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Blood–brain barrier protein recognized by monoclonal antibody

(immunocytochemistry/peroxidase–antiperoxidase/Langerhans cells/experimental allergic encephalomyelitis/retina)

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ABSTRACT **An IgG1 mouse monoclonal antibody produced in response to immunization with rat brain homogenate reacted with endothelial cells in the central and peripheral nervous system. Because antibody reactivity was associated with endothelia that have a selective permeability barrier, the antibody was called anti-endothelial-barrier antigen (anti-EBA). Paraffin sections of Bouins'-fixed rat tissue were used for initial screening and subsequent characterization of antibody reactivity. The antibody was generally unreactive with endothelial cells in other organs and with nonendothelial cells in or outside of the nervous system. Antibody binding was greatly reduced or absent in endothelia of the area postrema and choroid plexus, sites known to possess fenestrated blood vessels. In developing rat brain, anti-EBA binding to some microvessels was seen at 3 days postnatally. Anti-EBA reactivity outside the nervous system occurred in spleen and skin. Patchy reaction with portions of some spleen blood vessels and binding to some cells in the spleen were observed. In the skin, small cells, tentatively identified as Langerhans cells, which participate in Ia presentation, were stained. On immunoblots of rat brain microvessel preparations electrophoresed in Na-DodSO$_4$/polyacrylamide gels, anti-EBA reacted with a protein triplet of M_r 30,000, 25,000, and 23,500 components.**

Among a number of monoclonal antibodies with selective specificity for individual components of the central nervous system that we have developed (1), one is an endothelial cell antibody (anti-endothelial-barrier antigen) (anti-EBA) that reacts with central nervous system and peripheral nervous system endothelia. Brain or nerve microvessel endothelial cells are the site of the blood–brain or blood–nerve barrier important in determining the selective permeability characteristic of the nervous system (2). Little is known about the proteins associated with endothelia and endothelial-barrier properties. Monoclonal antibodies can serve as useful probes for elucidating the biochemical basis of the endothelial barrier.

A variety of interesting monoclonal antibodies (3–10) and an antiserum (11) have been reported that reacted with brain microvessels. Some of these antibodies reacted broadly with endothelia from other, non-nervous system organs (5–10). An antiserum, prepared against isolated bovine brain microvessels, reacted with a M_r 46,000 protein found in brain but not in heart, liver, and kidney capillaries (11). Rat-brain microvessels were used as the immunogen to produce a monoclonal antibody that bound to brain endothelia, the brush border of proximal kidney tubules, and bile canaliculi (3). A M_r 74,000 protein has been defined by a monoclonal antibody that is expressed on the surface of chick embryonic blood cells, brain endothelium, choroid plexus epithelium, and basolateral membranes of kidney tubules (4).

In the present study, we describe a monoclonal antibody directed against a triplet of endothelial proteins. This antibody is a useful probe for studying macromolecules related to blood–brain barrier function.

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MATERIALS AND METHODS

Monoclonal Antibody Production. Monoclonal antibodies were produced from BALB/c mice immunized with rat brain homogenates as described (12) by using the procedure of Kohler and Milstein (13). Immunocytochemistry on paraffin sections was used to select hybridomas producing antibodies to endothelia.

Immunocytochemistry. Lewis or Sprague–Dawley rats were perfused with Bouins' fixative. Organs were postfixed in the same fixative overnight, dehydrated, and embedded in paraffin. Seven-micrometer-thick sections of brain, peripheral nerves, optic nerve, retina, heart, lung, liver, intestines, thymus, lymph nodes, spleen, adrenal, skeletal muscle, skin, pancreas, pituitary, and pineal body were treated with the antibody or reagent controls. Unfixed, frozen brains, Vibratome sections of Bouins'-fixed brains, and paraffin-embedded brains, fixed in either buffered 4% paraformaldehyde, or buffered 2% paraformaldehyde with 2% glutaraldehyde, were also examined for ability to bind the antibody and for pattern of reactivity.

The sections were treated in the following sequence: (*i*) 3% normal sheep serum for 30 min; (*ii*) anti-EBA ascites fluid diluted 1:1000 to 1:500,000 for 1 hr to overnight; (*iii*) goat anti-mouse immunoglobulin, diluted 1:40 for 30 min; (*iv*) peroxidase–antiperoxidase complex (14, 15) prepared from monoclonal antiperoxidase, diluted 1:100 to 1:800 for 30 min; and (*v*) 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide for 8 min. Wash solutions were 0.05 M Tris, pH 7.6, in 1.5% sodium chloride (1.5 T). Diluents were the same solutions containing, in addition, normal sheep serum. Micrographs were made with Nikon–Nomarski optics in order to visualize background.

Microvessel Preparation. Brain microvessels were prepared from frozen rat brains by the methods described by Mrsulja *et al.* (16) or by Lidinsky and Drewes (17). Two millimolar EGTA, aprotinin (0.5 trypsin inhibitor unit/ml), leupeptin (10 μg/ml), 2 mM phenylmethylsulfonyl fluoride, and 1 mM bacitracin were present during homogenization and in subsequent steps to prevent possible degradation of EBA during the isolation procedure. Protein determination was done by the bicinchoninic acid assay (18).

Endothelial Cell-Membrane Preparation. The microvessels were suspended in distilled water, agitated at 4°C, and centrifuged at 15,000 × *g* for 10 min as described by Lidinsky and Drewes (17) to lyse endothelial cells and separate membranes from cytoplasmic materials.

Polyacrylamide Gel Electrophoresis. One-dimensional discontinuous gels were run in a vertical slab gel apparatus by the method of Laemmli (19) as has been described in detail previously (20). The stacking gel contained 4% acrylamide, and the separating gel contained 8% acrylamide. Samples

Abbreviation: anti-EBA, antibody to endothelial-barrier antigen.

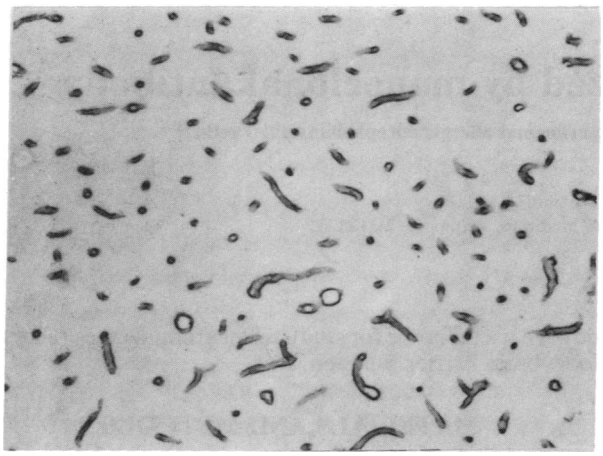

FIG. 1. Sagittal section of adult rat brainstem treated with anti-EBA. Antibody binding was limited to microvessels. (×120.)

were incubated for 3 min at 100°C in buffer containing 63 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol/0.001% pyronin Y and were used immediately. Molecular weight standards were run in adjacent lanes. Electrophoresis was done at constant voltage (150 V) for 4.5–5 hr with cooling. The gels were either stained in 0.125% Coomassie blue R-250 in 50% methanol/10% acetic acid and destained in 5% methanol/7% acetic acid or were used directly in electroblot.

Protein Electroblot Transfer to Nitrocellulose Paper. Separated proteins were transferred to nitrocellulose paper as described by Towbin *et al.* (21). Blots that were to be stained immunocytochemically were incubated in 3% (vol/vol) bovine serum albumin and 1% (vol/vol) normal sheep serum in 1.5 T buffer for 30 min while covered with aluminum foil to avoid exposure to light. The blots were rinsed carefully for 15 min in 1.5 T buffer and cut into strips corresponding to separate lanes on the original gel.

Immunocytochemistry. Strips of nitrocellulose paper were stained immunocytochemically by incubating with anti-EBA ascites fluids, diluted 1:1000, for 1 hr; goat anti-mouse IgG, diluted 1:40, for 30 min; mouse peroxidase–antiperoxidase prepared from monoclonal antibody, diluted 1:200, for 30 min; and 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide for 8 min. Each step was followed by careful rinsing for 15 min with several changes of 1.5 T buffer.

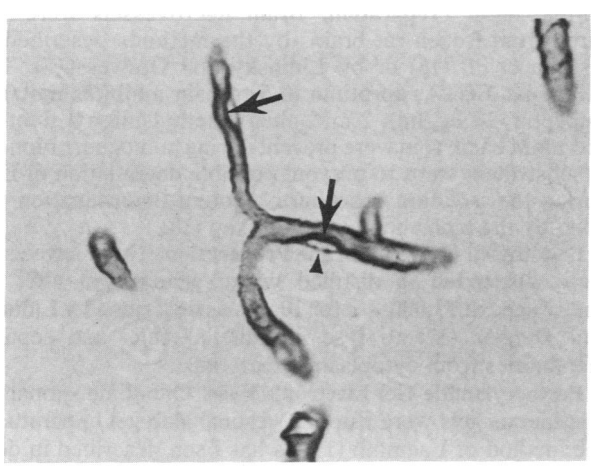

FIG. 2. Brainstem microvessels. Anti-EBA reaction appeared to be predominantly associated with the luminal surface of the endothelial cell (arrows). The occasional faint reaction with other areas of the cell is indicated by the arrowhead. (×480.)

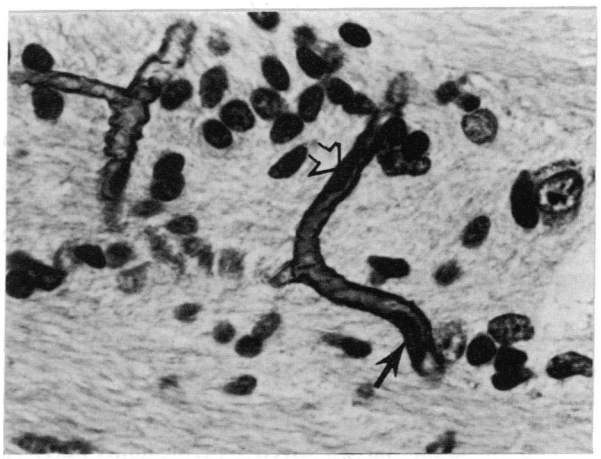

FIG. 3. Section treated first with anti-EBA, then counterstained with hematoxylin/eosin to show association of antibody reactivity with endothelial cell. Arrows indicate endothelial nuclei. (×480.)

All dilutions were made with 1.5× T buffer containing 1% normal sheep serum.

RESULTS

Anti-EBA reacted with microvessels in the adult rat brain (Fig. 1) and with vessels in the meninges that remained attached to the brain (data not shown). Reaction was confined to microvessels. This exclusive pattern of brain reactivity was retained in unfixed frozen, Bouins'-fixed Vibratome sections, and paraffin-embedded sections from brains that had been fixed in buffered 4% paraformaldehyde or fixatives containing 2% glutaraldehyde. At higher magnification antibody binding appeared to be primarily associated with the luminal surface of the endothelial cell (Fig. 2) with only occasional faint reaction seen in other cell areas. Immunocytochemical staining followed by histological counterstaining with hematoxylin/eosin (Fig. 3) confirmed that endothelia and not pericytes or basement membrane were reacting. Microvessel reactivity was still seen at anti-EBA dilutions of 1:300,000 or greater, depending upon the ascites fluid used. Additional sites of antibody binding examined were optic nerve, spinal cord, and retina (Fig. 4). Endothelial

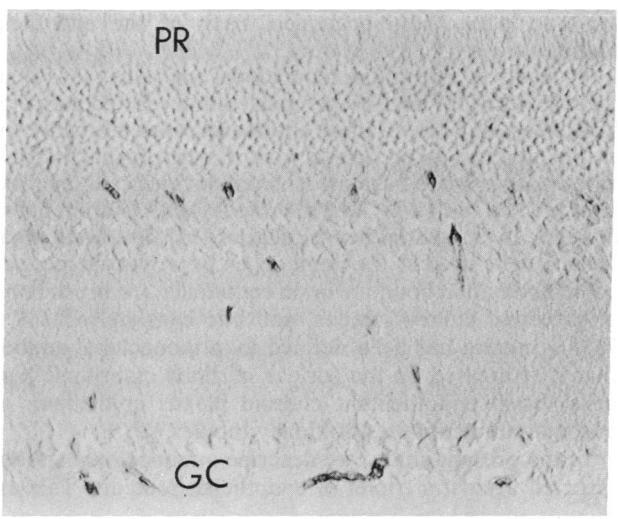

FIG. 4. Reaction of anti-EBA with neural retina. Ganglion cell layer (GC) is at the bottom of the micrograph, and photoreceptor layer (PR) is at the top. (×230.)

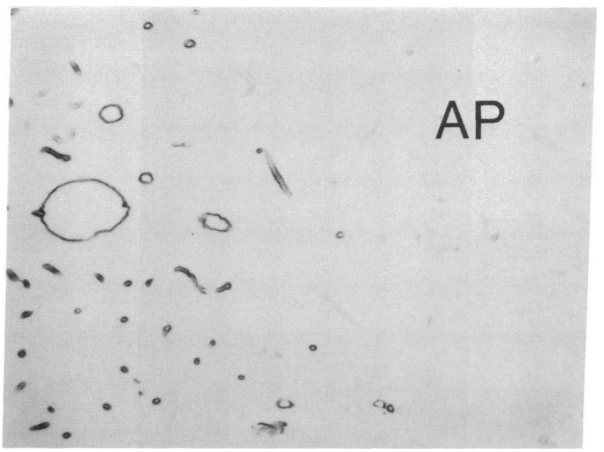

FIG. 5. Fenestrated blood vessels in the area postrema (AP), at the right in the micrograph, reacted poorly, or not at all with anti-EBA. Nonfenestrated vessels in surrounding brain reacted well. Antibody dilution 1:1000. (×120.)

cells in the peripheral nervous system, where a blood–nerve barrier is present, also reacted with anti-EBA.

Antibody binding to sites in the nervous system known to possess fenestrated blood vessels was absent, or detectable only at high concentrations of antibody. In the area postrema, one of the circumventricular organs, microvessels reacted poorly, or not at all, even at a 1:1000 dilution of anti-EBA (Fig. 5). Some vessels in the choroid plexus (Fig. 6), trigeminal ganglion, and pineal body were positive, but this reaction was quickly abolished by dilution of the ascites fluid to 1:10,000.

In the developing rat brain, sagittal sections from 0-, 3-, 6-, 11-, 15-, 20-, and 28-day rats were examined for appearance of EBA. Antibody reaction with some microvessels was seen at 3 days (Fig. 7), and the number of positive vessels increased rapidly with development. By 20 days, essentially all recognizable blood vessels reacted with anti-EBA. There was no rostral/caudal gradient of first-appearing reactivity.

Peripheral, non-nervous system tissues were examined also. Blood vessels and sinusoids in the liver (Fig. 8) and vessels in the heart, adrenal, skeletal muscle, intestine, thymus, lymph nodes, pancreas, thyroid, skin, and pituitary were unreactive. In the spleen, a patchy reaction was seen on some vessel walls or associated with cells sometimes seen on

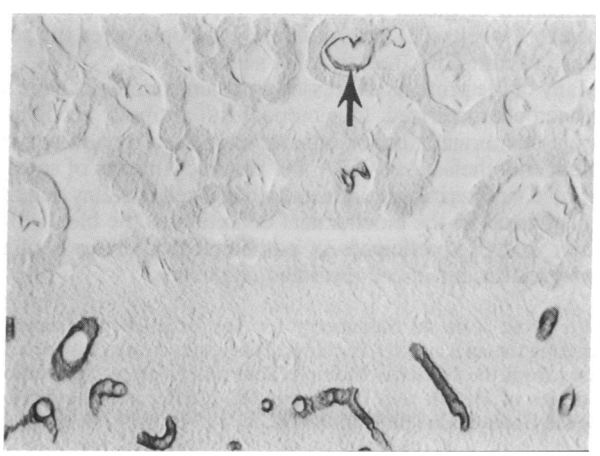

FIG. 6. Some endothelia of the choroid plexus were positive for anti-EBA reactivity at 1:5000 antibody dilution (arrow). Most choroid plexus endothelia were nonreactive at this dilution. Brain microvessels were strongly stained. (×230.)

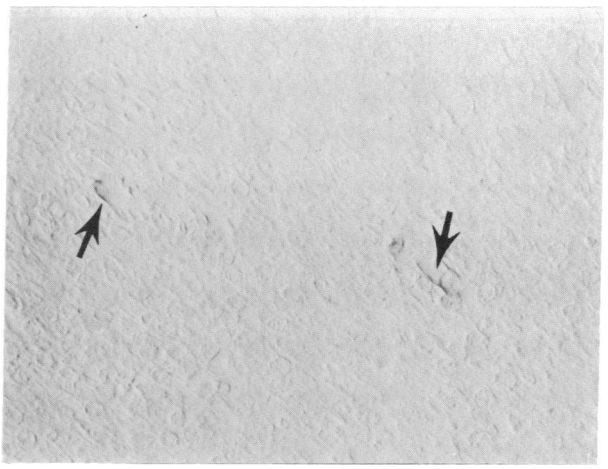

FIG. 7. At 3 days of age, reaction was seen in some microvessels of the developing brain. These vessels are in the cortex. (×230.)

the luminal surface (Fig. 9). In the epidermis of the skin, anti-EBA reacted with small cells with fine processes (Fig. 10). Reaction occurred at the cell surface and within the cytoplasm. These cells have tentatively been identified as Langerhans cells, a special kind of macrophage, which are Ia-presenting cells.

On nitrocellulose blots of electrophoresed preparations of brain microvessel membranes, the antibody identified three components with M_r 30,000, 25,000, and 23,500 (Fig. 11, lanes C, D, and E). The lower M_r band was less easily detected, and higher concentrations of the microvessel preparation were required to visualize this band. Anti-EBA did not react with proteins in the endothelial cytosol fraction (Fig. 10, lanes A and B). No bands were seen on blots of liver homogenates reacted with anti-EBA.

DISCUSSION

Anti-EBA recognized endothelial proteins present predominately in endothelia of the blood–brain and blood–nerve barriers. Although some fenestrated endothelia, such as the capillaries of the choroid plexus, bound the antibody to a limited extent, EBA appeared to be a minor component of these vessels. The function and biochemical nature of EBA are not yet known. It is possible that EBA proteins are components of microvessel tight junctions. Pardridge *et al.* (11) have suggested this function for a M_r 46,000 protein that had a ring-like pattern of endothelial reactivity similar to that

FIG. 8. Liver blood vessels and sinusoids did not react with anti-EBA. (×230.)

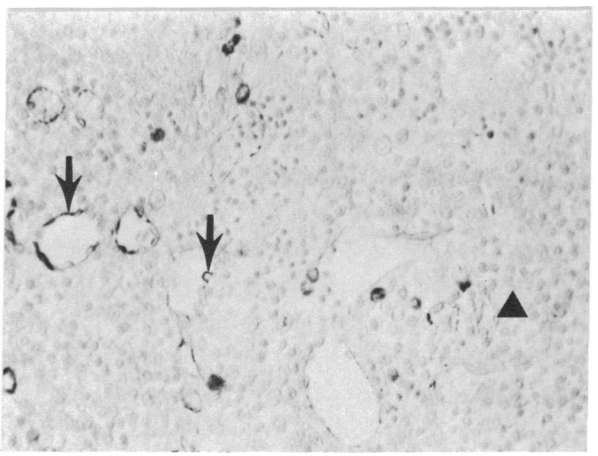

FIG. 9. In the spleen, antibody binding to portions of some blood vessels and some cells was seen (arrows). The diffuse background of non-anti-EBA-related staining (arrowhead), presumably due to Fc receptor binding, was also seen in control sections. (×120.)

seen with anti-EBA. However, this pattern suggests that, at the light microscopic level, reaction was associated with the entire luminal surface, rather than being restricted primarily to the lateral margins of contiguous endothelial cells. Alternatively, EBA may be associated with a receptor complex characteristic of nervous system endothelia.

Anti-EBA was distinct from other monoclonal antibodies that have been identified as reacting with brain endothelia (3–10). The unique patterns of reactivity on tissue sections and electroblots also eliminate its identification with known endothelial proteins such as factor VIII (22), transferrin receptor (23), and other proteins that are characteristic of all endothelial cells and have different molecular weights.

It is intriguing that anti-EBA was expressed on some cell types and tissues associated with the immune system. Endothelial cells are considered to play a major role in the development of cell-mediated immune responses (24). After inducible expression of class II major histocompatibility antigens (Ia), endothelia can act as antigen-presenting cells (25–27). Langerhans cells, in the epidermis of the skin, also function in Ia presentation and play a key role in contact sensitivity reactions in the skin (28). It was not clear from our preliminary observations on spleen sections what immune cell types were recognized by anti-EBA.

The bands reacting with anti-EBA on nitrocellulose blots

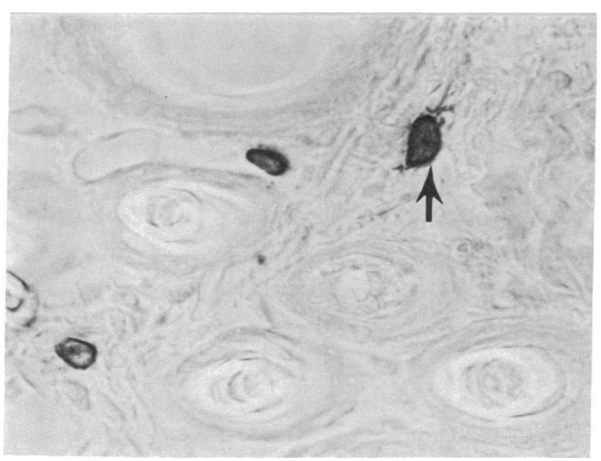

FIG. 10. Small cells with fine processes (arrow) were stained by anti-EBA in sections of rat skin. These cells may be Langerhans cells. Skin blood vessels did not react. (×480.)

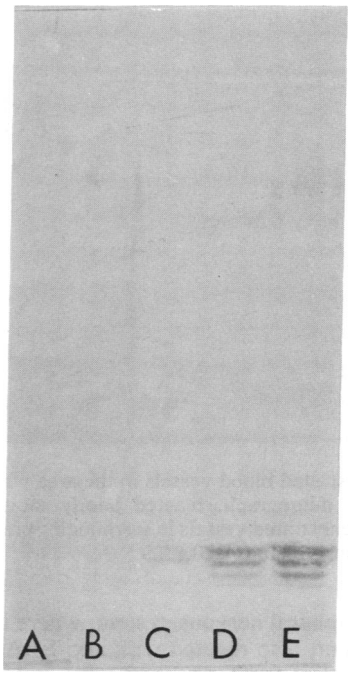

FIG. 11. Electroblot of isolated microvessel cytosol (lanes A and B) and membrane fraction (lanes C, D, and E). Lane A, 30 μg of cytosol fraction; lane B, 100 μg of cytosol fraction. Lanes C, D, and E were loaded with 40, 100, and 200 μg of membrane fraction. All lanes were treated with anti-EBA.

have apparent molecular weights similar to some populations of Ia-like molecules (29). However, the presence of EBA on normal brain endothelia and the absence of EBA on vessels in experimental allergic encephalomyelitis that are surrounded by inflammatory cell infiltrations (30) make it highly unlikely that the antibody is recognizing Ia antigens. Instead, these findings suggest that anti-EBA recognized an endothelial cell modification in inflammatory disease and that *in vivo* modification of EBA may be part of the local immune response.

At birth, the rat has poor vascularization of the brain. Vascularization proceeds by a budding or sprouting from existing vessels (31). There is evidence that a selective barrier for some molecules exists in the newborn rat and that this barrier increases in efficiency with age. The postnatal appearance of EBA may reflect the gradual biochemical development of permeability barriers that accompany the vascularization of brain. A complete vascular bed was established by 3 weeks (31), approximately the time when the adult pattern of anti-EBA reactivity is seen.

Many of the proteins expressed by brain endothelia have not yet been characterized. Our monoclonal antibody recognized plasma membrane components not expressed by non-nervous system endothelia. Anti-EBA will provide a means of separating and characterizing unique endothelial cell proteins that may (*i*) play a role in the biochemical definition of the blood–brain barrier and (*ii*) participate in pathological alterations of the barrier that occur in cell-mediated responses.

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