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Cloning of a Species-Specific Antigen of *Mycobacterium bovis*

ANTHONY J. RADFORD,[1]* BEATRICE J. DUFFIELD,[2] AND PHILIP PLACKETT[1]

Division of Animal Health, Commonwealth Scientific and Industrial Research Organisation, Parkville, Victoria 3052,[1] and Oonoonba Veterinary Laboratories, Townsville, Queensland 4810,[2] Australia

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A DNA library from a virulent strain of *Mycobacterium bovis* was constructed in the expression vector λgt11, and the library was probed with antisera to *M. bovis*. Clones expressing *M. bovis* antigens were isolated and characterized by using *M. bovis*-specific monoclonal antibodies that recognize a 22,000-molecular-weight protein (MPB70). MPB70 is a major protein antigen of the vaccine strain of *M. bovis* BCG and of virulent *M. bovis*, the causative agent of bovine tuberculosis. Of 32 clones selected by using polyclonal affinity-purified anti-*M. bovis* sera, 5 were recognized by the anti-MPB-70 monoclonal antibodies, and one monoclonal antibody, SB10, recognized all 5 clones. Characterization of these clones showed that one clone containing a 253-base-pair insert expressed a polypeptide bound by all of the MPB70-specific monoclonal antibodies. Western blots (immunoblots) showed that this cloned protein was recognized by sera from *M. bovis*-infected cattle, although not all cattle with bovine tuberculosis produced antibodies reactive to this clone. DNA sequencing of the clone showed that it coded for 84 amino acids from positions 17 to 114 of the 161-amino-acid protein, with a 16-peptide deletion between positions 79 and 94. Apart from this deletion, there were seven other variations between the cloned sequence and that deduced from *M. bovis* BCG MPB70.

Bovine tuberculosis (BTB) is a major disease of cattle worldwide. In the Americas alone it was estimated to have cost the cattle industry $83 million in 1977 (24). Several nations have mounted or are running campaigns to eliminate BTB, and although these campaigns have drastically reduced the incidence of the disease, none have been totally successful. In many parts of the world there is no concerted effort to control the disease, which poses human, as well as animal, health risks. The causative agent of bovine tuberculosis, *Mycobacterium bovis*, is closely related to *M. tuberculosis*.

Eradication of the disease from cattle has been hampered by the lack of sensitivity and specificity of the bovine skin test currently used for detection of infected animals. A simple serological test for BTB would be the preferred option, but there are two fundamental problems with any test for *M. bovis*-specific antibody. Infected animals generally have low levels of antibody to *M. bovis*, and many of the antibodies produced cross-react with antigens from other environmental mycobacterial or nocardial species (4). Enzyme-linked immunoassays using *M. bovis* protein extracts as antigens have been used to detect infected cattle (18, 23), but these tests using crude antigens appear to lack sufficient specificity or sensitivity to be acceptable for use in an eradication campaign (2). These problems are common to the serological diagnosis of all mycobacterial infections. In an attempt to overcome them for the diagnosis of leprosy and human tuberculosis, the antigens of *M. leprae* and *M. tuberculosis* have been cloned into the expression vector λgt11 (25, 27). This has enabled the isolation of recombinant antigens specific to these organisms and detailed mapping of specific *M. leprae* epitopes (12). T-cell-mediated immune responses have also been analyzed by using recombinant *M. tuberculosis* and *M. bovis* BCG antigens (5, 15).

In an analogous fashion, this work describes the cloning of *M. bovis* AN5 DNA into λgt11 and the detection of clones expressing specific antigens of *M. bovis*. With *M. tuberculosis* and *M. leprae*, a great deal of interest has centered on the 65-kilodalton antigen, since in both species this protein has been found to be a major stimulant of T-cell reactivity in patients infected with those organisms (5, 15). A large number of specific monoclonal antibodies (MAbs) raised against *M. tuberculosis* and *M. leprae* are directed against this antigen, although it also possesses epitopes cross-reactive with a wide range of other mycobacteria (6). However, in this study the antigen of primary interest was the protein MPB70 (7, 8, 13, 14). This major antigen forms up to 10% of the protein excreted by some *M. bovis* BCG strains in culture (8, 14) and possibly as much as 23% (1). Most of its amino acid sequence has recently been determined by using peptide sequence analysis on MPB70 obtained from a BCG strain (16). MPB70 contains epitopes specific for the *M. bovis-M. tuberculosis* complex, as determined by MAbs (P. R. Wood, J. Ripper, A. S. Radford, P. G. Bundesen, D. B. Rylatt, L. E. Cottis, M. John, and P. Plackett, submitted for publication), and a significant proportion of antibodies in *M. bovis*-infected cattle recognize MPB70 (T. Fifis, personal communication). Specific delayed-type hypersensitivity responses are also mounted against MPB70 in sensitized animals (14), and studies in our laboratory showed that MPB70 stimulates T-cell proliferation in peripheral blood lymphocytes from BTB-infected cattle, although with lesser efficiency than *M. bovis* purified protein derivative (PPD). Thus, isolation of recombinant antigens carrying the specific epitopes of MPB70 was a primary goal in the development of improved diagnostic tests for BTB in cattle. MPB70 is also a major immunogen of several *M. bovis* BCG strains (13); however, its protective significance in BCG-vaccinated individuals is unknown.

MATERIALS AND METHODS

Phage and bacteria. λgt11, *Escherichia coli* Y1089, and *E. coli* Y1090 have been previously described (26), as has the pEX expression vector (22). *M. bovis* AN5 was obtained from the Commonwealth Serum Laboratories, Parkville,

* Corresponding author.

Australia. The Commonwealth Serum Laboratories obtained the strain from the Ministry of Agriculture Weybridge Laboratories, England, in 1973. Subsequently, it has been stored in freeze-dried ampoules.

Enzymes. All enzymes were purchased from Promega Biotec. Except where specifically mentioned, all of the DNA techniques used were as described by Maniatis et al. (10).

MAbs. MAbs to *M. bovis* were a generous gift from Agen Biomedical Australia Ltd. The properties of MAbs SB1 to SB10 are to be published elsewhere (Wood et al., submitted).

Isolation of *M. bovis* DNA. *M. bovis* AN5 was grown on BAI medium (17) for 6 weeks, after which cells were harvested by centrifugation. DNA extraction was essentially by the method of Shoemaker et al. (21).

Construction of the *M. bovis* library in λgt11. *M. bovis* DNA was sonicated briefly (~3 s) at low power, giving DNA fragments ranging in size from 2 kilobase pairs to 200 base pairs (bp), as assessed by agarose gel electrophoresis. DNA methylation, flushing with T4 DNA polymerase, and addition of *Eco*RI linkers were by the protocol of Young et al. (27). Elimination of excess *Eco*RI linkers after linker ligation and *Eco*RI digestion was achieved by using gel filtration (Superose 12 column; Pharmacia FPLC), and the eluate was monitored by UV absorption. The DNA was then ethanol precipitated and suspended in TE buffer (10), and 0.5 μg was ligated with 1 μg of dephosphorylated *Eco*RI-digested λgt11 (Promega) overnight at 4°C. Phage packaging was in Stratagene gigapack extracts.

Preparation of affinity-purified antibody to *M. bovis*. An emulsion of approximately 2×10^9 heat-killed mycobacterial cells in Freund incomplete adjuvant was used for hyperimmunization of rabbits. Rabbits received two inoculations, 35 days apart. Blood was taken from the ear of each rabbit 10 days after the second inoculation. Sera were collected, and the titer to *M. bovis* PPD (Commonwealth Serum Laboratories) was determined by enzyme-linked immunosorbent assay. Rabbit sera used in affinity purification gave titers of 1:40,960, as determined by enzyme-linked immunosorbent assay. An *M. bovis* affinity column was prepared by coupling *M. bovis* PPD to Sepharose 4B by the method of March et al. (11). The column (20 ml) was equilibrated with 3 volumes of affinity buffer (0.1 M Tris, 0.5 M NaCl, 0.1% Tween 20), 5 ml of rabbit serum was pumped through, and the column was then washed with 3 volumes of affinity buffer to elute unbound material. Bound substances were then eluted with 2 M glycine hydrochloride (pH 2.5) into 2 M Tris, and the eluate was monitored by UV absorption. Eluted protein was concentrated by using an Amicon concentrator and washed with phosphate-buffered saline (PBS), and the protein concentration was determined by the method of Bradford (3).

Screening of the λgt11 library. Filters were taken from 85-mm-diameter phage overlay plates prepared as described by Young et al. (27). Immediately after removal from the plates, filters were washed for 10 min in PBS–0.1% Tween 20 (PBST) and then blocked in PBS–5% bovine lacto transfer technique optimizer (skim milk) for 1 h. Affinity-purified antibody to *M. bovis* was added to 3.5 μg ml^{-1}, and the filters were shaken gently overnight to 4°C. The filters were then sequentially washed for 10 min in PBS, PBST, and PBS, after which swine anti-rabbit horseradish peroxidase conjugate was added tin PBST (Daiko; 1/300) and the filters were incubated with gentle rocking for 2 h. Washing was repeated, and 4-chloro-1-naphthol substrate was added. Color development was complete after 15 min. The filters were then washed in distilled water and dried.

Plaques corresponding to reactive spots on the filters were picked off and placed in 1.0 ml of SM medium (10). Purification was by spotting of 5-μl samples from serial dilutions of the original phage suspensions on lawns of *E. coli* Y1090 for further antibody probing, after which single reactive plaques were picked, suspended, and streaked on lawns of *E. coli* Y1090.

Lysogens were made from purified phage preparations by mixing *E. coli* Y1089 with phage at a ratio of between 1:5 and 1:10, respectively, incubating the mixture at 30°C for 1 h, and then spotting 10 μl of the mixture on Luria-Bertani plates for overnight incubation at 30°C. Colonies were then picked and streaked, and lysogeny was checked by growing single colonies at 30 or 42°C. We prepared lysogen colonies for antibody probing by growing lysogens on nitrocellulose filters for 3 h at 30°C and then transferring the filters to a plate containing 10 mM isopropyl-β-D-thiogalactopyranoside and incubating them at 42°C for 3 h. The filters were then placed colony side up on blotting paper soaked with 1% sodium dodecyl sulfate for 15 min, followed by exposure to a chloroform-saturated atmosphere for 15 min, after which the filters were processed and probed as were the filters taken from induced phage spots.

When MAbs were used for analysis of isolated clones, preparation of filters was identical to the method described. MAbs were substituted for affinity-purified serum (1/2,000 ascites), and anti-mouse alkaline phosphatase conjugate (Promega; 1/5,000) was used for detection. Filters were developed in 5-bromo-4-chloro-3-indolyl-phosphate–Nitro Blue Tetrazolium substrate.

Protein electrophoresis and Western blotting (immunoblotting). Protein samples were solubilized by heating at 100°C for 5 min in Tris hydrochloride (pH 6.8) containing 2% sodium dodecyl sulfate, 5% (vol/vol) glycerol, and 0.002% (wt/vol) bromophenol blue. Vertical slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (9), in 12% acrylamide gels. Proteins were transferred to nitrocellulose by electroblotting overnight. Probing with MAbs (1/2,000) was done by the protocol used for phage spots. Probing with cattle sera was done at 1/50 dilution in PBST for 2 h, and the sera were absorbed with pEX-derived protein bound to nitrocellulose for 1 h before use. Bovine antibodies were detected by using a peroxidase-conjugated antibovine immunoglobulin MAb (Australian Monoclonal Development Ltd.).

Sequencing. Sequence analysis was done by the primer extension dideoxy termination method of Sanger et al. (19) after subcloning in M13tg130 (Amersham Corp.); the products of the sequencing reaction were analyzed on 6% polyacrylamide–8 M urea gels.

RESULTS

Construction of a λgt11 library of *M. bovis*. *M. bovis* AN5 DNA was extracted, sonicated, and cloned into λgt11. Australian PPD for use in the bovine caudal-fold skin test is isolated from *M. bovis* AN5, and since PPD is a proven diagnostic reagent, this was the strain chosen for extraction of DNA for cloning. The AN5 library contained 2.5×10^6 independent PFU, of which 90% were recombinant as assessed by β-galactosidase inactivation. The sizes of the inserts, as measured by restriction digestion and agarose gel analysis of the library, ranged from 200 to 900 bp.

Probing the *M. bovis* AN5 library with polyclonal sera. Twenty filters representing 5×10^4 PFU per filter (10^6 PFU

overall) were probed with affinity-purified polyclonal anti-*M. bovis* AN5 serum. Between two and eight reactive phage plaques were found on each filter, and these were picked off the plates and further purified. The signal strength of each plaque varied greatly, ranging from barely perceptible dots to conspicuous spots. Not all of the filter spots revealed further reactions on phage purification; of the 88 spots found on the original filters, 32 phage gave consistent signals and passed through primary and secondary purification steps. Very little background due to *E. coli* cross-reactive antibody was found by using the affinity-purified antibodies, in contrast to the situation when unpurified sera from either rabbits or cattle were used. Absorption with *E. coli* lysate either bound to nitrocellulose or added directly to serum was far less effective in reducing background signal than the use of affinity-purified antiserum.

Analysis of recombinant clones with MAbs. Purified phage preparations and lysogens of the λgt11 clones reactive with polyclonal antibody were probed with *M. bovis-M. tuberculosis*-specific MAbs SB1 to SB10 in phage spot and colony assays to check for the presence of the specific epitopes.

Of the 32 recombinant phage, 5 expressed antigens that were recognized by at least one of the MAbs when phage spots were probed, although as might be expected of antigens derived from a randomly cloned library, not all of these five clones were recognized by all of the MAbs (Table 1). Since MAbs SB1 to SB10 are known to recognize three separate epitopes on the protein antigen MPB70 (Wood et al., submitted), this is indicative that the recombinant antigens recognized by the MAbs are coded by clones derived from separate ligation events carrying inserts representing different sections of the MPB70 gene.

Of the five λgt11 clones recognized by the MAbs, two, designated pB3C and C4a, displayed affinity for all of the MAbs in the panel, with the exception of MAb SB9, which did not recognize clone pB3C. Agarose gel analysis showed that clone C4a had an *Eco*RI fragment insert of ~250 bp. This was the smallest inserted fragment found that carried all of the specific epitopes and was capable of coding for less than half of MPB70 (molecular weight, 22,000). As such, it was possible that this clone carried none of the cross-reactive epitopes present on MPB70 (7) and might prove useful as an antigen in serological diagnosis of BTB. Harboe et al. (8) found that MPB70 had only limited cross-reactivity, but previous work in our laboratory, using MPB70 derived from a culture of *M. bovis* as an enzyme-linked immunosorbent assay antigen, showed that it was not substantially more specific for detecting *M. bovis*-infected animals than was crude *M. bovis* culture filtrate.

TABLE 1. Recognition of *M. bovis* clones by SB MAbs[a]

MAb	Reactivity with the following clones:				
	pB2a	pB3c	C4a	XB2a	XC2a
SB1	−	+	+	−	−
SB2	−	+	+	+	−
SB3	−	+	+	−	−
SB4	−	+	+	−	−
SB5	−	±	+	−	−
SB6	−	+	+	+	−
SB7	−	+	+	+	+
SB8	−	±	+	−	−
SB9	−	−	+	−	−
SB10	+	+	+	+	+

[a] Reactivity was assessed by probing of phage spots. Drops (5 μl) containing ~10^3 phage were spotted on *E. coli* Y1090 lawns, and filters were prepared and probed as described in the text. The symbol ± indicates a weak reaction.

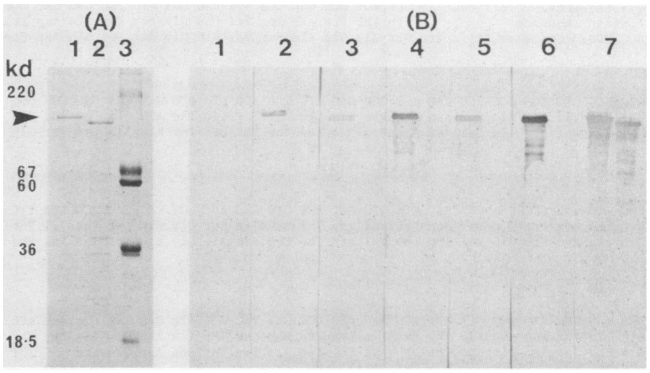

FIG. 1. Western blot probing pEX C4a fusion protein with sera from BTB-infected cattle. (A) Coomassie blue-stained gel. Lanes: 1, pEX C4a fusion protein (arrowhead); 2, pEX cro-β-gal protein; 3, molecular weight markers (Pharmacia; high molecular weight). (B) Western blots of the gel in panel A. Lanes 1 to 5 were probed with sera from naturally infected cattle. Lane 6 was probed with MAb SB10, and lane 7 was probed with an anti-β-gal MAb. Breakdown products of lower molecular weight can be seen reacting below the fusion protein band. kd, Kilodaltons.

Another factor which dictated the choice of clone C4a was the fact that λgt11 clones pB2a and pB3C had only one *Eco*RI cut site rather than the two that would enable clean excision and subcloning of the fragments.

To facilitate serological analysis, the C4a insert was cloned into the high-expression pEX vector (22), and expression was confirmed by immunoblotting of colonies with the SB10 MAb. The pEX vectors express cloned proteins as a cro-β-gal fusion protein, insoluble in most aqueous solutions, which allows for a relatively simple initial purification, eliminating most soluble proteins by washing in 1% Nonidet P-40–1% deoxycholate in PBS. After the washed pEX C4a fusion protein was dissolved in sodium dodecyl sulfate loading buffer, the mixture was run on an acrylamide gel before transfer to nitrocellulose. Sera from both *M. bovis*-infected and healthy cattle were used to probe the Western blots. These blots indicated that antibody reactive to the fusion protein was limited to infected animals. However, not all animals with the disease had a detectable antibody response to the cloned protein (Fig. 1). Neither pooled sera from BTB-free herds nor six sera from uninfected cattle in a BTB-infected herd showed reactions with the cloned protein, although it was necessary to absorb out anti-*E. coli* activity in the sera and to use a specific MAb antibovine immunoglobulin conjugate to lower cross-reactivity.

Sequence of clone C4a. Synthetic peptides containing the specific epitopes of MPB70 have diagnostic potential and should be totally free of endogenous cross-reactions. To facilitate this approach, the C4a insert was cloned into phage M13 and the DNA sequence was obtained from both strands. Figure 2 shows the DNA sequence and inferred protein sequence of *M. bovis* clone C4a and compares it with the MPB70 sequence obtained by amino acid analysis as described by Patarroyo et al. (16). The insert contains 253 bp of DNA, not counting the linkers, and the clone extends from amino acid 17 to amino acid 114 of the protein with the previously published sequence as a reference frame.

There are eight points of variation between the peptide sequence derived from the DNA sequence of clone C4a and that of the MPB70 protein sequence reported by Patarroyo et

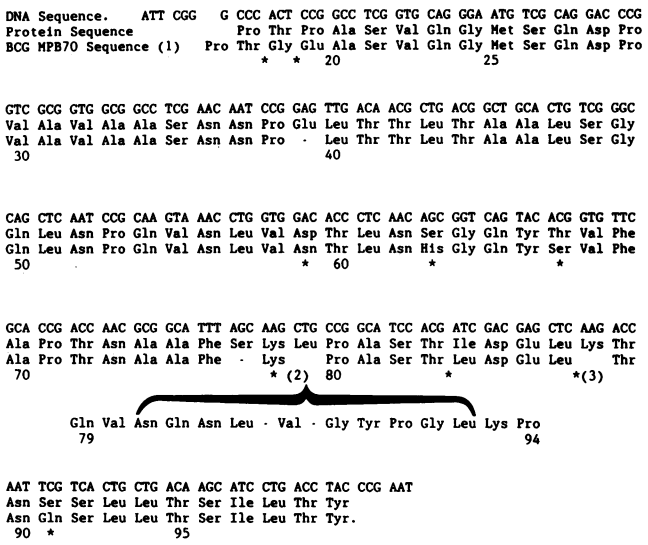

FIG. 2. Sequence of *M. bovis* AN5 of MPB70 from amino acid 16 to amino acid 100. (1), The sequence has been matched to the BCG MPB70 sequence of Patarroyo et al. (15). Asterisks mark points of divergence. (2), The sequence of amino acids 79 to 94 is not present in *M. bovis* AN5. (3), The lysine at position 88 is not found in the peptide sequence of the BCG strain.

al. (16), as well as two points where the DNA sequence deduces amino acids previously uncertain. The most significant difference is the complete absence of amino acids 79 to 94 in the C4a clone.

DISCUSSION

The DNA library described in this work contains inserts in the range of 200 to 900 bp, considerably smaller than the *M. tuberculosis* and *M. leprae* inserts cloned by Young et al. which were size selected during the cloning process (25, 27). Since this range is smaller than the size range of the sonicated DNA, it suggests preferential cloning of smaller fragments. Although this may have affected packaging efficiency, since λgt11 DNAs with inserts of this length are below the optimum packaging size, recombinant phage with smaller inserts still express immunologically reactive fusion proteins. A perceived advantage of having smaller inserts is that they reduce or eliminate the need to subclone DNA from recombinant phage for further analysis.

The λgt11 library described contains around 2.2×10^6 recombinant clones. Probing of 10^6 of these clones with polyclonal serum selected 5 clones that contained the SB10 MAb epitope and were very probably from the MPB70 gene. However, this does not necessarily indicate that this is the incidence of the gene in the library, since only clones that consistently express sufficient antigen to be detected with the antibodies described could be purified.

The lack of two *Eco*RI sites on either side of the inserted *M. bovis* DNA in some clones indicates that some genetic rearrangement other than that expected had occurred in the construction or propagation of the library. This appears to be a common occurrence with the λgt11 system for cloning chromosomal DNA, because Shinnick (20) reported a similar phenomenon when the *M. tuberculosis* library of Young et al. (25) was used.

While not strictly an *M. bovis*-specific antigen, MPB70 and its close relative MPB80 are produced in far greater abundance by *M. bovis* than by *M. tuberculosis*, and because of this quantitative difference, MPB70 can be considered specific to *M. bovis* (7). In skin tests, MPB70 has been found to be a strictly *M. bovis* BCG-specific antigen and elicited a delayed cutaneous reaction in guinea pigs sensitized with *M. bovis* BCG but not in those sensitized with *M. tuberculosis* H37Rv, *M. kansasii*, *M. intracellulare*, or *M. phlei* (14). However, not all strains of BCG produce large amounts of MPB70 (7). The identification of the region of the MPB70 gene that codes for epitopes limited to *M. bovis* is essential to the development of improved diagnostic tests for BTB.

The sequence of clone C4a described here contains at least three *M. bovis*-specific epitopes, and preliminary work has indicated that these epitopes are recognized by antibodies present in some, but not all, *M. bovis*-infected cattle. Because antibody responses to mycobacterial infections are notoriously uncertain and high levels of antibody are rare in the early stages of the disease, this inconsistency was not unexpected. It is unlikely that reactivity with only one antigen will ever provide accurate information in serological tests for mycobacterial infections. Nevertheless, it is possible that the epitopes found on clone C4a, in conjunction with other epitopes limited to pathogenic mycobacteria, such as those elucidated by Mehra et al. in *M. leprae* (12), will form the basis of a practical serological test for BTB.

However, given the variation of the serological response, it is unlikely that serological tests will give a definitive diagnosis of BTB infection. MPB70 also stimulates cell-mediated immune responses both in vivo (13) and in vitro, and it is foreseeable that either recombinant MPB70 or synthesized peptide fragments of MPB70 could function as antigens in assays for specific cell-mediated immune responses to BTB infection, possibly in conjunction with other antigens.

The sequence of MPB70 described here varies somewhat from that deduced by Patarroyo et al. (16), and it is possible that the C4a clone is not that of MPB70 but of the very closely related antigen MPB80, which may be a posttranslational variation of MPB70 (8). For the purposes of obtaining *M. bovis*-specific epitopes for use in a serological test for *M. bovis* infection, this distinction is largely irrelevant. It is, however, pertinent to an understanding of the immune response to *M. bovis* and BCG vaccinations, because there are notable differences between the molecules described by Patarroyo et al. (16) and that characterized in this work. If both are variants of MPB70, this suggests that there are wide variations between strains in the sequence of this major immunogen of *M. bovis*, since clone C4a was derived from the virulent *M. bovis* AN5, whereas the protein sequence of Patarroyo et al. (16) was obtained from *M. bovis* BCG. Further DNA sequencing of clones reactive with MPB70- and MPB80-specific SB MAbs should resolve this question.

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