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Immunoprecipitation and partial characterization of diphtheria toxin-binding glycoproteins from surface of guinea pig cells

(cell surface receptors/lentil lectin/lactoperoxidase iodination/mouse L cells)

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ABSTRACT ^{125}I-Labeled membrane glycoproteins that specifically interact with diphtheria toxin and CRM197 protein—but not with diphtheria toxoid, fragment A of diphtheria toxin, or cholera toxin—were detected by use of the lactoperoxidase labeling technique followed by an immunoprecipitation system. These glycoproteins, which adhere to lentil lectin-Sepharose columns, are present on the surface of diphtheria toxin-sensitive guinea pig lymph node cells but are completely lacking on the surface of diphtheria toxin-resistant mouse L cells. The major ^{125}I-labeled glycoprotein that interacts with diphtheria toxin exhibits anomalous behavior, characteristic of glycoproteins, when analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. This demonstration of the biochemical nature of specific diphtheria toxin binding membrane components raises the possibility that the detected components are diphtheria toxin receptors.

Diphtheria toxin (DT), a protein of molecular weight 63,000, is produced by *Corynebacterium diphtheriae* organisms that are lysogenic for phage carrying the *tox* gene. This toxin is synthesized as a single polypeptide chain that can be proteolytically cleaved to yield two fragments, A and B, which remain associated via a disulfide bond (reviewed in refs. 1 and 2).

Intoxication of susceptible cells by DT is a complex process that involves the binding of the toxin, through fragment B, to specific receptors on the cell surface, followed by the translocation of the enzymatically active fragment A into the cytoplasm. Fragment A then catalyzes the transfer of ADP-ribose from NAD^+ to elongation factor 2(EF-2), resulting in an inactive EF-2 and the cessation of protein synthesis (reviewed in refs. 1 and 2).

Sensitivity of a cell to DT apparently resides at the level of the cell membrane because fragment A can inactivate EF-2 in cell lysates of resistant cell types (3). In addition, mouse L cells, a resistant cell line, bind very little radioiodinated toxin, indicating a lack of receptors (4).

Information concerning the numbers of DT receptors and the chemical properties of such receptors on various animal cells is limited. Studies based on the binding of ^{125}I-labeled DT to cells *in vitro* have been limited by the small numbers of DT-binding sites on susceptible cells such as HeLa cells (estimated at 4000/cell), by difficulties in demonstrating saturability of toxin-binding to susceptible cells, by nonspecific binding of radioiodinated DT to cells, and by pinocytic uptake of DT by cells (1, 4, 5). Recently, Draper *et al.* (6) have provided evidence which suggests that the DT receptor may contain an oligosaccharide component. These authors reported that the lectins concanavalin A and wheat germ agglutinin and ovalbumin glycopeptides blocked the action of DT and suggested that DT may have the properties of a lectin.

In this communication, direct evidence is provided for the binding of DT to specific glycoproteins from the plasma membranes of guinea pig lymph node cells that are susceptible to the action of DT. These glycoproteins are not detectable in the cell membranes of mouse L cells that are resistant to DT. In addition, the binding of DT to these cell membrane glycoproteins is dependent on the presence of a functional fragment B with receptor-blocking activity. All of these observations suggest that the specific DT-binding glycoproteins detected may be physiologically relevant plasma membrane receptors for DT.

MATERIALS AND METHODS

Reagents. All chemicals utilized were of the highest purity available. Lactoperoxidase was obtained from Calbiochem. Carrier-free ^{125}I was purchased from Amersham/Searle. The nonionic detergent Nonidet P40 (NP-40) was obtained from Shell Oil Co. Crystalline bovine serum albumin (Miles–Pentex) was acetylated with [^{3}H]acetic anhydride [0.5 Ci/mmol (1 Ci = 3.7×10^{10} becquerels), Amersham/Searle] and then cross-linked by the method of Carpenter and Harrington (7).

Purified cholera enterotoxin (choleragen) (8) was the generous gift of Richard Finkelstein (Department of Microbiology, University of Texas Health Science Center at Dallas) and the rabbit anti-cholera toxin was that described previously (9). Partially purified DT was purchased from Connaught Laboratories (Ontario, Canada), and highly purified DT and fragment A of DT were prepared according to published methods (10, 11). The enzymatically inactive CRM197 protein produced by *C. diphtheriae* strain C7 ($\beta^{tox\text{-}197}$) was purified by the method of Uchida *et al.* (12). Diphtheria toxoid was prepared as described by Linggood *et al.* (13).

Antiserum to DT was produced in New Zealand White rabbits by hyperimmunization with diphtheria toxoid. The resulting antiserum reacted with DT, fragment A, CRM197 protein, and diphtheria toxoid as determined by radioimmunoassay methods (unpublished data).

Protein A-bearing *Staphylococcus aureus* (Cowan strain 1), obtained from E. Rosenblum (Department of Microbiology, University of Texas Health Science Center at Dallas), were grown and processed by the method of Kessler (14).

Lentil lectin was affinity-purified from lentil beans and was covalently coupled to Sepharose 4B by utilizing cyanogen bromide (15). The resulting conjugate contained 2 mg of protein per ml of beads.

Cell Preparations. Lymph node cells were obtained from

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Abbreviations: DT, diphtheria toxin; EF-2, elongation factor 2; NP-40, Nonidet P40; P_i/NaCl, phosphate-buffered saline; Tris/NaCl, Tris-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate.

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normal female Hartley guinea pigs (Charles River Breeding Laboratories) as described (16). Mouse L cells (L-60 and L-929) were obtained from C. Y. Kang and P. Black (Department of Microbiology, University of Texas Health Science Center at Dallas) and J. W. Streilein and M. Nelles (Department of Cell Biology, University of Texas Health Science Center at Dallas).

Iodination of Cells. Single-cell suspensions, 2×10^8 cells per 2 ml of phosphate-buffered saline (P_i/NaCl; 0.01 M sodium phosphate/0.15 M NaCl, pH 7.2), were iodinated by the lactoperoxidase method essentially as described by Haustein (17). Each iodination procedure utilized 8 mCi of carrier-free ^{125}I and the entire procedure was carried out at 4°C. The iodinated cells were washed in an excess of Pi/NaCl and then lysed in 0.5% NP-40 in Tris-buffered saline (Tris/NaCl; 0.01 M Tris·HCl/0.15 M NaCl, 0.02% NaN_3, pH 7.4) at a ratio of 5×10^7 cells per ml of 0.5% NP-40. Nuclei were removed by centrifugation, and the supernatant fraction was dialyzed for 3 hr at 4°C against Tris/NaCl.

Fractionation of ^{125}I-Labeled Cell Lysates. The ^{125}I-labeled cell lysates were subjected to lentil lectin affinity chromatography at 4°C (15). The nonadherent fraction was collected and saved. After extensive washing of the lentil lectin-Sepharose columns with Tris/NaCl containing 0.1% NP-40 and 0.1 mg of bovine serum albumin per ml, the adherent ^{125}I-labeled glycoprotein fraction was eluted with 0.2 M α- methylmannoside in the same buffer. The eluted glycoprotein fraction was concentrated by vacuum dialysis against Tris/NaCl.

Immune Precipitation. Aliquots of radiolabeled lysates and nonadherent or adherent fractions of cell lysates were treated with rabbit IgG plus goat antirabbit Fc (9) to remove labeled components that bind to immune complexes. The process was repeated a total of three times. The resulting supernatant fractions were then treated twice with 0.5 ml of a 10% suspension of heat-killed Cowan strain 1 *S. aureus* to remove residual immune complexes and nonspecific binding components. The resulting supernatant fractions were then diluted in the Tris/NaCl buffer containing NP-40 and albumin to a concentration of $0.5–3 \times 10^7$ cell equivalents per ml. Appropriate aliquots (1 ml) were incubated with DT (or fragment A, CRM197 protein, or toxoid) or cholera toxin for 1 hr at 37°C, followed by incubation with the respective antitoxin for 1 hr at 4°C. The resulting immune complexes were removed by centrifugation after addition of 0.25 ml of a 10% suspension of *S. aureus*. The immune precipitates were washed twice with Tris/NaCl buffer containing NP-40 and albumin and finally with Pi/NaCl. The washed precipitates were assayed for ^{125}I in a Nuclear Chicago model 1185 spectrometer.

Precipitated proteins were solubilized by boiling for 2 min in 0.2 ml of 1% sodium dodecyl sulfate ($NaDodSO_4$)/0.1 M Tris·HCl/8 M urea, pH 8.4. After boiling, the insoluble *S. aureus* was removed by centrifugation and the supernatant was boiled again (2 min) without or with 1.2 M 2-mercaptoethanol. Just prior to the second boiling step, aliquots of crosslinked [^3H]albumin were added to each sample to yield internal marker proteins with apparent molecular weights of 68,000 136,000, and 204,000 (7).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed as described by Shapiro *et al.* (18). Solubilized immune precipitates were applied to 5% polyacrylamide gels and the gels were developed at 6–7 mA per gel for 15–16 hr. In some experiments, agarose /2.5% polyacrylamide or 7.5% polyacrylamide gels were used; these gels were developed at 7 mA per gel for 8 hr or at 7 mA per gel for 18 hr, respectively. The gels were fractionated by utilizing a Savant Auto gel divider and analyzed for ^{125}I in a Nuclear Chicago model 1185 spectrometer. ^3H was detected in the same samples by transfer of the gel to scintillation vials, addition of 7 ml of toluene/Liquifluor/Biosolv, and assay in a Beckman liquid scintillation counter, model LS-350. Values were corrected for ^{125}I crossover into the ^3H channel.

RESULTS

Guinea pigs are extremely sensitive to the effects of DT (1, 19, 20). Furthermore, guinea pig lymphoid tissue has been demonstrated to be sensitive to the toxic effects of DT *in vitro* (20). These observations suggested to us that guinea pig lymphoid cells could be utilized to detect membrane components which specifically interact with DT.

Interaction of DT with Membrane Components of Lymph Node Cells. When unfractionated NP-40 lysates of iodinated guinea pig lymph node cells were assayed for the presence of DT binding components by the immunoprecipitation method, no specific components could be detected. However, when lysates were first fractionated on a lentil lectin column into adherent and nonadherent fractions, different results were obtained. The nonadherent fraction behaved essentially as the unfractionated lysate. In contrast, as the results in Table 1 indicate, specific radiolabeled membrane components that bound to DT could be detected when the adherent fraction was used in the assay system. An 8-fold greater number of ^{125}I counts per minute were precipitated from this fraction when both DT and anti-DT were included in the reaction mixture, as compared to controls which contained DT alone or anti-DT alone. In addition, material was also not immunoprecipitated when cholera toxin (an unrelated bacterial toxin) and anti-cholera toxin were substituted for DT and anti-DT.

If the membrane material, reactive with DT, were indeed specific for fragment B, the binding component of the toxin (21, 22), then this material should react with CRM197 protein which has a normal binding subunit but an altered enzymatic subunit (12, 23). In addition, the membrane material should not react with the enzymatic fragment A alone or with diphtheria toxoid because these proteins have been shown not to interfere with either cytotoxicity (12, 21, 24) or the binding of DT to cells (1). These predicted findings were obtained (Table 2). That is, the substitution of CRM197 protein for DT in the complete reaction mixture led to immunoprecipitation of labeled membrane components, but fragment A and toxoid could not substitute for DT in the immunoprecipitation of labeled membrane components, although both fragment A and toxoid were immunoprecipitable by the antiserum used.

Table 1. Interaction of toxins with iodinated membrane components

System	Toxin,* μg	Antitoxin,† μl	cpm immunoprecipitated
Diphtheria	20	20	31,810
	20	—	2,530
	—	20	3,640
Cholera‡	20	20	6,180

A lysate of ^{125}I-labeled guinea pig lymph node cells was prepared, fractionated, precleared with immune complexes and specifically immunoprecipitated as described in the text. Each assay contained an amount of lysate equivalent to 3×10^7 cells.

* In this system, 20 μg of DT is saturating (see Table 3).

† As determined by radioimmunoassay, 20 μl of anti-DT completely precipitates 20 μg of DT.

‡ Cholera toxin and anti-cholera toxin were substituted for the DT and anti-DT; 20 μl of anti-cholera toxin completely precipitates 20 μg of cholera toxin.

Table 2. Interaction of DT-related proteins with iodinated membrane components

CRM-197, μg	Fragment A, μg	Toxoid, μg	Anti-toxin, μl	cpm immuno-precipitated
20	—	—	20	71,260
20	—	—	—	7,140
—	20	—	20	8,310
—	—	20	20	8,710

The conditions of the assays were the same as in Table 1, except that proteins related to DT were substituted for DT. Each assay contained an amount of lysate equivalent to 3×10^7 cells.

Therefore, it was concluded that the interaction of the membrane component(s) with DT was via interaction with the binding subunit (fragment B). In addition, the finding that these membrane component(s) adhere to lentil lectin and are eluted with α-methylmannoside indicates that the reactive components are glycoproteins that have accessible carbohydrate residues with the mannopyranoside configuration (25).

Gel Electrophoretic Analysis of DT-Binding Glycoproteins from Lymph Node Cells. To investigate the molecular nature of the DT-binding membrane glycoproteins, the immune precipitates from the experiments in Tables 1 and 2 were dissociated, reduced, and analyzed by $NaDodSO_4$/5% polyacrylamide gel electrophoresis. The ^{125}I-labeled glycoproteins immunoprecipitated with DT consisted of one major peak and two or three minor peaks of radioactivity (Fig. 1*A*). The size of the major component, based on five experiments, was calculated to be 168,000 ± 5,600 daltons. As can be seen in Fig. 1*B*, the same material was reactive with CRM197 protein. In addition, Fig. 1 also illustrates the finding that reagent controls, fragment A, or diphtheria toxoid immune precipitates did not contain this major peak of radioactivity. It should also be noted that, although immune precipitates obtained with cholera toxin and anti-cholera toxin contained more ^{125}I than did other controls (Table 1), no apparent peak of radioactivity could be detected (Fig. 1*A*).

Further experiments demonstrated that the electrophoretic profile of the DT-binding glycoproteins was essentially unaltered if the treatment with 2-mercaptoethanol was omitted, indicating that the 168,000-dalton peak was not a subunit of a larger disulfide-linked glycoprotein. Analysis of reduced immune precipitates on $NaDodSO_4$/agarose/2.5% polyacrylamide gels and on $NaDodSO_4$/7.5% polyacrylamide gels indicated that the apparent molecular weight of this material was strongly influenced by the concentration of polyacrylamide in the gels. The apparent molecular weight on agarose/2.5% polyacrylamide gels was greater than 250,000 whereas a value of 145,000 was obtained from 7.5% polyacrylamide gels. This anomalous behavior is characteristic of heavily glycosylated proteins (26) and is consistent with our conclusion, based on binding to lentil lectin columns, that the DT-binding components from the labeled membranes are glycoproteins.

Characterization of the Interaction of DT with Lymph Node Cell Membrane Components. DT-sensitive eukaryotic cells contain a finite number of receptor sites for this bacterial toxin, and the binding of labeled DT to such cells is a saturable process (4, 22). Therefore, if the detected membrane component described above were indeed the receptor for DT, then it should be possible to saturate the immunoprecipitation system with respect to DT as well as with respect to the membrane receptor.

Using a constant amount of iodinated membrane material (1×10^7 cell equivalents) and varying the concentration of DT

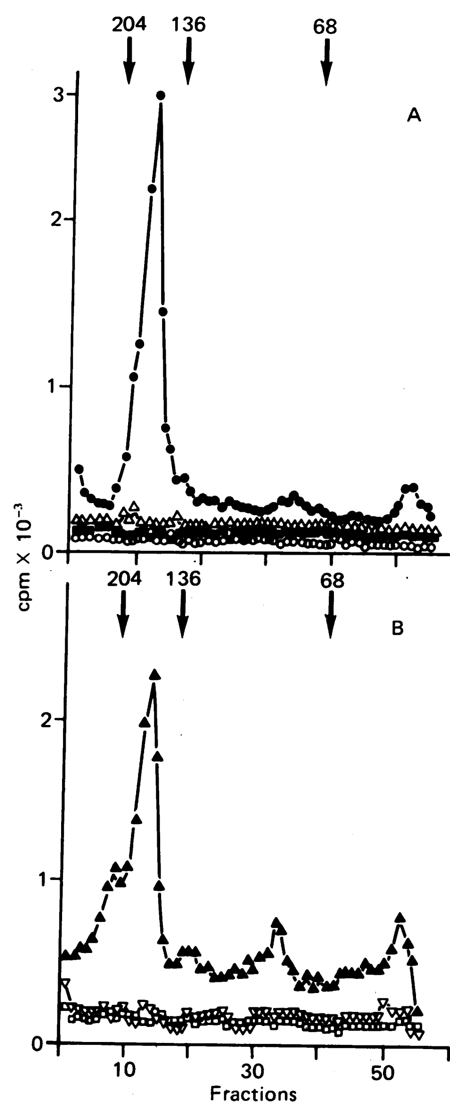

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of DT-binding glycoproteins. (*A*) This analysis was performed on the following immune precipitates described in Table 1: DT plus anti-DT (●); DT alone (○); anti-DT alone (■); and cholera toxin plus anti-cholera toxin (△). These immune precipitates were dissociated, reduced, and applied to $NaDodSO_4$/5% polyacrylamide gels. The gels were electrophoresed at 6 mA per gel for 15.5 hr. The gels were sliced and each fraction was analyzed for ^{125}I and ^3H. The arrows indicate the molecular weights ($\times 10^{-3}$) of the internal ^3H-labeled markers: bovine serum albumin monomer (68,000); crosslinked albumin dimer (136,000); and crosslinked albumin trimer (204,000). (*B*) This analysis was performed on the following immune precipitates described in Table 2: CRM 197 protein plus anti-DT (▲); diphtheria toxoid plus anti-DT (▽); and fragment A plus anti-DT (□). These immune precipitates were analyzed as in *A*.

in the precipitation system led to increased amounts of iodinated material that could be immunoprecipitated until the membrane binding capacity was saturated (Table 3, Exp. A). The converse experiment, in which a constant amount of DT (20 μg) was added to the reaction and the number of cell equivalents of ^{125}I-labeled material was varied from 0.5 to 3×10^7 cell equivalents, indicated that the number of ^{125}I-labeled molecules reactive with DT was finite and proportional to the ^{125}I input until the DT was saturated (Table 3, Exp. B). The lower limit for the detection of the DT-binding component was approximately 0.5×10^7 cell equivalents.

Table 3. Saturability of the DT–membrane glycoprotein system

Cell equivalents $\times 10^{-7}$	cpm immunoprecipitated 0 μg	4 μg	10 μg	14 μg	20 μg	30 μg
			Exp. A			
1	2020	4320	7390	8100	9,250	10,280
			Exp. B			
0.5					9,430	
1					16,150	
2					27,880	
3*					29,470	

The experimental procedure was the same as described in Table 1, except that 30 μl of anti-DT was used to precipitate the fraction with 30 μg of DT. The amount of DT was varied in Experiment A; the cell equivalents were varied in Experiment B. Experiments A and B were performed with different glycoprotein preparations, and the difference observed is due to the variation in radiolabeling efficiency between experiments.

*Control immune precipitates, with 3×10^7 cell equivalents, in which anti-DT was omitted or DT was omitted contained 2530 or 3640 cpm, respectively

Interaction of DT with Mouse L Cell Membrane Components. In contrast to guinea pigs, which are extremely sensitive to DT, mice and rats are resistant to the action of this toxin (1). *In vitro* analysis of the effect of DT on murine cell lines, such as L cells, has verified the *in vivo* findings (27, 28). L cells are very resistant to the biological effects of DT, presumably because they lack specific receptors for the toxin (4). Therefore, if the above-described guinea pig membrane glycoproteins that interact with DT were relevant to intoxication by this toxin, they should be lacking from a cell line such as mouse L cells.

To test this hypothesis, mouse L cells (L-60 and L-929) were radioiodinated, and the labeled cell lysates were analyzed for the presence of membrane components that specifically interact with DT. For both strains of L cells, no binding of ^{125}I-labeled membrane components to DT or any of its derivatives could be detected when cell lysates, the lectin nonadherent fraction, or the lectin adherent glycoprotein fraction was utilized. That is, no specific ^{125}I could be immunoprecipitated and $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of the immune precipitates did not reveal the presence of any peaks of radioactivity. These experiments were carried out with up to 3×10^7 cell equivalents. Therefore, if L cells do contain the glycoprotein component detected in the guinea pig system, then it is present at a concentration no more than one-sixth that on guinea pig lymph node cells, where the glycoprotein was readily detected with 0.5×10^7 cell equivalents.

DISCUSSION

Many bacterial and plant toxins exert their biological effects on eukaryotic cells via modification of regulatory enzymes, such as adenyl cyclase, or via modification of protein synthesis (1, 29). Studies of the biochemical mechanisms of such cytoplasmic alterations have been particularly successful in elucidating events that occur after the primary interaction of toxins with the plasma membrane.

In contrast to the biochemical mechanism of intoxication, little is known about the primary events dealing with the interaction of these toxins with the cell membranes and the internalization processes. Exceptions to this generalization are cholera toxin and tetanus toxin, for which the receptors on the cell membrane are believed to be the gangliosides G_{M1} and G_{D1b}, respectively (30). Other toxins such as abrin and ricin behave as lectins, specific for nonreducing terminal galactose residues, and can be shown to interact with a subpopulation of membrane glycoproteins (31, 32).

Unlike cholera and tetanus toxins, the cytotoxic activity of DT cannot be blocked by glycolipids (1). In addition, it has been difficult to demonstrate that DT binds to specific glycoproteins on sensitive cells. This failure to detect specific membrane components has been attributed to the relatively low concentrations of such components even on sensitive cells (4).

Our detection of DT binding components from the membranes of guinea pig lymph node cells was only possible when cell lysates were first fractionated on lentil lectin-Sepharose columns to yield an enriched glycoprotein fraction. This step not only decreased the nonspecific radioactivity in the control precipitates but also afforded an approximately 30-fold purification of the starting material and permitted detection of the DT-binding component, which represented only 0.1–0.2% of the ^{125}I incorporated into cellular components. Assuming that all membrane components label equally, this very low percentage of ^{125}I in the DT-binding components would be consistent with a small number of such molecules per cell.

Our results indicate that the DT-binding component from guinea pig cells is a glycoprotein. This conclusion is based both on adherence to the lentil lectin-Sepharose column and the anomalous behavior on $NaDodSO_4$/polyacrylamide gels. By analogy with previous experiments (33) it is unlikely that the interaction of DT with the membrane glycoprotein could be due to a tightly associated but unlabeled glycolipid. We have not yet determined whether the carbohydrate or the protein components of the membrane glycoprotein are responsible for the interaction with DT. Draper *et al.* (6) postulated that DT could be interacting with carbohydrate configurations on cell membranes. This latter hypothesis was based on the findings that concanavalin A, wheat germ agglutinin, and ovalbumin glycopeptides blocked intoxication of cells by DT. Furthermore, they concluded that DT has the properties of a lectin. Because concanavalin A and lentil lectin have similar carbohydrate specificities (15), this could be a possibility. However, our finding of one major DT-binding component on gel electrophoresis (Fig. 1), compared to the large number of peaks obtained with the whole lentil lectin-Sepharose adherent fraction (unpublished data), distinguishes DT from the toxic plant lectins abrin and ricin which bind to several membrane glycoproteins (32). Thus, it is unlikely that DT is behaving as a classical lectin. Therefore, the question of the relevant component, carbohydrate or protein, of the DT-binding molecule remains unanswered.

Finally, a question of primary importance is the relationship of the DT-binding component reported here to the functional DT receptor on sensitive cells. Because the binding component is specific for fragment B of DT and can be detected on DT-sensitive cells (guinea pig lymph node cells) but not on DT-resistant cells (L cells), at present there is complete correlation between biological and biochemical results. However, until a larger number of sensitive and resistant cell types are studied and more extensive correlative data are obtained, the possibility that the DT-binding membrane glycoprotein *is* the DT receptor must remain a hypothesis.

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