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# THE DIAGNOSTIC SIGNIFICANCE OF THE DIRECT ANTIGLOBULIN TEST (DAT) IN ANEMIC DOGS

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ABSTRACT

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The direct antiglobulin test (DAT) was positive in 134 (36.1%) of 371 anemic dogs with internal diseases. Four principal types of reaction were recognized: IgG alone in 15 (11.2%), IgG + C' in 41 (30.6%), C' alone in 74 (55.2%) and IgM + C' in 2 (1.5%). Rarely, IgM and/or IgA reactions occurred in association with strong IgG + C' reactions. In 2 (1.5%) DAT-positive dogs the type of reaction was not clear.

One or more symptoms of hemolysis, such as hemoglobinemia, indirect type hyperbilirubinemia, increased red cell osmotic fragility, and increased fecal urobilinogen excretion, were demonstrated in 84 DAT-positive dogs. These consisted of 10 of 15 dogs with IgG type DAT, 36 of 41 dogs with IgG + C' type DAT, 36 of 74 dogs with C' type DAT and 2 of 2 dogs with IgM + C' type DAT.

Most dogs with IgG + C' type reactions had severe hemolysis, whereas "primary" or "associated" diseases were recognized in only 26 of 56 cases. IgG type incomplete warm antibody, reacting with pooled donor cells, was demonstrated in red cell eluates in each of 3 dogs with IgG type DAT and in 6 of 7 dogs with IgG + C' type reactions. This indicates that dogs with IgG or IgG + C' reactions usually have autoimmune hemolytic anemia.

In dogs with C' type DAT, indications of hemolysis were frequently minimal or absent. Symptoms almost always indicated some "primary" disorder. Diagnoses mainly included infections, inflammatory and neoplastic (especially myelo- and lymphoproliferative)diseases. In only 7 (9.5%) of 74 dogs with C' type DAT no diagnosis other than (transient peracute) hemolytic anemia was made. The results of tests for antibodies in the serum and red cell eluates were always negative in dogs with C' type DAT.

In one dog with hemolytic anemia and C' + IgM type DAT, there was a high titer of IgM cold agglutinins in the serum and in heat eluates.

It is concluded that a positive DAT with anti-IgG antiserum is a strong indication of autoimmune hemolytic anemia but that a reaction of the C' alone type is a rather common phenomenon in canine internal diseases which is seldom associated with serious hemolysis.

## INTRODUCTION

(Auto)immune hemolytic anemia is a proces wherein red cells are destroyed because of their interaction with (auto)antibody. This interaction results in the fixation of the antibody (IgG, IgM, or IgA) and/or components of complement (C') to the red

cell, which can be demonstrated by Coomb's direct antoglobulin test (DAT) (Coombs et al., 1945; Loutit and Mollison, 1946). In this test antibodies, produced in other animals (heteroantibodies) against immunoproteins of the species to be tested, are reacted with washed red cells of the patient. If immunoproteins are fixed to the red cell membrane, agglutination will occur (Rosse, 1975).

By the use of specific antisera containing antibodies to a single immunoprotein, it is possible to discriminate which immunoprotein(s) is/are fixed to the red cell. This knowledge may be of clinical significance since different immunoproteins may indicate different pathogenesis (Chaplin, 1974). As yet, monospecific antiglobulin sera have not been used in dogs. Moreover, positive DAT in dogs has only been described in selected cases in which there was a clinical suspicion of autoimmune hemolytic anemia (e.g. Lewis et al., 1963; Mackenzie, 1969; Bull et al., 1971; Avolt et al., 1973; Kelly and Farrow, 1974; Schalm, 1975, Schalm et al., 1975 pp. 729-734). It is not known if positive DAT in dogs is also associated with conditions other than autoimmune hemolytic anemia or if a positive DAT may be considered evidence of hemolysis.

This study was undertaken to ascertain the incidence of positive DAT in anemic dogs with internal disease, the types of immumoproteins involved and their clinical significance.

## MATERIALS AND METHODS

The normal dogs consisted of 35 healthy experimental dogs of various breeds, including 24 dogs of mixed breeding, aged 1 to 9 years.

The patients consisted of 371 dogs with anemia (packed cell volume less than 40%) not related to trauma, referred to the Small Animal Clinic of the University of Utrecht because of a wide variety of internal diseases. The diagnoses reported herein were established either clinically or at postmortem examination.

Any of the following were considered to be evidence of in vivo hemolysis: hemoglobulinemia (plasma Hb > 3.5 µmol/l), indirect type hyperbilirubinemia (serum bilirubin > 3.4 µmol/l and total-direct (5 min) bilirubin ratio < 0.59) (Slappendel 1978, pp. 45-60), increased osmotic red cell fragility (more than 10 % lysis at 162 mOsm/l) and increased hemolytic index (HI > 0.35) (Slappendel, 1978, pp. 27-44). HI measurements could be performed in 186 of the patients.

The total and direct (5 min) serum bilirubin, the osmotic red cell fragility, and the hemolytic index (HI), defined as the amount of fecal urobilinogen excreted during 24 hours divided by the product of the body weight and the venous Hb, were estimated as described previously (Slappendel, 1978). The blood hemoglobin concentration (Hb) was measured by the cyanmethemoglobin method (Dacie and Lewis, 1970, p. 37) the packed cell volume (PCV) by microhematocrit centrifuge and the plasma hemoglobin (plasma Hb) by a benzidine method (Crosby and Furth, 1956).

The direct antiglobulin test (DAT) was performed in the following manner: Blood was collected in polystyrene tubes containing ethylene-diamine tetra acetic acid (EDTA, 1.5 mg/ml) and within 20 minutes was centrifuged at 1200 g for 10 minutes at 22°C. The red cells were separated and washed four times. Washing was performed with phosphate buffered saline (PBS) consisting of 0.0073 M $Na_2HPO_4$, 0.0013 M $NaH_2PO_4$, and 0.140 M NaCl, pH 7.5. An aliquot of 0.05 ml of a 4-5 % suspension of washed red cells in PBS was added to each of a series of agglutination tubes containing 0.05 ml of doubling dilutions of antiglobulin serum in PBS, prepared by use of a semi-automatic micro pipette. The contents of the tubes were thoroughly mixed and incubated at 37°C for 2 hours. One tube containing red cells in PBS without serum was used as a control for autoagglutination.

Agglutination was evaluated macroscopically and, when necessary, by microscopy. Agglutination occurring in less than 5 consecutive tubes and/or reactions that were barely visible to the naked eye were designated as "weak". The following antisera were used simultaneously in parallel series: anti-dog (total) serum protein, anti-dog (non-heavy chain specific) immunoglobulin G (IgG), anti-dog $C_{3b}$-complement (C'), anti-human (heavy chain specific) immunoglobulin M (IgM) and, in a limited number of cases, anti-dog (heavy chain specific) immune globulin A (IgA). All antisera, except for anti-IgA serum, were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and had been prepared in rabbits. Anti-IgA serum was produced in rabbits immunized with a canine IgA myeloma globulin (Goudswaard et al., 1975). The anti-human IgM serum gave strong precipitation at the IgM position in immunoelectrophoresis with normal dog serum. In order to eliminate hetero-agglutinins, all antiglobulin sera were absorbed with pooled canine red cells before use. After absorption reagents were tested against the red cells of 3-5 randomly selected normal dogs and DAT was always found to be negative.

Cold and warm agglutinating or hemolysing antibodies were demonstrated by adding 1) washed patient red cells, 2) canine blood group A-positive donor red cells and 3) blood group A-negative donor red cells to series of agglutination tubes containing doubling dilutions in PBS of the patient's serum, collected and separated from the cells at 37°C. Three parallel series of tubes were prepared for each type of cells. Agglutination and hemolysis were evaluated macroscopically after 1 hour of incubation of each type of cells at 0-4°C, 16°C and 37°C, respectively (Engelfriet et al., 1968)

In man, warm reactive hemolysis can seldom be detected without pretreatment of the red cells with enzymes such as papaine and bromeline (Engelfriet et al., 1974). In dogs however, even normal red cells are hemolyzed by enzyme pretreatment. Hence this procedure had to be abandoned.

The autoimmune character of warm-reacting incomplete antibodies was investigated as follows: Possibly cell-fixed antibodies were eluted from the patients red cells by ether (Rubin, 1963). 0.05 ml of pooled red cells was incubated for 1 hour at 37°C with 0.25 to 0.50 ml of the eluate. The cells were then washed 4 times with

PBS and tested as in the DAT. When the test could not be performed immediately, the eluates were stored at -25°C. Heat eluates were prepared for the identification of cold reacting antibodies. The technique was performed in 2 dogs and described in detail elsewhere (Slappendel et al., 1975).

RESULTS

Normal dogs

DAT was negative with anti-total serum protein reagent and with monospecific anti-IgG, anti-IgM, anti-IgA and anti-C' sera in all 35 normal dogs tested.

Cold/warm reactive agglutinins/hemolysins

The majority of the sera from the normal dogs agglutinated autologous red cells at 0-4°C but never in dilutions exceeding 1:8. At 16°C a few sera caused agglutination but only when undiluted. No agglutination was observed at 37°C. Hemolysis was usually visible at 37°C in dilutions up to 1:4, but not at low temperatures.

Patients

DAT was positive in 134 of 371 anemic patients (Table I). A weak positive DAT with anti-total serum protein, but without concurrent reactions with anti-IgG, anti-IgM, or anti-C' reagent, was observed in 2 patients with lymphoid neoplasma, one of which also had a seminoma and a Sertoli cell tumour. IgA antibody was not assayed in these dogs because anti-IgA serum was not yet available.

In the remaining 132 DAT-positive dogs, four main types of reaction were recognized: IgG alone, C' alone, IgG + C' and IgM + C' (Table 1).

Sporadically, positive reactions with anti-IgM and/or anti-IgA sera were observed in dogs with very strong IgG + C' reactions. These disappeared within few days, whereas the IgG + C' reactions remained positive for longer periods. The phenomenon was observed for IgM and IgA in 2 dogs with idiopathic immune hemolytic anemia and for IgM in one dog with demodicosis and multiple abscesses.

In 2 dogs, weak transient IgM reactions occurred in combination with strong C' type DAT (Table 1). The serum of these patients agglutinated both autologous and homologous red cells at low temperatures. In one of these dogs, blood group A-positive as well as blood group A-negative donor cells were agglutinated at 0-4°C in serum dilutions up to 1:64, but not at 16°C. This was observed on two consecutive days but had disappeared few days later. The first day, autologous red cells were agglutinated by the serum in dilutions up to 1:16.000 at 0-4°C but this could not be

TABLE I

The incidence of various types of DAT and their relation to in vivo hemolysis in 371 anemic canine patients

| | | DAT | | hemolysis | |
|---|---|---|---|---|---|
| | | strong | weak | yes | no |
| negative DAT (anti total) | 237 | - | - | 77 | 160 |
| positive DAT (anti total) | 134 | 95 | 39 | 84 | 50 |
| anti IgG | 15 | 12 | 3 | 10 | 5 |
| anti IgG + C' | 41 | 37[×] | 4 | 36 | 5 |
| anti C' | 74 | 44 | 30 | 36 | 38 |
| anti IgM + C' | 2 | 2 | - | 2 | - |
| undefined | 2 | - | 2 | - | 2 |

Chi-square test (with Yates correction) for in vivo hemolysis:

neg. DAT vs pos. DAT : $P < 0.005$ neg. DAT vs anti C' : $P < 0.025$

neg. DAT vs anti IgG : $P < 0.025$ anti IgG vs anti IgG + C' : ($P > 0.05$)

neg. DAT vs anti IgG + C' : $P < 0.005$ anti C' vs anti IgG + C' : $P < 0.005$

[×] incidently associated with anti IgM and/or anti IgA reactions.

reproduced the next days. Moreover, agglutination was absent at 16°C and attempts to isolate cold reactive antibodies from heat eluates were unsuccessful. In the other dog, an IgM type cold autoantibody could be demonstrated unequivocally. The clinical, immunohematological and pathological data on this patient have been presented elsewhere (Slappendel et al., 1975).

Sera of 47 dogs with C' type DAT were also tested for cold and warm agglutinating and hemolysing antibodies. Occasionally agglutination was seen at 0-4°C in sera diluted 1:16 and 1:32, but none of these sera showed positive reactions at 16°C and they were therefore considered negative. There was also no significant hemolysis.

Warm and cold agglutinating/hemolysing antibodies were also assayed in 18 dogs with IgG + C' type DAT and in 5 dogs with IgG type DAT. The results were negative.

Spontaneous agglutination of thoroughly washed patient red cells at 37°C in PBS occurred in 3 dogs. Such agglutination obviates the interpretation of the DAT, but in all cases the phenomenon subsided within a few days and DAT was then invariably of the IgG + C' type.

Incomplete warm auto-antibodies were demonstrated in the ether eluates in 3 of 3 dogs with IgG alone type DAT and in 6 of 7 dogs with IgG + C' type DAT but not with C' type DAT.

The incidence of in vivo hemolysis in dogs with positive DAT was 63%. The relative incidence of hemolysis in dogs with various types of DAT is presented in Table I. Hemolysis was the main clinical problem in the majority of dogs with IgG or IgG + C' type DAT. In patients with the C' alone type reactions, the clinical picture was usually dominated by symptoms directly related to the primary disease; there were no symptoms of hemolysis in 51% of these dogs and in the remainder they were

often minimal. However, the incidence of hemolysis in dogs with C' type DAT was still significantly higher than in DAT-negative dogs (Table I).

TABLE II

Incidence of positive DAT in 371 anemic dogs with internal diseases.

| | n | pos. DAT | type of reactions IgG | IgG + C' | C' | IgM + C' | UN* |
|---|---|---|---|---|---|---|---|
| (Sub)tropical infectious diseases | 18 | 15 | 2 | 1 | 12 | | |
| Other infectious, inflammatory and granulomatous diseases | 74 | 24 | 3 | 7 | 13 | 1 | |
| Lymphoid neoplasms | 49 | 17 | 2 | 3 | 11 | | 1 |
| Myeloid neoplasms | 4 | 3 | | | 3 | | |
| Mast cell tumours | 4 | 1 | | | 1 | | |
| Other malignancies | 40 | 8 | | 2 | 6 | | |
| Benign cysts and tumours | 5 | 0 | | | | | |
| Unidentified tumours | 6 | 2 | | | 2 | | |
| SLE and SLE-like diseases | 7 | 4 | 1 | 1 | 2 | | |
| Endocrine disorders | 14 | 0 | | | | | |
| Splenic torsion/thrombosis | 2 | 2 | | | 2 | | |
| Thrombocytopenic purpura | 16 | 1 | | | 1 | | |
| Pure red cell aplasia | 6 | 1 | | 1 | | | |
| Panmyelopathy | 4 | 0 | | | | | |
| (Presumed autoimmune) hemolytic anemia | 37 | 32 | 6 | 18 | 7 | 1 | |
| Various and complicated diseases | 22 | 6 | 1 | 2 | 2 | | 1 |
| No diagnosis (tropical residency) | 15 | 11 | | 5 | 6 | | |
| No diagnosis (others) | 48 | 7 | | 1 | 6 | | |
| | 371 | 134 | 15 | 41 | 74 | 2 | 2 |

* UN = unspecified (positive reaction with anti-total dog protein antiserum only).

Positive DAT frequently accompanied infectious, inflammatory and/or granulomatous diseases (Table II). DAT was positive in 15 of 18 patients with (sub)tropical infections, including piroplasmosis, leishmaniasis, dirofilariasis and probably Erhlichiasis. In addition, positive DAT was seen in 11 of 15 dogs classified under "no diagnosis", which had been referred because of malaise following residency in (sub)tropical regions (Table II). Most of these dogs had symptoms suggesting occult parasitic infections. Two dogs with strongly positive IgG + C' DAT and severe hemolytic anemia had just returned from a short stay in a subtropical region in which piroplasmosis is endemic. At least one had been infestated with ticks but piroplasmas were not detected in the blood. These dogs have therefore been classified in Table II in the"(presumed autoimmune) hemolytic anemia" category.

The category of SLE and SLE-like diseases consisted of 7 dogs and includes 4 dogs with four or more major criteria of SLE, including positive LE-cell tests and anti-nuclear antibodies (ANA). In the other 3 dogs,three or even more criteria of SLE

were present but repeatedly performed LE-cell tests and ANA assays were negative. Two of 4 dogs with definite SLE showed weak positive DAT which was of the C' alone type.

Dogs in the "no diagnosis" category had anemia and other signs of disease but in vivo hemolysis was insignificant.

Hemolytic anemia without associated disease was diagnosed in 37 of the 371 dogs studied. DAT was positive in 32 of these 37 dogs. C' type reactions occurred in 7, IgG type reactions in 6, IgG + C' type reactions in 18 and an IgM + C' type reaction in 1 dog. DAT was negative in 5 dogs. The 7 dogs with the C' type DAT all recovered within 10 days. The duration of hemolytic anemia in dogs with IgG or IgG + C' reactions ranged from several days to 3 years.

Follow-up studies in patients with positive DAT showed that the type of reaction was very constant in a given patient, even if the reaction was positive for several months or years. In dogs with IgG + C' type DAT, either the IgG type reaction or the C' reaction sometimes became negative before DAT became entirely negative. Very rarely changes of the reaction type were observed in the active stage of the disease but this may have been due to allo-antibody formation following blood transfusions.

## DISCUSSION

The present study was undertaken to determine the incidence of positive DAT in anemic canine patients with internal diseases and to evaluate its clinical significance and that of the immunoproteins involved.

The incidence of positive DAT in the 371 anemic dogs studied was relatively high (36.1%).The immunoproteins involved were almost exclusively IgG and complement, either alone or associated.

Warm type IgM and IgA reactions were rare and occurred exclusively in association with strong IgG + C' type DAT. This may have reflected residual anti-IgG or anti-C' activity in the anti-IgM and anti-IgA reagents, but this is unlikely because both the anti-IgA and anti-IgM sera were made heavy chain specific and because IgA and IgM reactions were negative in the great majority of dogs that had strong IgG + C' type reactions. The rare occurrence of IgM and IgA autoantibodies, mostly associated with IgG + C' type DAT, has also been reported in man (Dacie and Worlledge, 1969; Mueller-Eckhardt and Kretschmer, 1972; Engelfriet et al.,1974; Habibi et al., 1974). Their clinical significance is not clear.

In two dogs, IgM type DAT, although poorly reproducible, was observed in association with strong C' type DAT. In both cases, autologous and heterologous red cells were agglutinated at $4^{\circ}C$ at high serum dilutions, but the presence of cold reacting (IgM) antibody was unequivocally demonstrated in only one of these dogs, as was described elsewhere (Slappendel et al., 1975).

Since IgM cold antibody will elute from the cells at $37^{\circ}C$, C' alone type DAT,

rather than reactions with anti-IgM serum, may indicate cold agglutinating antibody. Tests for cold agglutination or hemolysis were negative, however, in all 47 dogs with C' type DAT tested. It must be concluded therefore that significant titers of cold antibodies are very rare in anemic canine patients.

Cold agglutination tests were also negative in 18 dogs with IgG + C' type DAT and in 5 dogs with IgG type DAT that showed spontaneous agglutination of red cells. Hence it must be also concluded that the spontaneous agglutination that can often be observed in dogs with AIHA is not due to cold agglutinins, as has been suggested (Schalm, 1975; Schalm et al., 1975, p. 443), but is caused by high titers of incomplete warm reacting antibodies, as has been described in man (Swisher, 1972).

In DAT-positive dogs, C' alone type DAT was most frequent, but reactions were often weak (Table I). In dogs with this type of reaction, signs were mainly related to the primary disease and hemolysis was frequently minimal or absent. The complement may have been fixed to the cell membrane by antibodies or antigen-antibody complexes that only temporarily and perhaps accidently contacted the red cell and thus activated the complement system insufficiently to cause significant cell damage. Alternatively, antibodies may have been present on the red cells, but in concentrations too low to be detected by DAT. This is suggested by the finding that C' alone type reactions and IgG + C' type reactions often occurred in the same categories of patients (Table II).

In dogs with anti-IgG or anti-IgG + C' type DAT, perceptible hemolysis was more frequent than in dogs with the C' alone type reaction (Table I). This is consistent with results of experiments in man that demonstrated that C' sensitized red cells, in contrast to IgG coated cells, can normally survive in the circulation (Rosse, 1974).

Demonstration of acquired hemolytic anemia associated with immunoglobulin coating of the red cells may be considered evidence of autoimmune hemolytic anemia, provided that the immunoglobulins have been fixed to the red cells by real autoimmune mechanisms. Antibody may also be fixed to the erythrocyte by exogenous antigen, especially by certain drugs (Petz and Garratty, 1975), but this is difficult to establish. Drug induced immune hemolytic anemia has not been recognized thus far in dogs. Many DAT-positive dogs examined in this study had been treated with drugs, especially antibiotics, before entering the Clinic. In the majority of cases, the history indicated that the hemolytic disease had been present before drug administration was started. In 3 dogs, penicillin-dependent red cell sensitization was suspected, but tests for penicillin antibodies in the serum were negative. Other drugs known to be associated with immune hemolytic anemia in man (Petz and Garratty, 1975) had, to our best knowledge, not been received by our patients, except for phenacetin in one and phenylbutazone in 2 cases.

In man positive DAT may also result from T-antigen activation, a rare phenomenon characteristically associated with sepsis or bacterial contamination of the blood

to be tested. Normal rabbit serum contains anti-T and therefore antiglobulin serum may agglutinate T-activated red cells. Since DAT was always performed in fresh blood, in vitro contamination can be excluded as a cause of postive DAT. Although sepsis was diagnosed in some DAT-positive dogs, it is unlikely that the positive reactions were caused by T-activation. The results with anti-IgM and anti-IgA sera were invariably negative, except for a few temporarily positive reactions in which persisting IgG + C' type DAT indicated that even in these cases anti-T was not involved.

Positive DAT has also been described as a result of aspecific reactions with transferrin attached to the surface of reticulocytes (Petz, 1976). This was not the case in our tests, since DAT was invariably negative in dogs with reticulocytosis associated with nonhemolytic anemia.

Ether eluates were positive for incomplete warm type IgG antibody in 9 of 10 cases in which IgG or IgG + C' type reactions had occurred. This indicates that in an anemic dog a positive DAT with anti-IgG reagent usually indicates a true autoimmunity, and does not imply the fixation of non-immunological protein or exogenous antigen or the modification of an autologous antigen.

## CONCLUSIONS

This study revealed a high incidence (36.1%) of positive DAT in 371 anemic dogs with internal diseases. In 56 (15.1%) of these patients, warm incomplete type IgG antibody was demonstrated alone or in combination with complement. Hemolysis was diagnosed in 46 (82%) of the latter cases and was usually severe. This indicates that a positive DAT of the anti-IgG type alone or in combination with an anti-C' reaction is a strong indication of hemolytic anemia.

In the majority (55.2%) of patients with positive DAT, reactions were of the C' alone type. "Primary" or associated disease was diagnosed in 90.5% of these cases. Of the latter, more than 50% did not have symptoms of hemolysis and in the remainder hemolysis was usually very mild and needed no specific treatment. This implies that a positive DAT caused by C' coating of the red cells is rarely of clinical significance in relation to the diagnosis of immune hemolytic anemia.

It is uncertain whether the 7 dogs with positive C' alone type DAT associated with severe hemolysis of unknown etiology had (auto)immune hemolytic anemia, since (auto)antibodies could not be demonstrated.

There was a high incidence of C' alone type reactions in dogs with infections, inflammatory, granulomatous, myeloproliferative and SLE-like disorders (Table 2) which also included many dogs with immune or chronic nephritis. This suggests that C' alone type reactions may possible be of help in the detection of immune complex diseases, but it is questionable whether antiglobulin sera with both antiglobulin and potent anti-C' activity should be used to confirm the diagnosis

of autoimmune hemolytic anemia, as has been proposed (Swisher, 1972). The use of such sera is probably justified only if monospecific antisera are then used in order to specify the type of immunoprotein involved. Otherwise a high percentage of positive DATs will be encountered and their relation to autoimmune hemolytic anemia will be very uncertain.

Complement fixing IgM cold autoantibodies were demonstrated in one dog, demonstrating that this type of antibody does indeed occur in the dog. Consequently, C' type reactions may indicate autoimmune hemolytic anemia caused by cold agglutinins, but this is probably rare because cold agglutinins could not be demonstrated in the sera of any of the 47 dogs with C' type DAT which were tested.

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