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Isoform Specificity of Monoclonal Hybridoma Antibodies to Quail Skeletal Muscle Myosin Subunits

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SUMMARY Monoclonal antibodies to adult quail breast muscle myosin (QBM) have been prepared and characterized using a solid phase enzyme linked immunosorbent assay and immunoblot procedures. One antibody (QBM-1) is directed against an epitope in the rod portion of the myosin heavy chain while another (QBM-2) binds exclusively to a conserved portion of the two alkali light chains of fast muscle myosin. Both of these antibodies cross-react with myosin from myotubes cultured in vitro but do not recognize non-muscle myosin. The application of these antibodies to the study of myogenesis is discussed.

Myosin is a multimeric protein composed of heavy and light chain subunits, which is found in most non-muscle as well as muscle cells. Two dimensional gel electrophoresis (1), peptide mapping (2,3) and DNA sequence analysis (4) have all been used to show that these subunits exist in a variety of sequence divergent forms (isoforms) which, although related, are products of different members of multigene families. These isoforms generally have a specific distribution at discrete developmental stages and in different tissues (5,6,7).

The ubiquitous distribution of non-muscle myosin has complicated the application of immunochemical probes to the study of myosin synthesis during myogenesis since many serum polyclonal antibodies fail to discriminate between non-muscle and muscle-specific myosin isoforms. Although affinity chromatography has been used successfully to obtain anti-myosin antibodies of defined

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ABBREVIATIONS USED: QBM, quail breast myosin; MLC, myosin light chain; MHC, myosin heavy chain; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbant assay; SaC, Staphylococcus aureus, Cowan strain 1; M_r, molecular mass; ALD, anterior latissimus dorsi.

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specificity (7,8,9) this approach is laborious and often results in antibodies of low titer. As an alternative approach to this problem, we have used the hybridization technique of Köhler and Milstein (10) as modified by Oi and Herzenberg (11) to produce hybridoma cell lines which secrete monoclonal antibody to adult quail muscle myosin. Characterization of these antibodies using solid phase ELISA and immunoblot analysis reveals that they are highly specific for muscle myosin subunits.

MATERIALS AND METHODS

Protein purification and digestion. Myosin was purified from adult quail breast muscle (QBM) using a standard high salt extraction (12) except that 2 mM $MgCl_2$ and 5 mM ATP were added during the purification. Papain digestion of insoluble myosin was carried out as described by Margossian and Lowey (13). Platelet myosin was isolated from outdated human platelets as described by Pollard et al (14). Actomyosin from quail heart, gizzard, pectoralis and anterior latissimus dorsi muscle and human platelets was extracted with 0.6 M KCl, 15 mM TRIS HCl (pH 7.4), 1mM dithiothreitol (2) and twice precipitated by lowering the ionic strength through a five fold dilution with distilled water. Electrophoresis of protein samples was carried out on SDS polyacrylamide slab gels as described by Laemmli (15)

Production and screening of hybridoma cell lines. Our method for the preparation of hybridoma monoclonal antibodies to the contractile proteins, including myosin, has been previously described in great detail (16). Wells containing positive hybridoma growth were tested for specific antibody after 20 days using a solid phase procedure using ^{125}I Protein A (Amersham). Specifically, QBM myosin in 0.1% SDS was bound to each well of a 96 well plate (MicroELISA Removawell, Dynatech Labs) at an initial concentration of 5 ug/well and incubated overnight at room temperature. After blocking the plate with 5% BSA in PBS for 2 hrs at room temperature, wells were washed with 1% BSA in PBS and incubated with 100 ul of hybridoma culture supernatant overnight at 4°C. Wells were subsequently washed with 1% BSA in PBS and then incubated with ^{125}I Protein A overnite at 4°C. After extensive washing, the wells were removed and counted in a Beckman Gamma 4000 counter. Wells containing five times the background radioactivity were considered positive. Cells from positive wells were recloned and again analysed for specific antibody production at least 3 additional times to assure the establishment of a homogeneous population with a stable genome. Monoclonal antibodies were collected from the supernatant of large volume cultures and concentrated by several cycles of ammonium sulfate precipitation. Further purification of each hybridoma antibody was accomplished by affinity chromatography on Protein A-Sepharose (Sigma) (17). Each line was also injected intraperitoneally into pristane-primed (Aldrich) BALB/C mice and the resulting ascites fluid samples containing hybridoma antibody were collected, clarified and frozen at -70°C.

Characterization of epitope specificity. The antigen binding characteristics of each antibody were determined using immunoblot analysis. Proteins electrophoresed on polyacrylamide gels were electrophoretically transferred onto nitrocellulose paper as described by Towbin et al (18), except that 10% SDS was used in the backing filter to facilitate the transfer of the high molecular weight proteins. These nitrocellulose replicas were either stained with amido black (0.1% in 45% methanol, 10% acetic acid) or reacted with monoclonal antibody and horse radish peroxidase conjugated anti-mouse IgG second antibody (Tago, CA). O-dianisidine HCl was used as substrate.

ELISA assays were performed essentially as described for the ^{125}I Protein A screening procedure, except that an alkaline phosphatase conjugated anti-mouse $F(Ab)_2$ (Litton Bionetics) was used as the second antibody (19).

Phosphatase activity was assayed using Sigma 104 phosphatase substrate (p-Nitrophenyl phosphate, 5 mg/ml) in 10% diethanolamine buffer and reaction product was measured at 414 nm in a Titertek Multiscan photometer (Flow Labs).

Immunoprecipitation of antigens from ^{35}S methionine labelled crude cell extracts was carried out using formalin-fixed SaC (Calbiochem-Behring) essentially as described by Kessler (20). Quail myoblasts cultured as previously described (21) were allowed to fuse and were then pulse labelled with ^{35}S methionine and lysed in 0.5% SDS. Total myotube protein (3 x 10^4 CPM) was incubated overnite with anti-myosin monoclonal antibody QBM-1 in incubation buffer (0.3 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 30 mM TRIS pH 7.6 and 0.1% BSA) then reacted for 5 min with 50 ul 10% SaC in incubation buffer. Following several washes with incubation buffer, the SaC were extracted with SDS sample buffer for 5 min in a boiling water bath, pelleted and reextracted. The supernatants were pooled, assayed for radioactivity, and characterized on SDS polyacrylamide gels using fluorography (22).

RESULTS

Since the myosin which we used as immunogen is composed of a heavy chain as well as 3 light chain subunits, we wanted first to map the epitope for each antibody to a single subunit. Immunoblot analysis of quail myosin (Fig 1) indicates that QBM-1 binds strongly to the 200,000 M_r heavy chain of myosin.

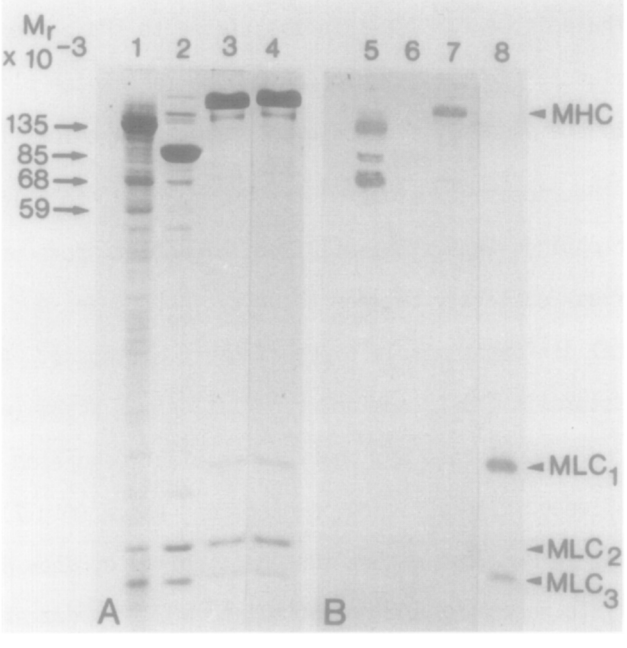

Figure 1. The subunit specificity of each antibody was determined using immunoblot analysis. Insoluble myosin rod peptide fragments (1) or soluble HMM S-1 fragments (2) were generated by papain digestion of insoluble myosin (3), prior to electrophoresis on 12.5% polyacrylamide SDS gels. Myosin (3, 4) and myosin fragments were transferred electrophoretically onto nitrocellulose and incubated with a 1/50 dilution of either QBM-1 (5-7) or QBM-2 (8). Binding of QBM-1 to myosin rod fragments (5) and QBM-2 to MLC_1 and MLC_3 (8) was demonstrated using peroxidase-conjugated second antibody. A = gel stained with Coomassie blue; B = the nitrocellulose immunoblot.

QBM-2 binds to the myosin light chain 1 (MLC_1) and light chain 3 (MLC_3) subunits, but not to MLC_2. In order to map the epitope for QBM-1 within the MHC subunit, synthetic myosin thick filaments were digested with papain and the resulting peptides analysed on immunoblots (Fig 1). Papain digestion of quail myosin cleaves off the globular head of the molecule to generate a soluble S-1 fragment of about 85,000 M_r which is easily separated by centrifugation from the 135,000 M_r insoluble rod portion of the molecule. The insoluble myosin rod is further digested to yield a light meromyosin (LMM) fragment (68,000 M_r) and a 59,000 M_r S-2 fragment in a fashion similar to that described for rabbit myosin (13). Immunoblot analysis of these fragments with QBM-1 indicates no binding of the antibody to the S-1 myosin head fragment. Both the intact myosin rod and its LMM subfragment are recognized by QBM-1, however, as is a 89,000 M_r peptide in the insoluble fraction which may be analogous to the "long LMM" fragment (23). This binding pattern indicates that the epitope for QBM-1 is restricted to the carboxy-terminal third of the molecule (ie the LMM portion of the rod) which plays an important role in the assembly of myosin molecules into thick filaments.

In light of the conserved nature of the MHC rod and, to a lesser extent, the alkali light chains, we further utilized immunoblot procedures to assess the degree of cross-reactivity between these two antibodies and myosins prepared from several different muscle types of the adult quail. Actomyosin prepared from pectoralis (fast) and anterior latissimus dorsi (a predominantly slow muscle) as well as gizzard and heart, were electrophoresed on gradient SDS gels and challenged with antibody as described above (Fig 2). While QBM-1 binds equally well to MHC from either fast pectoralis, or slow ALD, it binds with low affinity to heart MHC and not at all to MHC from gizzard (panel P). QBM-2 is even more discriminating, since it fails to bind to MLC from slow ALD, gizzard or heart muscle under conditions which afford maximal binding to pectoralis fast MLC. Although QBM-2 binds to a 25,000 M_r minor protein component of ALD actomyosin, the M_r of this component is significantly different from ALD MLC_1 (1), as seen in the stained gel (panel A). Since it

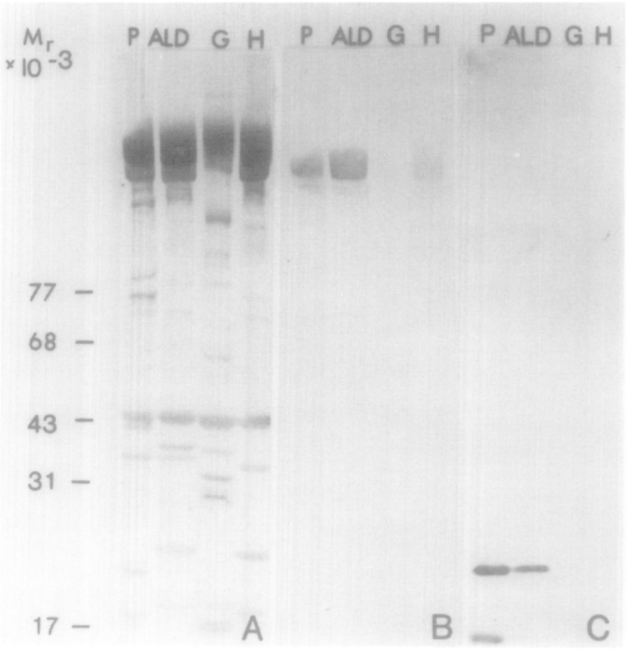

Figure 2. The ability of each antibody to cross-react with myosin from different muscle types was determined using immunoblot analysis. Actomyosins prepared from the pectoralis (P), anterior latissimus dorsi (ALD), gizzard (G) and heart (H) muscle of the same adult quail were electrophoresed on 5-15% gradient SDS gels and electrophoretically transferred onto nitrocellulose. The nitrocellulose was stained with amido black (A) and duplicate strips were incubated with a 1/50 dilution of either QBM-1 (B) or QBM-2 (C).

has been shown that in both the chick and quail the ALD is actually a mixed muscle containing approximately 10% fast muscle fibers (24), this band probably represents binding to a fast myosin MLC contributed by this minor fiber type.

Because of our interest in using these antibodies to study myogenesis *in vitro*, they were examined to determine whether they could discriminate between the non-muscle myosin isoform of undifferentiated myoblasts and the muscle specific isoform of myotubes cultured *in vitro*. This is particularly important in light of recent reports that cultured myotubes contain embryonic forms of both MHC (5,25) and MLC (7,26) which, in some cases, are not recognized by monoclonal antibodies raised to adult myosin (27). Although myosins from all "non-muscle" sources are not identical, available evidence suggests that the myosin components of platelets, fibroblasts and myoblasts are very similar at least with respect to subunit composition and peptide maps (2,28). Since

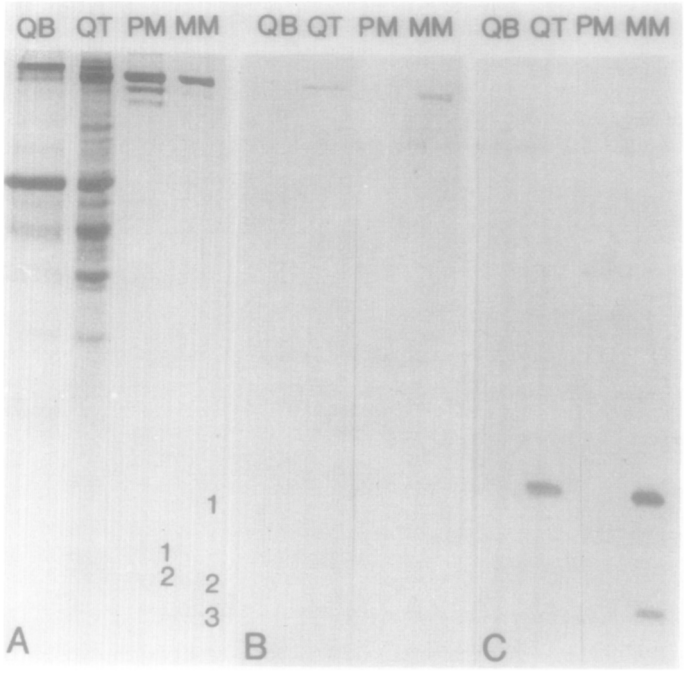

Figure 3. The specificity of the antibodies for muscle-specific myosin was examined using immunoblot analysis of actomyosin and crude cell extracts. Cultures of quail myoblasts (QB) and myotubes (QT) were scraped and homogenized in SDS sample buffer and co-electrophoresed on 12.5% polyacrylamide gels with myosin prepared from human platelets (PM) and adult quail breast muscle (MM). Following electrophoretic transfer, the nitrocellulose replicas were incubated with a 1/50 dilution of either QBM-1 (B) or QBM-2 (C). Panel A shows the stained gel.

large amounts of starting material were needed, human platelets were used as a source of non-muscle myosin and actomyosin. As demonstrated in figure 3, QBM-1 binds equally well to MHC from both cultured myotubes and adult quail breast muscle. It does not, however, cross react with platelet myosin nor with crude extracts of cultured myoblasts. QBM-2 binds equally well to MLC_1 from both cultured myotubes and adult muscle, but exhibits no affinity for platelet myosin or quail myoblast total protein. The absence of binding to MLC_3 confirms earlier reports (1,29) that MLC_3 is absent (or present in very low levels) in avian myotubes cultured *in vitro*.

To determine the degree of cross-reactivity with platelet myosin over a wide range of antibody dilutions, we repeated these studies using a solid phase ELISA. This approach provides a degree of quantitation not easily achieved with the immunoblot analysis and confirms our immunoblot data.

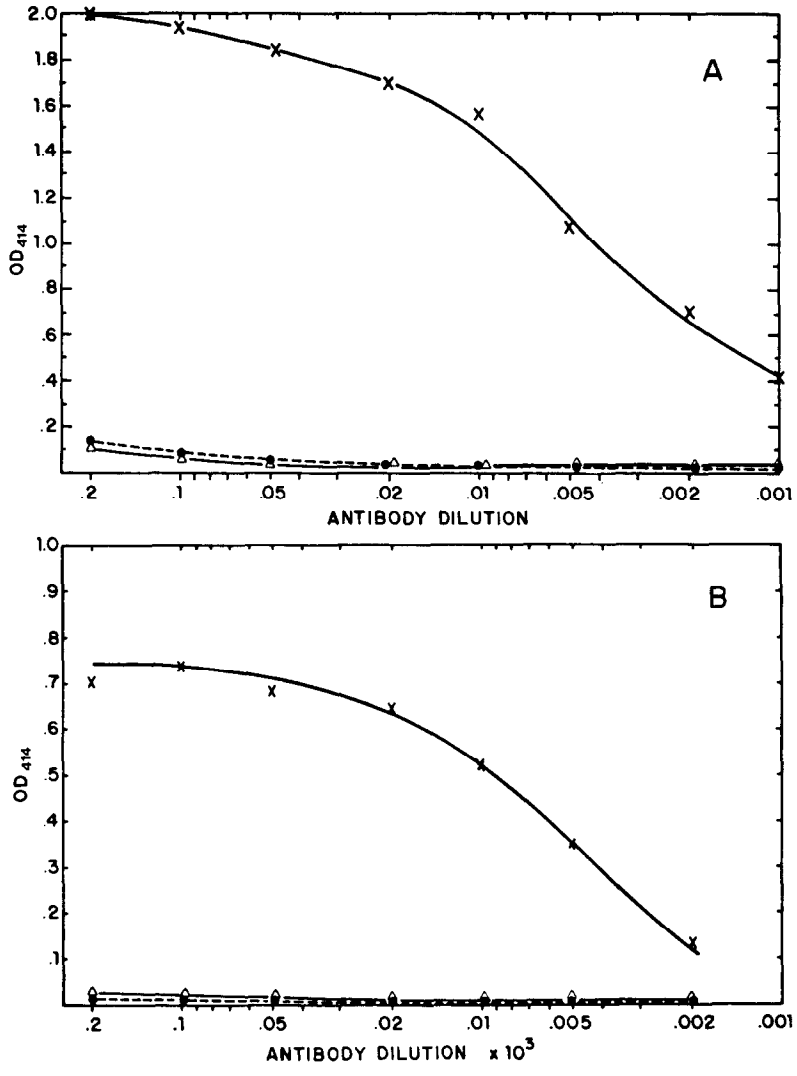

Figure 4. A solid phase, enzyme linked immunosorbent assay (ELISA) was used to quantitate the relative binding of each antibody to purified myosin from human platelets (●) or adult quail breast muscle (x). Wells of a 96 well plate were incubated with 1 g of either antigen, washed and subsequently incubated with dilutions of either QBM-1 (A) or QBM-2 (B) ranging from 1/5 to 1/1000. Monoclonal antibody binding was determined using an alkaline phosphatase-conjugated second antibody. The binding of both monoclonal antibodies to platelet myosin was similar to that obtained with comparable dilutions of IgG from a non-immunized mouse(△).

Neither QBM-1 or QBM-2 bind to platelet myosin to an extent significantly different from that observed with pre-immune mouse IgG (Fig 4).

In order to employ these highly specific monoclonal antibodies in on-going studies of myosin heavy chain synthesis during myogenesis *in vitro* (see ref 30), we wanted to determine whether QBM-1 particularly could be used to quan-

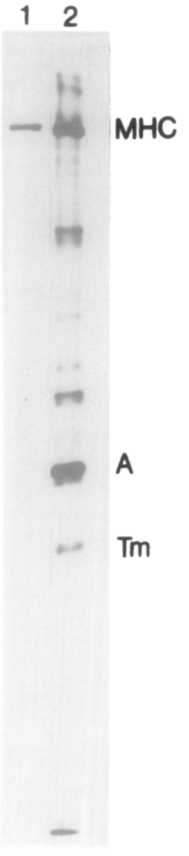

Figure 5. QBM-1 was incubated with extracts of ^{35}S methionine labelled myotube cultures in the presence of 1% Triton X-100, 1% deoxycholate and 0.1% SDS. Immune complexes were adsorbed with formalin-fixed SaC, solubilized and electrophoresed on 8% polyacrylamide gels. Fluorography of the QBM-1 immune complex (1) reveals that only MHC subunit is immunoprecipitated. Lane 2 of the fluorogram contains 10^{-3} CPM of crude myotube extract used in this analysis.

titatively precipitate myosin heavy chain from soluble extracts of myotubes pulse-labelled with ^{35}S-methionine. Using the SaC immunoprecipitation protocol, we find that QBM-1 selectively precipitates MHC from myotube extracts (Fig 5). This precipitation is also quantitative, since the incorporation into MHC by differentiated myotubes as a % of total incorporation determined using this approach is similar to that previously reported using rabbit polyclonal antibody (30).

DISCUSSION

Our finding that QBM-1 is directed against an epitope in the rod portion of the MHC was somewhat unexpected, since this alpha helical region of the

molecule is generally considered to be a poor immunogen (31,32). Based on epitope location, however, it is not surprizing that QBM-1 should cross-react with cardiac MHC, since sequence analysis of cDNA probes to both cardiac and skeletal muscle myosin has suggested strong amino acid sequence homology between these two isoforms in this region (4). Since QBM-1 does not bind to non-muscle myosin under conditions which permit recognition of MHC from slow, fast and cardiac muscle, this antibody can be applied to the analysis of MHC synthesis and accumulation in each of these three muscle types. Of even greater importance, is QBM-1's ability to recognize and selectively precipitate MHC from quail myotubes cultured in vitro. We are currently investigating the cell cycle dependence of the activation of MHC synthesis during myogenesis in vitro (30), and the quantitative precipitation of the MHC subunit from extracts of pulse-labelled cells should allow the selective purification and the rapid quantitation of MHC synthesis. Our previous studies have required densitometry of autoradiograms or the excision of the MHC subunit from the gels to achieve quantitation.

Our conclusion that QBM-2 recognizes an epitope common to MLC_1 and MLC_3 is supported by the sequence homology of these two subunits which has been demonstrated in the chicken and rabbit. Frank and Weeds (33) have shown that myosin alkali light chains of rabbit fast skeletal muscle have identical sequences over their carboxy-terminal 141 residues and that there is an additional sequence of 41 residues at the amino-terminal end of MLC, which accounts for the difference in M_r between the two light chains on SDS gels. A similar homology between alkali light chains has also been reported in the chicken (34). On the basis of the total conservation of the carboxy-terminal sequences between MLC_{1f} and MLC_{3f} in chicken and rabbit, Matsuda et al (34) have proposed that the constant portion of these proteins originates from a single gene, with the different amino terminal portions of MLC_1 and MLC_3 resulting from differential splicing. Cross-reactivity of QBM-2 with both MLC_1 and MLC_3 indicates that the alkali light chains of quail skeletal muscle are also closely related and that the common epitope may lie in the

conserved carboxy-terminal portion of the proteins. The alkali light chains also share significant sequence homology with the myosin DTNB (or MLC_2) light chain and troponin C (35), but QBM-2 obviously discriminates between these other members of the troponin C superfamily (36) from skeletal muscle. The inability of QBM-2 to recognize the analogous light chains from gizzard, heart and slow muscle, as well as from platelets, suggests that although related, these isoforms do not have the same conserved epitope shared by MLC_1 and MLC_3. In fact, recent sequence data from the chicken indicates that MLC_1 from skeletal muscle, gizzard and heart are substantially different (37,38). Because of the remarkable specificity of this antibody, QBM-2 may be very useful in answering questions about the light chain heterogeneity of native myosin , the accumulation of MLC_3 in myotubes cultured *in vitro* , and the switch in fast/slow MLC isoforms which occurs during normal maturation of muscle *in vivo*, but not in cultured cells.

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