Characterization and Use in Immunoradiometric Assay of Monoclonal Antibodies Directed Against Human Proinsulin

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SUMMARY

Proinsulin in human serum is heterogenous. Existing assay methods do not distinguish between the various forms intermediate on the pathway of processing from proinsulin to insulin. We report the production of mouse monoclonal antibodies against human proinsulin with biosynthetic human proinsulin (produced by recombinant DNA technology) as immunogen. Four monoclonal antibody–producing cell lines were obtained from two separate fusions. Two of the antibodies had affinity constants for intact proinsulin of 3.1 × 10^{10} and 5.6 × 10^{10} L/mol and bound all forms of proinsulin and insulin. Cross-reactivity with intact proinsulin was greater than with 65-66 and 32-33 split proinsulins and des 64-65 and des 31-32 proinsulins. The third antibody had an affinity constant for intact proinsulin of 6.3 × 10^{10} L/mol and reacted only with intact proinsulin, 65-66 split proinsulin, and des 64-65 proinsulin; there was no reaction with 32-33 split proinsulin, des 31-32 proinsulin, or C-peptide. The fourth antibody reacted only with intact proinsulin and had an affinity constant of 4.1 × 10^{8} L/mol. We report the use of two antibodies in a sensitive two-site immunoradiometric assay for intact proinsulin. Insulin, C-peptide, and 32-33 split proinsulin did not react in the assay up to a concentration of 100 pM. The 65-66 split proinsulin and des 64-65 proinsulin reacted with a potency of ~55% relative to intact proinsulin. Serum proinsulin concentrations measured with this assay were compared with those determined by an alternative method that detects only split proinsulins. The results suggest that both intact and split proinsulins occur in human serum but that the forms differ in their kinetics of release and removal in response to an oral glucose load. *Diabetes* 36:684–88, 1987

Proinsulin, the precursor of insulin, is hydrolyzed via several intermediate forms in the β-cell of the pancreas to form insulin (1). The putative enzymes responsible for this processing possess trypsin-like and carboxypeptidase B–like specificity. It is thought that the trypsinlike enzyme hydrolyzes positions 32-33 and 65-66 and the carboxypeptidase B–like enzyme removes the basic residues 64-65 and 31-32 to produce insulin and C-peptide. It is therefore apparent that there are several possible partially hydrolyzed forms of proinsulin intermediate on this pathway, e.g., 32-33 and 65-66 split proinsulins and des 64-65 and des 31-32 proinsulins (Fig. 1). We have reported that an indirect two-site immunoradiometric assay that employs polyclonal antisera directed against insulin and C-pepide appears only to measure partially hydrolyzed proinsulins and not intact proinsulin (2). Proinsulin concentrations in human serum measured with this and other methods (4,5) are similar, suggesting that circulating human proinsulin immunoreactivity may be predominantly if not entirely due to hydrolyzed proinsulins.

There are no direct assays available that readily differentiate between the possible forms of proinsulin. Separation techniques such as high-performance liquid chromatography (HPLC) are unsuitable for processing large numbers of serum samples. We have raised monoclonal antibodies against biosynthetic human proinsulin and characterized them in terms of epitope specificity and cross-reactivity with the possible forms intermediate on the pathway of conversion of proinsulin to insulin. Given monoclonal antibodies of suitable specificity and affinity, it should be possible to establish assays specific for the various forms of proinsulin. We report a two-site immunoradiometric assay for intact

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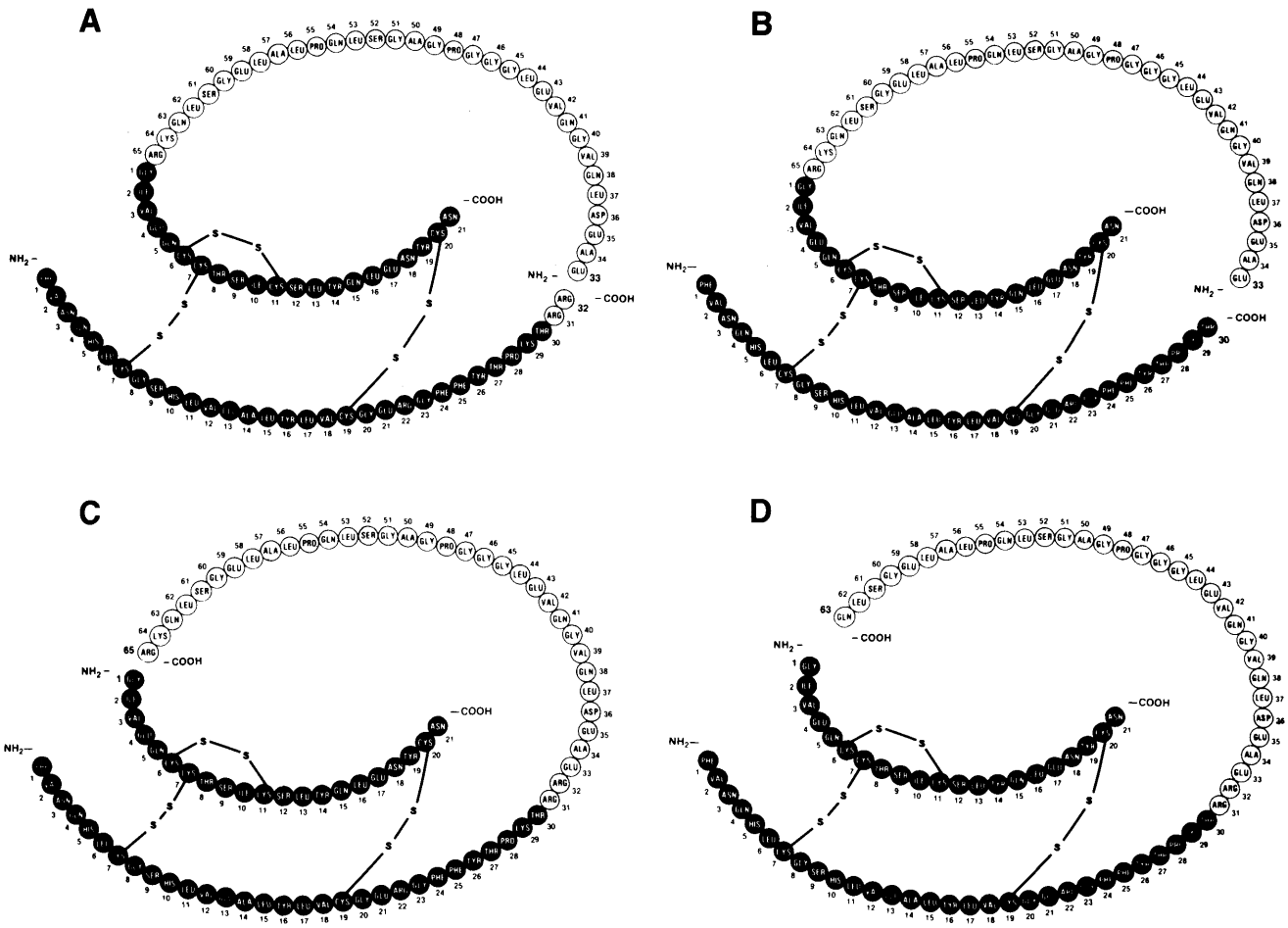

FIG. 1. Structures of various proinsulins. *A*: 32-33 split proinsulin. *B*: des 31-32 proinsulin. C: 65-66 split proinsulin. *D*: des 64-65 proinsulin.

proinsulin with monoclonal antibodies and its use to measure proinsulin in human serum. Comparison is made between the results obtained with this assay and those obtained with an assay that does not detect intact proinsulin.

MATERIALS AND METHODS

Biosynthetic human proinsulin and human C-peptide were produced with recombinant DNA technology (6). Biosynthetic 65-66 and 32-33 split human proinsulins were prepared by subjecting human proinsulin to limited tryptic hydrolysis followed by subsequent purification with preparative reverse-phase HPLC. The structure of each derivative was verified by amino acid analyses and by amino acid sequencing. Biosynthetic human proinsulin was iodinated and isolated with previously described procedures (7). The proinsulin tracer was monoiodinated on the 79 tyrosine and had a specific activity of 210 μCi/μg. Insulin was iodinated to a specific activity of 150–200 μCi/μg (8). Sheep anti-murine immunoglobulin was produced after monthly subcutaneous injection of 1 mg IgG in Freund's adjuvant. Crude IgG fractions from the sheep serum were prepared by ammonium sulfate precipitation (9) and coupled to aminocellulose by diazotization (10).

Immunization, hybridization, and tissue culture. BALB/c mice (Bantin and Kingman, Hull, UK) were immunized with 10 μg biosynthetic human proinsulin subcutaneously in complete Freund's adjuvant (Behringwerke AG, Marburg, FRG). After 6 wk a similar amount of proinsulin in 150 mM NaCl was administered intravenously in phosphate-buffered saline as a booster. Fusions of spleen cells with NSO myeloma cells were carried out 4 days later, and hybrids were grown and cloned twice at limiting dilution according to previously described methods (11,12). Five million cloned cells were administered intraperitoneally to pristane-primed BALB/c mice [2,6,10,14-tetramethylpentadecane (Aldrich, Milwaukee, WI)], 0.5 ml/animal, to produce monoclonal antibody–containing ascites fluid. Antibodies were partially purified from such fluid by precipitation with 40% saturated $(NH_4)_2SO_4$ (9).

Screening assays. Ten to 50 microliters of culture supernatant were incubated with 100 μl iodinated proinsulin or insulin (~50 pg) in 50 mM veronal buffer, pH 8.0, containing 1 mg/ml bovine serum albumin. After 30 min at room temperature, 0.5 mg sheep anti-murine immunoglobulin immunoadsorbent was added in 20 μl veronal buffer and allowed to react for 30 min at room temperature. After addition of 1 ml buffer, the tubes were centrifuged for 10 min at 2000 × *g*. Supernatants were decanted, and the radioactivity in the pellets was determined in an NE 1600 γ-counter (Nuclear Enterprises, Berkshire, UK).

TABLE 1
Affinity constants and cross-reactivities of monoclonal antibodies directed against proinsulin

Antibody no.	Affinity constant (L/mol)	Relative cross-reactions (%)						
		Intact proinsulin	32-33 proinsulin	65-66 proinsulin	des 31-32 proinsulin	des 64-65 proinsulin	Insulin	C-peptide
3B1	3.1×10^{10}	100	44	58	100	58	75	<0.01*
3B7	5.6×10^{10}	100	56	81	100	100	93	<0.01*
2E6	6.3×10^{10}	100	<0.01*	34	<0.01*	26	<0.01*	<0.01*
1H1	4.1×10^{8}	100	<1*	<1*	<1*	<1*	<1*	<1*

Affinity constants are those for intact proinsulin at 4°C as determined by Scatchard analysis (13). Cross-reactivities, expressed as relative concentrations causing 50% inhibition of binding of ^{125}I-proinsulin in liquid-phase assay, are means of 3 experiments.
*Highest concentrations at which there was no detectable reaction.

Measurement of affinity and specificity. The dilution of ascites fluid that half-maximally bound the added iodinated proinsulin or insulin in the screening assay described above was determined. Fifty microliters of this dilution of antibody were incubated for 30 min at room temperature or overnight at 4°C with 100 μl of various concentrations of intact proinsulin, 65-66 and 32-33 split proinsulins, des 64-65 and des 31-32 proinsulins, insulin, or C-peptide and 100 μl (~50 pg) ^{125}I-proinsulin, all in veronal/albumin buffer. Thereafter, 0.5 mg sheep anti-murine immunoglobulin immunoadsorbent was reacted for 30 min at room temperature. One milliliter buffer was added, the tubes were centrifuged at 2000 × *g* for 10 min, and the supernatant was decanted. The pellet was washed further with 1 ml buffer and then counted for radioactivity. Affinity constants were calculated by Scatchard analysis (13).

Epitope analysis. The wells of flexible microtiter plates (Falcon Microtest 111, Becton Dickinson, Oxnard, CA) were coated with a solution of 100 μg/ml partially purified antibody in 50 mM sodium bicarbonate buffer, pH 9.6, for 24 h at 4°C. The excess antibody was aspirated and retained for further use. The wells were washed three times with veronal/albumin buffer (200 μl/well), and the plates were stored at −20°C until required. To block a given proinsulin epitope, labeled proinsulin (~200 pg) was reacted separately in solution with an excess of each monoclonal antibody overnight at 4°C in 400 μl veronal buffer. Thereafter, 100 μl of each mixture was added in triplicate to antibody-coated wells and reacted at 4°C overnight. After washing three times with 200 μl cold veronal buffer/well, the wells were separated, and the bound radioactivity was counted.

Two-site assay. Anti-proinsulin monoclonal antibodies were partially purified from mouse ascites fluid by $(NH_4)_2SO_4$ precipitation (9). A monoclonal antibody (3B7) that cross-reacted with insulin was immobilized on diazotized cellulose (10). The binding capacity of the preparation determined from the amount of proinsulin bound by 100, 50, 20, 10, and 5 μg of adsorbent with an excess of proinsulin (8 ng/tube) was 820 pmol proinsulin/mg cellulose. A proinsulin-specific antibody (2E6) was iodinated by the iodogen method (14) to a specific activity of 20 μCi/μg. Standard (1–1000 pM biosynthetic human proinsulin in 200 μl) or sample was incubated with the solid-phase immobilized antibody (50 μg immunoadsorbent plus 250 μg finely divided cellulose in 100 μl) for 24 h at 4°C. The mixture was then centrifuged at 2000 × *g*, and the pellet was washed twice with 1 ml veronal buffer. Iodinated antibody (50,000 cpm in 200 μl buffer) was added, and the pellet was resuspended and incubated for a further 24 h at 4°C. After two washes the pellet was counted in an NE 1600 γ-counter. Identical response curves were obtained whether standards were added in veronal buffer or in serum from which endogenous proinsulin had been removed by prior incubation with immobilized rabbit polyclonal anti-insulin immunoglobulin.

Subjects. Fasting proinsulin levels and the response to a 75-g glucose load were determined in nine normal, nonobese, healthy subjects (7 men, 2 women) with an age range of 20–24 yr. Levels were determined according to the methods described above (monoclonal assay) and a previously described method (indirect assay) (2,15).

RESULTS

Properties of monoclonal antibodies. Four different monoclonal antibodies directed against proinsulin were isolated from two independent hybridizations. Binding of ^{125}I-proinsulin or ^{125}I-insulin was investigated in the presence of various concentrations of unlabeled proinsulin or insulin, respectively. Affinities of the monoclonal antibodies for intact proinsulin varied from 4.1×10^{8} to 6.3×10^{10} L/mol at 4°C, the Scatchard plots being, within experimental error, linear. Two antibodies (3B1 and 3B7) bound both insulin and proinsulin with similar affinities, whereas the remaining two (2E6 and 1H1) bound only proinsulin (Table 1).

The ability of various concentrations of intact proinsulin, 65-66 and 32-33 split proinsulins, des 64-65 and des 31-32 proinsulins, insulin, and C-peptide to compete with ^{125}I-proinsulin for binding to antibody was tested in a liquid-phase assay. Results are shown in Table 1. All antibodies reacted somewhat more avidly with intact proinsulin than with par-

TABLE 2
Analysis of epitope specificity of monoclonal antibodies directed against proinsulin

Solid-phase antibody	Liquid-phase antibody			
	3B1	3B7	2E6	1H1
3B1	2	4	66	80
3B7	2	2	70	85
2E6	100	92	4	80
1H1	96	95	90	3

Binding of antibody-bound ^{125}I-proinsulin to antibodies immobilized on solid phase is shown. Binding expressed as percentage of maximum bindable ^{125}I-proinsulin in the absence of antibodies in the liquid phase.

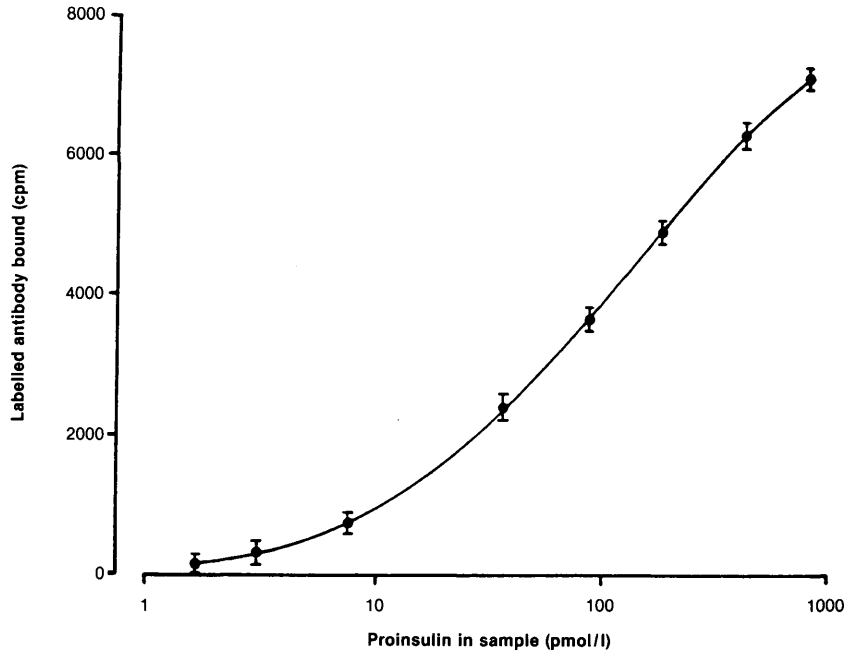

FIG. 2. Standard curve for immunoradiometric assay of proinsulin. Experimental details described in MATERIALS AND METHODS. *Points* represent means ± SE for 40 standard curves. Nonspecific binding (0 proinsulin) was ~1% of total radioactivity and has been subtracted.

tially hydrolyzed proinsulin. None cross-reacted with C-peptide. Antibody 2E6 cross-reacted only with intact proinsulin and 65-66 split proinsulin, whereas antibody 1H1 reacted only with intact proinsulin.

Epitope analysis showed that the two antibodies that bound both insulin and proinsulin, and had similar patterns of cross-reaction, also mutually inhibited binding of ^{125}I-proinsulin and ^{125}I-insulin (results not shown) to the same antibodies immobilized on solid phase (Table 2). Neither of the two proinsulin-specific antibodies inhibited binding of proinsulin to other antibodies immobilized on solid phase, showing that they reacted with different parts of the molecule. Thus, three distinct epitopes on intact proinsulin were identified by the four antibodies.

Use of antibodies in proinsulin assay. Two compatible antibodies of high affinity (3B7 and 2E6) were used to establish a two-site immunoradiometric assay for proinsulin. The standard curve obtained with intact proinsulin as standard is shown in Fig. 2. The working range, defined by a within-assay coefficient of variation of ~10% or less, was 3–200 pM, and the sensitivity, defined as the concentration giving a response equivalent to 2.5 times the SD at zero dose, was 1 pM. Insulin at a concentration of 500 mU/L did not affect binding in the assay and serum samples diluted in a linear manner (results not shown). Insulin, C-peptide, and 32-33 split proinsulin did not react in the assay at concentrations up to 1000 pM, whereas 65-66 split proinsulin and des 64-65 proinsulin reacted with a potency of ~55% relative to intact proinsulin.

Fasting levels of proinsulin of 15 normal young adults as measured with this assay were 5.3 ± 0.7 pM. The corresponding basal proinsulin level with the indirect assay for partially hydrolyzed proinsulins was 9 ± 4 pM (2). Proinsulin levels in response to a 75-g oral glucose load in a separate group of nine subjects are shown in Table 3. An increase in immunoreactive material was detected in both assays, but this was more marked in the indirect assay, and there were also differences in the relative levels especially at late time points.

DISCUSSION

We report the production of four mouse monoclonal antibodies directed against biosynthetic human proinsulin and their use in a two-site immunoradiometric assay. Low-affinity monoclonal antibodies to proinsulin have been produced previously with proinsulin conjugated to bovine thyroglobulin (16). Our results show that it is unnecessary to conjugate proinsulin as an immunogen and that high-affinity antibodies can be produced with unconjugated proinsulin. Two of the antibodies we obtained bound to both proinsulin and insulin, a fact that is not surprising in view of the large amount of amino acid sequence in common. The other two antibodies did not react with insulin or C-peptide and had generally little cross-reaction with split proinsulins. Antibody 2E6 prob-

TABLE 3
Proinsulin concentrations during 75-g oral glucose tolerance test by two assay methods

	Time (min)							
	0	15	30	45	60	90	120	150
Monoclonal assay (pM)	5.3 ± 0.4	8.6 ± 2.0	10.4 ± 2.5	14.8 ± 4.1	16.2 ± 4.1	16.4 ± 2.3	17.0 ± 3.6	16.4 ± 3.4
Indirect assay (pM)	9 ± 4	20 ± 2	31 ± 3	38 ± 5	42 ± 3	40 ± 3	35 ± 5	21 ± 7

Monoclonal assay as described herein and indirect assay as described in ref. 2. Values are means ± SE of concentrations measured in 9 normal subjects.

ably binds to an epitope in the region of the molecule where the B-chain of insulin joins C-peptide and thus reacts to some extent with 65-66 split proinsulins. The epitope for antibody 1H1 is more difficult to identify but probably reflects a conformation of a part of C-peptide that is only obtained in intact proinsulin.

The assay reported uses a combination of two monoclonal antibodies and measures intact proinsulin and to a lesser extent proinsulin derivatives in which the linkage of C-peptide to the B-chain of insulin is intact. Serum levels measured with this assay (monoclonal assay) are lower than the levels of proinsulin measured with the indirect assay (2), which detects partially hydrolyzed proinsulin derivatives equally but not intact proinsulin. Although it is not yet possible to exclude or confirm the presence of intact proinsulin in the fasting normal subject, it has become possible to set limits on the range of intact proinsulin. Three situations may be considered. These results are compatible with the presence of undetectable (<1 pM) amounts of intact proinsulin in serum from fasting normal subjects. However, for this to be consistent with the results of the two assays of different specificity, virtually the entire proinsulin detected by the indirect assay (~9 pM) would have to consist of 65-66 split proinsulins. These show ~50% reactivity in the monoclonal assay and could then account for the 5.3 pM measured by this method. On the other hand, if processing were random, the 9 pM in the indirect assay would represent an equal contribution from 32-33 and 65-66 split products. In this case the 65-66 split products would account for ~2.3 pM (50% of 4.5 pM) of the immunoreactivity detected in the monoclonal assay, giving an intact proinsulin concentration of 3 pM (25% of the total proinsulin-like material present in serum). The other limiting possibility is that all of the material detected in the monoclonal assay is intact proinsulin, in which case we can place an absolute upper limit for the amount of intact proinsulin as 5.3 pM or ~37% of the total. However, for this upper limit to be reached, we again have to assume the exclusive presence of a single type of processing (at the 32-33 position).

Studies in which proinsulin-like material has been separated by HPLC suggest that the possible split derivatives of proinsulin are not equally represented in plasma (17). Eighty percent or more of the hydrolyzed proinsulin is derived from the 32-33 split. Because this material does not cross-react in our monoclonal assay, the results of this method must be predominantly due to intact proinsulin. Cohen et al. (18) published results obtained with a radioimmunoassay of human proinsulin. In this assay, 65-66 split proinsulins reacted almost as well as intact proinsulin, whereas 32-33 split proinsulins were much less reactive. It was necessary to extract and concentrate the proinsulin-like material present in human plasma taken in the fasting state. Despite the greater cross-reactivity of the split forms of proinsulin in the assay of Cohen et al., their results are similar to ours (fasting proinsulin 5.9 pM compared to 5.3 pM in our study). Sixty minutes after 75 g oral glucose the values were 22.5 and 16.2 pM, respectively. The relatively good agreement between these two assays and the slightly lower values obtained in our study would be consistent with our monoclonal assay detecting mainly intact proinsulin.

After stimulation by an oral load of 75 g glucose, the proportion of intact proinsulin (monoclonal assay) to partially hydrolyzed proinsulins (indirect assay) remains constant until 90 min (Table 3). Thereafter the proportion of intact proinsulin increases considerably, indicating perhaps that "immature" insulin granules are being secreted. These findings may also reflect a longer half-life of intact proinsulin. Because partially hydrolyzed proinsulins are more active biologically than intact proinsulin, the clearance of these derivatives is probably accelerated in relation to intact proinsulin (3). It therefore seems likely that different kinetics of release and different half-lives of intact and split proinsulins are responsible for the differences between the results with the two assays. The ability to distinguish between intact and split insulin provides a valuable new approach to the investigation of insulin release in novel and pathological situations.

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