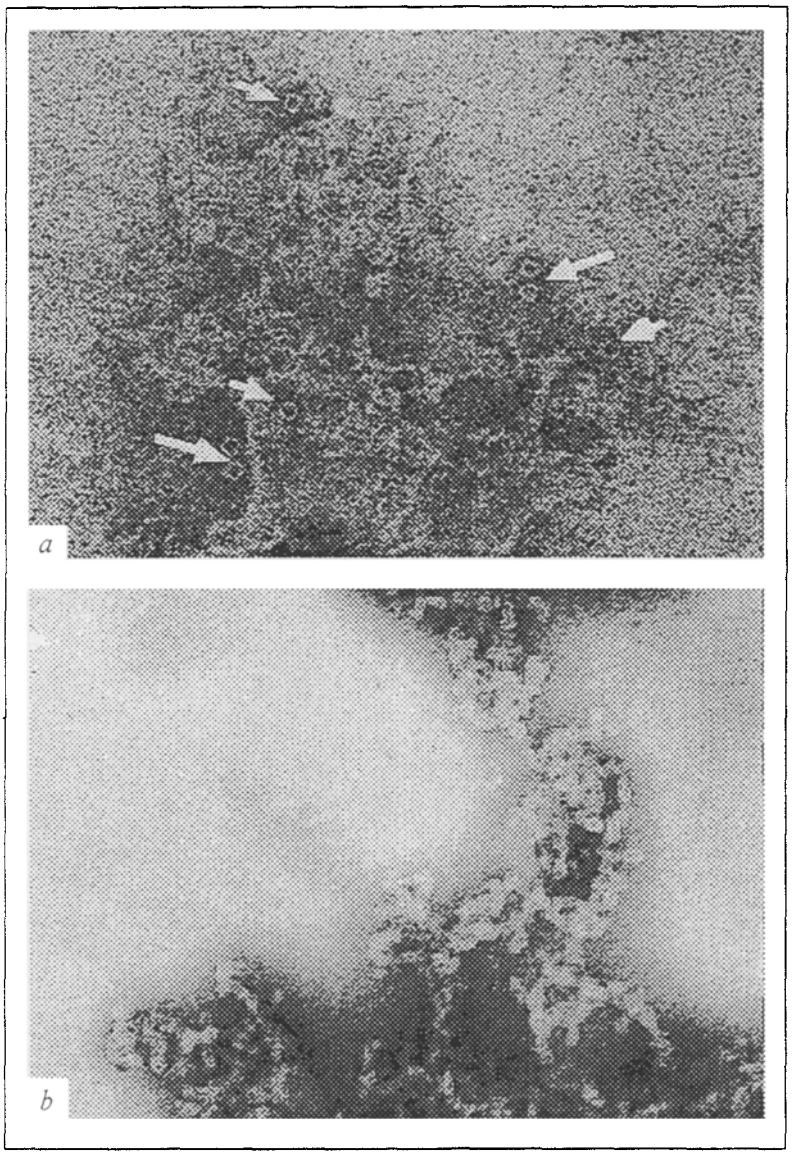

Fig. 1. Morphology of spherical globular structures isolated from the brain of patients who had died of AL. *a*) solitary (short arrow) and pairs of (long arrow) globular structures isolated from supernatant, fraction S (2). ×200,000. *b*) accumulation of globular structures isolated from 18–20 kD protein strip after electrophoresis in 12.5% PAGE. ×140.000. Negative contrast staining with 1% uranyl acetate.

was eluted from the gel with 30% hydrogen peroxide during 16 h at 60°C, and the suspension was diluted with PSB, pH 7.4, in the ratio 1:20, and stored at -20°C.

Electron microscopic analysis was carried out on grids covered with Formvar-carbon backing for negative contrast staining with 1% uranyl acetate or 2% phosphotungstic acid (pH 6.7). The preparations were examined under a JEM-100 CX-11 electron microscope (Japan).

Infectivity was assessed in primary dissociated cultures of human embryo brain and in animals. To 12-14-day cultures routinely prepared [1] 0.2 ml of 10-fold dilutions of the tested material (from 10^{-1} to 10^{-5}) per ml of culture medium was added. Four cultures per dilution were used. The presence of agent was indicated by the appearance of characteristic cytopathic changes (CPC) in the cultures [7] and the loss of adhesive properties by some cells. If necessary, the infected cultures were passaged two or three times until specific signs of CPC appeared.

Eight outbred guinea pigs weighing 300 to 400 g were used in animal experiments. The tested infectious material was heated in a water bath at 100°C for 15 min and administered twice retrobulbarly in a dose of 0.25 ml into both eyes. Group 1 (2 animals) consisted of controls administered a placebo (culture fluid from intact guinea pig brain culture), group 2 (4 animals) was the agent control group infected with a nonclassical virus, strain AL-D (GKV-N2206 deponent) obtained from guinea pig brain culture, and group 3 (2 animals) was the experimental group, infected with 18-20 kD protease-resistant protein of patient D isolated from gel (see above). The animals were followed up for 5.5 to 6 months from the time of infection to death.

RESULTS

Homogeneous globular formations 10-12 nm in diameter were found on negatively stained preparations in fractions S (1) and S (2) of both patients' brain specimens (Fig. 1, *a*). No rods or scrapie-associated filamentlike structures were detected in the preparations. The globules were situated alone or in pairs in each cell of the examined grids. The number of globules in different preparations was virtually the same and did not depend on the duration of treatment with proteinase K.

Electrophoresis in 12.5% PAGE revealed in these fractions the only unique protein with a molecular weight of approximately 18-20 kD. Negligible amounts of this protein still remained on the apex of the 5% focusing gel. Negative contrast staining of the material eluted from protein strips with molecular weight 18-20 kD revealed only globular structures (Fig. 1, *b*) which were morphologically similar but not identical to those found in fractions S (1) and S (2) before electrophoresis. Numerous globules were seen in each grid cell and formed huge conglomerates of a spongy sub-

stance. No other aggregations of proteins and/or filamentous structures specific for nonclassical viruses were seen.

Further studies were aimed at assessment of the infectivity of the resultant globular structures in the brain cell culture and in animals.

The addition of 10-fold dilutions of fraction S (2) and 18-20 kD protein purified by electrophoresis into primary dissociated human embryo brain culture resulted first in culture survival without manifest morphofunctional changes. But as early as days 6-12 postinfection small foci of proliferating and solitary degenerating cells (up to 5%) were detected in the culture, this being characteristic of CPC development in a persistent infection caused by the AL agent [4,7]. In addition, the accumulation of round but live cells which had lost their adhesive properties and capacity to adhere to a substrate was observed in the growth medium in this period. The counts of these cells gradually increased, attaining the maximum on days 10-12 postinfection: 3-5% of all monolayer cells. After 2-3 passages of infected cultures the number of morphologically altered cells increased to 7-10% in the culture, whereas the count of cells which had lost their adhesive properties was virtually constant. The presence of specific CPC in the cultures together with the phenomenon of loss of adhesive properties by infected cells suggests that infectivity of the examined samples under the conditions of our experiment varied from 103 to 104 log ID_{50}/ml, the agent titer in fraction S (2) being apparently higher than the infectivity of 18-20 kD protein by approximately 0.5-1.0 log.

Infection of animals with an original AL strain, AL-D (group 2), and with 18-20 kD protease-resistant protein of fraction S (2) (group 3) resulted in the simultaneous development in both groups 3.5-4 months later of a disease with the clinical picture of AL (fur loss, atrophy of the fore- and hind limbs). All animals died 5.5-6 months after the infection.

Hence, we isolated for the first time specific globular structures characterized by a high resistance to various physicochemical factors and by infectivity from the brain of victims of AL. These globules apparently contain infectious low-molecular 18-20 kD protease-resistant protein. Our findings are somewhat contradictory to generally acknowledged facts. Some workers investigating Creutzfeld-Jakob disease and scrapie reported the presence of 2 to 7 protease-resistant proteins cross-reacting with antiserum to PrP 27-30, including up to 4 proteins with a low molecular weight different from that of PrP 27-30, in human and animal brain homogenates [11]; but these proteins were detected only parallel with the major prion protein PrP 27-30 and were undetectable separately (without it). The infectivity of these proteins is not proven. Our findings allow us to assume that the infectivity of AL agent is related not only to straight filament-shaped structures and/or scrapie associated filamentlike structures, whose main component is protein PrP with a molecular weight of 27-30 kD [6,8], but also to globular structures 7-8 nm in diameter, which apparently contain the major 18-20 kD protein. Further research will show whether we succeeded in destroying protein PrP 27-32 or whether AL is an individual nosologic entity in the group of subacute spongioform encephalopathies. The latter hypothesis seems the more probable, for there are marked differences in the pathogenesis of amyotrophic leukospongiosis and Creutzfeld-Jakob disease. Pathological shifts in AL are manifested mainly in the death of spinal anterior horns motoneurons with marked spongiosis of the anterior and lateral stems of the white matter [3,4], whereas in Creutzfeld-Jakob disease neuronal loss with status spongiosis development is observed mainly in the cortical gray matter [15]. Hence, the available data are indicative of a unique nature of the agent of AL in comparison with other members of the "unconventional viruses" family and disclose new aspects in the pathogenesis of AL.

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