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Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin

(monoclonal antibody/serum factor)

IAN S. TROWBRIDGE AND M. BISHR OMARY

Department of Cancer Biology, The Salk Institute for Biological Studies, P. O. Box 85800, San Diego, California 92138

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ABSTRACT **A cell surface glycoprotein antigen with an apparent molecular weight of about 100,000 that is selectively expressed on proliferating cells was purified from deoxycholate-solubilized membranes of a cultured human leukemic thymus-derived (T) cell line by affinity chromatography on a monoclonal antibody-Sepharose column. A conventional xenoantiserum prepared by immunization with the affinity-purified glycoprotein was found to contain antibodies against a serum component that bound tightly to cultured cells. This molecule was shown to be specifically associated with the cell surface glycoprotein purified by immunoprecipitation from lysates of cells. We have identified the serum component as transferrin and conclude that the membrane glycoprotein is the cell surface transferrin receptor.**

We previously identified, by means of a monoclonal antibody designated B3/25, a human cell surface antigen that was found on most cultured human hematopoietic tumor cell lines and a variety of nonhematopoietic tumor cell lines but was not readily detectable on the normal tissues tested (1). The antigen was shown to be a glycoprotein with an apparent molecular weight of about 100,000 in reducing conditions on $NaDodSO_4$/polyacrylamide gels. It probably exists as disulfide-bonded dimers in the membrane, because in nonreducing conditions its apparent molecular weight was 200,000. Expression of the glycoprotein appears to be associated with cell proliferation. Although not detected on normal resting lymphocytes, the glycoprotein was found on mitogen-stimulated cells (1). Also, the glycoprotein was lost from the surface of HL-60, an inducible human promyelocytic tumor cell line (2), after *in vitro* exposure to dimethyl sulfoxide and other inducers that cause the cells to undergo functional and morphological maturation and stop growing.

During further studies, the cell surface glycoprotein was purified from a human thymus-derived (T) leukemic cell line by affinity chromatography of detergent-solubilized membranes on a B3/25 monoclonal antibody-Sepharose column. Characterization of a xenoantiserum against the affinity-purified glycoprotein revealed that it unexpectedly contained antibodies against a serum component derived from the culture medium in which the cells were grown. This observation led to the experiments reported here that show that the B3/25 glycoprotein specifically binds the serum component, which was identified as transferrin. The membrane glycoprotein appears, therefore, to be the cell surface transferrin receptor.

MATERIALS AND METHODS

Cells. Human hematopoietic cell lines were maintained in RPMI 1640 medium and murine hematopoietic cells were grown in Dulbecco's modified Eagle's medium. Either 10% horse serum or 5–10% fetal calf serum was used to supplement the culture medium. RPMI 8402, CCRF-CEM, and HPB-ALL are human T leukemic cell lines (3, 4) and BW5147 is a murine T lymphoma cell line (5). Peripheral blood mononuclear cells were isolated from defibrinated human blood by Ficoll/Hypaque gradient centrifugation (6).

Purification of B3/25 Glycoprotein. B3/25 glycoprotein was purified from CCRF-CEM cells grown in 8-liter spinner flasks in RPMI 1640 medium supplemented with 10% horse serum. All purification steps were carried out at 4°C. Cells were harvested, washed with 0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.4), resuspended in 0.13 M NaCl/0.02 M Hepes buffer (pH 7.2), and homogenized by nitrogen cavitation at 800 pounds/inch^2 (5.5 MPa) for 5 min at 4°C in an Artisan cavitation apparatus. Nuclei and unbroken cells were removed by centrifugation at 450 × *g* for 15 min and a crude membrane pellet was obtained by centrifugation at 100,000 × *g* for 60 min. Membranes obtained from 6×10^9 cells were solubilized in 20 ml of 2% sodium deoxycholate/0.01 M Tris·HCl (pH 8.0) containing Trasylol at 100 units/ml by homogenization in a Dounce homogenizer, and insoluble material was removed by centrifugation at 100,000 × *g* for 60 min. The detergent extract was then applied to a 20-ml column of B3/25 monoclonal antibody-Sepharose. The column was washed extensively with 0.5% sodium deoxycholate/0.05% $NaDodSO_4$/0.01 M Tris·HCl (pH 8.0) and then the bound material was eluted with 0.1% sodium deoxycholate/0.05 M diethylamine·HCl (pH 11.5) and immediately neutralized to pH 8.5 by the addition of solid glycine. Approximately 370 μg of protein estimated by the method of Lowry (7) was recovered after the affinity chromatography step.

Immunological Reagents. Monoclonal antibodies against B3/25 glycoprotein, designated B3/25 and T56/14, respectively, were obtained as described previously for B3/25 monoclonal antibody (1). T29/33 monoclonal antibody specific for the human homologue of murine T200 cell surface glycoprotein has been described elsewhere (8). Ascitic fluid containing monoclonal antibodies was obtained from pristane-primed BALB/c mice injected intraperitoneally with $1–2 \times 10^7$ hybridoma cells. Monoclonal antibody-Sepharose containing 5–10 mg of antibody per ml of settled beads was prepared by the cyanogen bromide coupling method (9), using antibodies purified from ascitic fluid by precipitation with 50% saturated ammonium sulfate. The B3/25 monoclonal antibody was further purified by ion-exchange chromatography on DEAE-cellulose. A xenoantiserum against affinity-purified B3/25 glycoprotein was prepared in a New Zealand White rabbit by two intradermal injections, spaced 4 weeks apart, of approximately 70 μg of the affinity-purified glycoprotein emulsified in complete Freund's adjuvant and divided among multiple sites on the back.

Cell Binding Assays. ^{125}I-Labeled horse serum, human serum, and human transferrin (Miles) were prepared by the chloramine-T method (10). Labeled proteins were separated from $Na^{125}I$ by chromatography on Bio-Gel P-10 (Bio-Rad).

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Assuming complete recovery of protein, specific activities of the ^{125}I-labeled proteins were 0.2–1 μCi/μg (1 Ci = 3.7×10^{10} becquerels). For binding assays, cells grown in fetal calf serum were washed three times in serum-free RPMI 1640 medium prewarmed to 37°C. Cells (1×10^7 in 1.0 ml of RPMI 1640 medium) were then incubated with 50 μl of ^{125}I-labeled protein for 60 min at 37°C in a CO_2 incubator. Afterwards, cells were washed three times with 1.0 ml of 0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.2) at 4°C by centrifugation for 5 sec in an Eppendorf microcentrifuge and solubilized in 1.0 ml of 1% Nonidet P-40/0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.2) for 10 min at 4°C. After removal of nuclei by centrifugation for 2 min in a microcentrifuge, 100-μl samples of the cell lysate were incubated with 50 μl of antibody-Sepharose beads for 15 min. The beads were then washed three times with 0.4 ml of 0.5% deoxycholate/0.5% Nonidet P-40/0.05% $NaDodSO_4$ in 0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.2).

For binding assays of ^{125}I-labeled serum to antibody-Sepharose beads pretreated with lysates of cells, 50 μl of beads was incubated for 30 min at 4°C with 100 μl of Nonidet P-40 lysate of 5×10^6 RPMI 8402 cells and then washed as just described. After incubation with 10 μl of ^{125}I-labeled serum for 30 min at 4°C in 200 μl of washing buffer, the beads were again washed three times with the same buffer.

Miscellaneous Procedures. Cell surface iodination, by the glucose-oxidase modification of the lactoperoxidase technique (11), immunoprecipitation procedures using fixed *Staphylococcus aureus* to isolate antibody–antigen complexes, and $NaDodSO_4$/polyacrylamide gel electrophoresis employing the discontinuous buffer system of Laemmli (12) were performed as described (13).

RESULTS

Characterization of the Rabbit Anti-B3/25 Glycoprotein Antiserum. A rabbit antiserum against B3/25 glycoprotein was prepared as described in *Materials and Methods*. The glycoprotein preparation used for immunization contained a major component with an apparent molecular weight of 95,000 corresponding to the B3/25 glycoprotein together with small amounts of lower molecular weight species (Fig. 1). By fluorescence-activated cell analysis, the specificity of the xenoantiserum appeared to be similar to that of the B3/25 monoclonal antibody. While cultured T leukemic cells were strongly stained by the xenoantiserum, peripheral blood mononuclear cells and induced HL-60 cells were negative. Immunoprecipitation studies, however, showed that the xenoantiserum precipitated an 80,000 M_r species from lysates of iodinated T leukemic cells in addition to B3/25 glycoprotein (Fig. 2). In some experiments, small amounts of this lower molecular weight component could also be detected in immunoprecipitates prepared with B3/25 monoclonal antibody (Fig. 2), and for this reason we initially thought that the 80,000 M_r protein was a structural variant of B3/25 glycoprotein that was precipitated more efficiently by the xenoantiserum than by the monoclonal antibody. Subsequent experiments showed this was not the explanation. First, the 80,000 M_r protein could not be metabolically labeled whether short or long labeling periods were used. Second, the xenoantiserum, in contrast to the monoclonal antibody, reacted strongly with many murine lymphoma cells. However, only murine cells grown in horse serum were positive. Because the preparation of B3/25 glycoprotein was derived from human cells cultured in horse serum, these results raised the possibility that the 80,000 M_r molecule may be a horse serum component. The reaction of the rabbit anti-B3/25 antiserum with a horse serum component adsorbed to cells was confirmed by showing

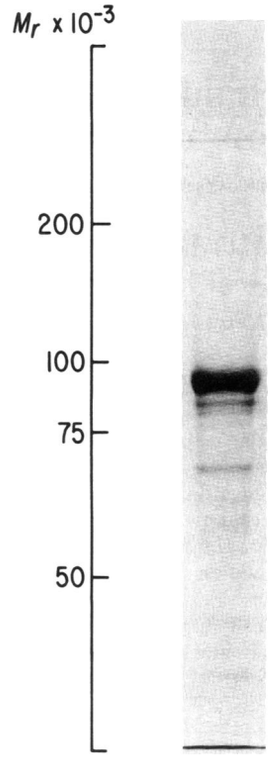

FIG. 1. $NaDodSO_4$/polyacrylamide gel analysis of affinity-purified B3/25 glycoprotein. B3/25 glycoprotein was purified from a deoxycholate extract of CCRF-CEM cells by affinity chromatography on a monoclonal antibody-Sepharose column. A sample of the glycoprotein preparation (15 μg) was run on a $NaDodSO_4$/7.5% polyacrylamide gel in the presence of 2-mercaptoethanol and the gel was stained for protein with Coomassie blue.

that horse serum specifically blocked the binding of the antiserum to murine cells and by direct immunoprecipitation of the 80,000 M_r protein from ^{125}I-labeled horse serum.

B3/25 Glycoprotein is the Cell Surface Receptor for the 80,000 M_r Serum Component. Several observations suggested that further investigation of the relationship between the 80,000 M_r serum component and B3/25 glycoprotein might be worthwile. Foremost was that sometimes the 80,000 M_r protein was found in small amounts in immunoprecipitates prepared with the B3/25 monoclonal antibody, because the specificity of this reagent was assured. We were also impressed by the fact that the serum protein not only was tightly bound to cells but also was apparently retained in sufficient amounts on the monoclonal antibody-Sepharose column to elicit antibodies when the affinity-purified B3/25 glycoprotein was injected into a rabbit. It seemed improbable that these interactions were entirely nonspecific. Although it was possible that B3/25 monoclonal antibody may have a low affinity for the horse serum component, this would not account for the avid binding of the serum component to cultured cells. We were led, therefore, to consider the alternative hypothesis that B3/25 glycoprotein may be the cell surface receptor for the serum factor and that the monoclonal antibody did not directly interact with the serum protein but instead bound receptor–80,000 M_r protein complexes that survived after solubilization of cells in detergent.

To test this idea, RPMI 8402 cells and BW5147 cells were incubated with ^{125}I-labeled horse serum, rapidly washed, and solubilized in detergent, and then fractions of the lysate were assayed for binding of radioactivity to monoclonal antibody-Sepharose beads. After extensive washing, similar amounts of radioactivity were associated with both cell types (Table 1).

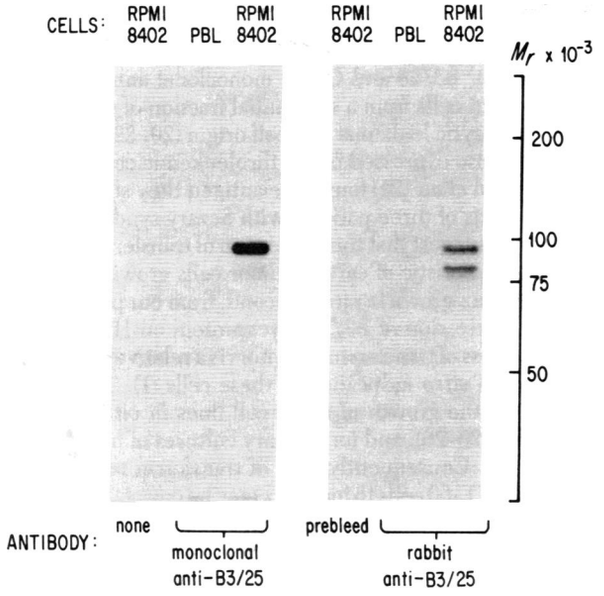

FIG. 2. $NaDodSO_4$/polyacrylamide gel analysis of immunoprecipitates prepared with rabbit anti-B3/25 glycoprotein antiserum and B3/25 monoclonal antibody from lysates of RPMI 8402 cells and peripheral blood mononuclear cells (PBL). Cells were labeled by lactoperoxidase-catalyzed iodination and immunoprecipitates were prepared from lysates of the cells. Serum obtained from the rabbit prior to immunization with B3/25 glycoprotein was used as a control (prebleed). Immunoprecipitates were analyzed on a 7.5% polyacrylamide gel and the autoradiograph was exposed with an intensifying screen for 24 hr.

However, when detergent extracts of the cells were incubated with monoclonal antibody-Sepharose beads, high amounts of radioactivity were bound only to B3/25 monoclonal antibody incubated with extracts of the human cell line. A second monoclonal antibody against B3/25 glycoprotein, designated T56/14, gave similar results. As a specificity control, cell extracts were also incubated with T29/33 antibody-Sepharose beads. Little radioactivity was bound by this monoclonal antibody, which reacts with a high molecular weight glycoprotein of human hematopoietic cells distinct from B3/25 glycoprotein (8). As shown in Fig. 3, the radioactivity associated with the monoclonal antibodies against B3/25 glycoprotein incubated with the human cell extract was the result of the specific binding of the 80,000 M_r horse serum component.

To confirm that the serum protein was binding to the monoclonal antibodies in association with B3/25 glycoprotein, anti-

Table 1. Binding of ^{125}I-labeled horse serum to B3/25 glycoprotein purified by immunoprecipitation

Cell lysate*	Antibody	^{125}I-labeled horse serum bound, cpm x 10^{-4}
RPMI 8402	T29/33 (control)	0.14
	B3/25	10.3
	T56/14	9.0
BW5147	T29/33	0.05
	B3/25	0.05
	T56/14	0.05

* Cells were preincubated with 2.25 x 10^8 cpm of ^{125}I-labeled horse serum and washed, and cell lysates were prepared. After washing, the radioactivity associated with BW5147 cells was 1.6 x 10^6 cpm and with RPMI 8402 cells it was 1.5 x 10^6 cpm. Samples of lysate were then incubated with antibody-Sepharose beads and the radioactivity bound to the beads was determined.

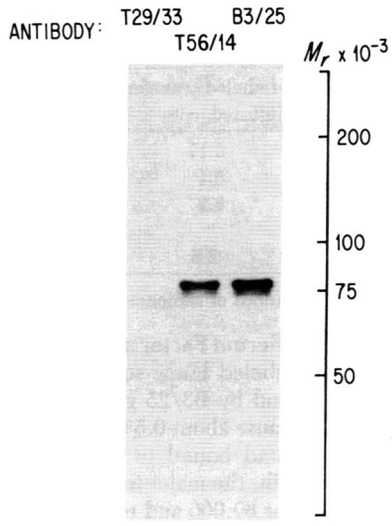

FIG. 3. The 80,000 M_r serum component bound to the cell surface is specifically associated with B3/25 glycoprotein. $NaDodSO_4$/polyacrylamide gel analysis of the ^{125}I-labeled horse serum protein bound to RPMI 8402 cells and immunoprecipitated by monoclonal antibody-Sepharose beads in the experiment described in Table 1 is shown. The ^{125}I-labeled horse serum protein was eluted from the antibody-Sepharose beads by boiling in 200 μl of electrophoresis sample buffer containing 5% (vol/vol) 2-mercaptoethanol and analyzed on a 7.5% gel. Exposure of the autoradiograph was for 6 hr.

body-Sepharose beads either were directly incubated with ^{125}I-labeled horse serum or were first incubated with a lysate of RPMI 8402 cells, washed extensively, and then incubated with ^{125}I-labeled horse serum. As shown in Fig. 4, the monoclonal antibodies against B3/25 glycoprotein bound only the horse serum component after incubation with the cell lysate.

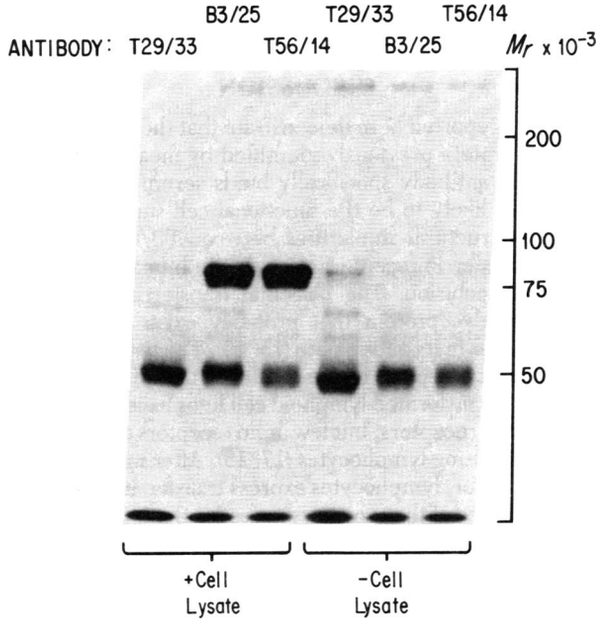

FIG. 4. The 80,000 M_r serum component binds to B3/25 glycoprotein adsorbed to antibody-Sepharose beads. Monoclonal antibody-Sepharose beads were incubated with a lysate of RPMI 8402 cells, washed, and then incubated with ^{125}I-labeled horse serum. Antibody-Sepharose beads that had not been preincubated with cell lysate were used as controls. The ^{125}I-labeled horse serum protein bound was eluted and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Exposure of the autoradiograph with an intensifying screen was for 18 hr.

Table 2. Binding of ^{125}I-labeled human transferrin to B3/25 glycoprotein

Antibody	^{125}I-Labeled transferrin precipitated, cpm × 10^{-4}	% of cell-associated radioactivity*
T29/33 (control)	0.17	0.7
B3/25	6.1	28.5
T56/14	6.2	28.9
Rabbit anti-B3/25 glycoprotein	2.8	13.0

* In this experiment, the input of radioactivity was 5.2×10^7 cpm.

Identification of the Serum Factor as Transferrin. The binding studies with ^{125}I-labeled horse serum suggested that the 80,000 M_r protein bound by B3/25 glycoprotein was a major serum component, because about 0.5% of the total ^{125}I-labeled serum protein was found bound to the antibody-Sepharose beads. Serum transferrin, the major iron transport protein, has an apparent M_r of about 80,000 and represents about 3–5% of the total serum protein (14). We decided therefore to directly test whether transferrin binds to B3/25 glycoprotein. Because horse transferrin was not available and preliminary studies showed that human serum also contained an 80,000 M_r protein bound by B3/25 glycoprotein, binding studies were carried out with human transferrin.

As shown in Table 2, human transferrin was bound to the surface of RPMI 8402 cells and could be isolated from lysates of the cells by monoclonal antibodies against B3/25 glycoprotein. Approximately 4% of the added labeled transferrin was bound to the cells after 60-min incubation at 37°C. Of this cell-associated radioactivity, more than 25% was recovered in association with B3/25 glycoprotein bound to monoclonal antibody-Sepharose beads. This was more than 50-fold the radioactivity bound to T29/33 monoclonal antibody-Sepharose beads used as a control. $NaDodSO_4$/polyacrylamide gel analysis confirmed that the ^{125}I-labeled protein bound was transferrin (data not shown).

DISCUSSION

The results reported here demonstrate that the human cell surface glycoprotein previously identified by means of the B3/25 monoclonal antibody specifically binds serum transferrin and is therefore likely to be the functional cell surface transferrin receptor. Structural similarities between B3/25 glycoprotein and the transferrin receptor isolated from human placenta support this conclusion. The placental transferrin receptor is a 90,000 M_r glycoprotein that probably exists as a disulfide-bonded dimer in its native state (15, 16). The tissue distribution of transferrin receptors is also similar to that of the B3/25 glycoprotein. Transformed lymphoid cell lines have large numbers of transferrin receptors, but few or no receptors can be detected on normal resting lymphocytes (17, 18). After mitogenic stimulation, however, lymphocytes express transferrin receptors and the appearance of the receptors precedes the initiation of DNA synthesis (17). Transferrin receptors have also been detected on cultured human cell lines derived from a variety of tissues but not on normal human liver or kidney membranes (19).

The identification of B3/25 glycoprotein as the cell surface transferrin receptor thus brings together two sets of independent observations concerning the relationship of the cell surface to cell proliferation. Studies of the expression of B3/25 glycoprotein with monoclonal antibodies extend previous knowledge about the relationship of transferrin receptors to cell growth in two respects. First, in addition to the monoclonal antibodies we have obtained, the OKT9 monoclonal antibody described by Reinherz *et al.* (20) and a monoclonal antibody (kindly provided by Tom Cotner) against a human cell surface antigen described by Judd *et al.* (21) both react with B3/25 glycoprotein (unpublished results). B3/25 and OKT9 monoclonal antibodies react with malignant cells from a substantial fraction of patients with acute lymphocytic leukemia of T cell origin (20, 22). B3/25 glycoprotein is also expressed on erythroleukemic cells (1, 22). In addition, Judd *et al.* (21) found the antigen they studied on the leukemic blasts of three patients with Sezary syndrome. These observations suggest that the expression of transferrin receptors may be characteristic of certain tumor cells growing *in vivo* as well as cell lines grown *in vitro*. Second, from our previous studies of the expression of B3/25 glycoprotein on HL-60 cells, it is clear that loss of transferrin receptors is a relatively early event during the *in vitro* induction of these cells (1). Transferrin is required for the growth of many cell lines *in vitro*, including HL-60 cells (23–26), and for primary cultures of hematopoietic cells (27, 28). Consequently, loss of transferrin receptors after exposure of HL-60 cells to inducers may be one of the regulatory events leading to terminal differentiation. However, because HL-60 cells are still inducible when provided with exogenous iron in the form of ferrous sulfate or ferric–fructose complexes (24), it is unlikely that loss of transferrin receptors is the only factor limiting cell growth (unpublished data).

Despite the presence of transferrin receptors on some normal tissues, such as placental trophoblast and maturing erythroid cells (29, 30), the relative lack of receptors on other normal tissues suggests that monoclonal antibodies against the transferrin receptor may be useful in the detection of some classes of tumor cells. Little is known about the properties of cell surface receptors of mammalian cells that bind polypeptide hormones and growth factors. The transferrin receptor is particularly amenable to study with the monoclonal antibodies now available and may provide information relevant to other receptors as well as specific details about transferrin binding and the mechanism by which iron enters the cell.

Finally, B3/25 glycoprotein is a cell surface molecule that was initially identified by means of a monoclonal antibody and for which a biological function was subsequently determined. The development of the monoclonal antibody technique has provided almost unlimited opportunity for immunological analysis of the cell surface. The demonstration that such studies can lead to the association of a cell surface molecule with a specific function confirms the value of this approach to investigate functional as well as structural aspects of the cell surface.

Note Added in Proof. Sutherland *et al.* (31) have independently shown that OKT9 monoclonal antibody reacts with the human transferrin receptor.

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