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Carbohydrate Catabolism in Adult *Onchocerca volvulus:* An Immunohistochemical Study

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Abstract: *Onchocerca volvulus* is a parasite responsible for Onchocerciasis whose main pathology is blindness. Existing treatment and control approaches are not entirely successful, with some, fraught with safety challenges. Due to these problems, the need for developing safer and effective drugs to combat the disease has become imperative. However O. volvulus materials are restricted by ethical concerns due to its strict human preference. To overcome these concerns some researchers use animal models of closely related species to obtain biological information on O. volvulus and drugs developed from these sources of information have so far failed to kill the adult O. volvulus. Realistic targets for drug development against O. volvulus could be detected directly in O. volvulus rather than its closely related species. We performed immunohistochemical detection of three major enzymes (G6PD, LDH and PDHK2) involved in carbohydrate metabolism on paraffin processed archival O. volvulus nodules. We observed that up to 64.5% of worms in the paraffin processed nodules had detectable LDH, 61.1% had G6PD and 56.7% had PDHK2 and that most of the enzymes were stored in the muscles of the adult worm. These observations suggest that the adult O. volvulus can operate the glycolytic, Pentose and Entner-Douhoroff pathways either independently or concurrently suggesting that any drug aimed at preventing the adult worm from utilising carbohydrates must target all three enzymes.

Keywords: Antibodies, Antigens, Carbohydrate catabolism, Onchocerciasis, Nodules, Enzymes

1. Introduction

Onchocerciasis, or River Blindness, is a neglected tropical disease caused by the parasitic worm Onchocerca volvulus. *Onchocerca volvulus* [1, 2]. It is one of the major neglected diseases in Sub-Saharan Africa and is estimated to affect approximately 37 million people in the tropics [1]. O. *volvulus* is detected in two forms in humans: the larval stage (microfilaria) responsible for the blindness and the adult male or female stages (macrofilariae). Whereas the larvae (measuring between 220 and 360 um) move freely in the dermis of the skin the much larger adults (measuring up to 5cm for males and about 80cm for females) are encapsulated in host fibrous tissues in the form of nodules [3, 4].

The treatment and eradication of Onchocerciasis has not been a complete success. The ideal treatment requires drugs which will kill the adult worms (macrofilarici Zdes) or permanently eliminate all microfilariae (microfilaricides) including those in gravid adult female worms in the infected individual. There are suggestions that a successful treatment of Onchocerciasis can be achieved by macrofilaricides with or without microfilaricides [5, 6]. Suramin is the most effective macrofilaricide to date. However, the complex administrative regimen and the many side effects associated with the drug do

not encourage its liberal administration as required for onchocercal treatment [7]. An alternative to suramin chemotherapy is large scale surgical removal of nodules containing the adult worms but this procedure is impractical especially where transmission is ongoing [8]. The only safe filaricide is ivermectin which is a microfilaricide and can only control onchocerciasis when it is administered once a year throughout the life span of the adult worm. This approach could take up to 15 years or more considering the reported long life-spans of adult filarial worms [9]. There are two major drawbacks to the administration of ivermectin which could soon affect its effectiveness in the control of onchocerciasis. Firstly, it is anticipated that the repeated administration of ivermectin for several years could lead to parasite resistance since animal models of Onchocerca species have been reported to have developed resistance to ivermectin [10]. Secondly, there are reports that there are complications associated with the administration of ivermectin to individuals co-infected with *O. volvulus* and *Loa loa* [10, 11].

As a result of these difficulties and the suspension of WHO sponsored Onchocerciasis control programmes in endemic areas since 2002 there appears to be a resurgences of the disease in some tropical foci resulting in onchocerciasis being included among 17 diseases in the renewed global interest in a "so called" neglected tropical diseases [12]. These observations suggest that the search for a safe macrofilaricide against *O. volvulus* would continue to be a priority as it has been for the last two decades [12, 13].

One of the factors considered in the selection of compounds for development into drugs against worms is the identification of biological targets within the worms which when inhibited by a candidate drug can lead to the death of the worm. Metabolic pathways in the parasite have not been extensively studied, partly because of the technical problems associated with parasite cultivation and partly because of limited availability of parasite materials. O. volvulus is a strict human parasite and it has been suggested that the limited availability of material from the parasite has contributed to limited availability of direct knowledge of O. volvulus biology for drug development [4]. A number of researchers obtain biological information on O. volvulus from other species of the parasite that can affect animals [14,15]. However, parasites belong to different phyla and the effect of one drug against a particular parasite does not necessarily imply that other parasites will respond similarly to the drug. Several drugs selected based on their effect on animal models or entirely different parasitic species turn to be less effective when used against O. volvulus [16, 17]. It is our view that a direct appraisal of biological targets within the O. volvulus itself could suggest biological targets for inhibition.

We had earlier on demonstrated that long-time inappropriately stored paraffin embedded Onchocerca nodules retain enough antigenicity for immunohistochemical demonstration of some enzyme epitopes [18]. In this study we used immunoperoxidase techniques on stored formalin-fixed paraffin embedded *O. volvulus* nodules to demonstrate the presence and anatomic location of some metabolic enzymes involved in the

breakdown of carbohydrates for the provision of energy in adult *O. volvulus*. The enzymes are lactate dehydrogenase (LDH), pyruvate dehydrogenase kinase-2 (PDHK2) and glucose-6-phosphate dehydrogenase (G6PD). These enzymes are essential in the utilization of glucose in most living organisms and could be the biological targets for inhibition by suitable candidate drugs. This study was part of a larger project which was granted ethics clearance from the protocol review and ethics committee of the University of Ghana Medical School, Accra, Ghana.

2. Materials and Methods

2.1. Test Materials

Thirty paraffin embedded *O. volvulus* nodules were obtained from Onchocerciasis Chemotherapy Research Centre (OCRC) in the Volta Region of Ghana, cleaned and re-embedded using fresh paraffin wax (Leica histowax, mp 57-58°C; Leica Microsystems GmbH, Germany) in freshly labeled cassettes as described in our previous publication [18]. All nodules had previously been fixed in 10% phosphate buffered formalin, dehydrated through ascending grades of ethanol and cleared in xylene. Sections from the re-embedded blocks were stained by haematoxylin and eosin method and examined by light microscopy to confirm the presence of adult *O. volvulus* tissues in the sections (Fig. 1). Further sections were taken from selected nodule blocks for immunohistochemical staining of enzymes A/BHD, SDH and ME1 as well as for negative controls.

2.2. Reagents

Tris-Buffered Saline (TBS) x10 concentrate (Sigma T5912), 3, 3' dimethylaminobenzidine Tetrahydrochloride (DAB) (Sigma D 3939) consists of liquid buffer A (Sigma D6190) and liquid chromogen B (Sigma 6065), 30% hydrogen peroxide (Sigma 95321) and Streptavidin-horseradish peroxidase polymer, Ultrasensitive (Sigma S2438)

2.3. Buffers and Solutions

Citrate buffer (pH 6.0) (Sigma C2488), TBS (pH 7.4) were obtained. Working solution of TBS contained: one part TBS x10 concentrate (Sigma T5912) and nine parts distilled water. Peroxidase-block solution (0.03% H_2O_2 in 95% ethanol): 1 l contains 3ml of 30% H_2O_2 and 997ml of 95% ethanol.

2.4. Primary Antibodies

Mouse anti-lactate dehydrogenase (Sigma HPA L7016), Rabbit anti-glucose-6-phosphate dehydrogenase (Sigma A9521) and Rabbit anti-pyruvate dehydrogenase kinase-2 (Sigma HPA 008287). All reagents, buffers and antibodies were obtained from Sigma-Aldrich Chemie GmbH, (Taufkirchen, Germany).

2.5. Staining Procedure

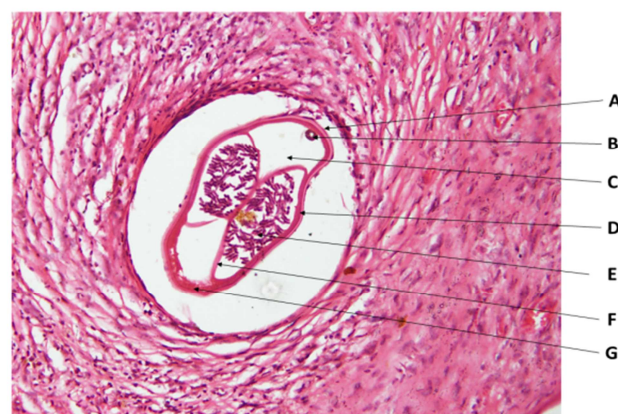The three sets of nodule sections labeled for G6PD, LDH

and PDHK2 and one negative control each were brought down to water and their antigens retrieved in citrate buffer (pH 6.0) using an ordinary floatation water bath at 65°C for 10 minutes [19]. Endogenous peroxidase was blocked for 5 minutes in a freshly prepared peroxidase block solution. They were placed on a humidified staining rack which has been described in our previous publication [19] and reagents applied in the following sequence: TBS (5 minutes), diluted primary antibodies (30 minutes), streptavidin-peroxidase polymer (30 minutes) and freshly prepared working DAB solution (10 minutes). The primary antibodies were applied to their appropriately labeled slides excluding the negative slides where TBS was maintained to avoid drying. The slides were rinsed and flooded with two changes of TBS for 5 minutes each after each application except after DAB where tap water was used to rinse the slides. The nuclei were stained with Mayer's haematoxylin for one minute and blued in tap water for five minutes. The sections were dehydrated in ethanol, cleared in xylene and mounted in DPX.
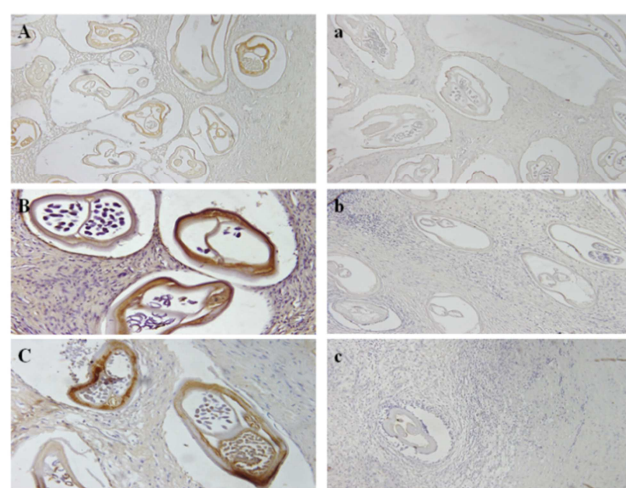
2.6. Examination of Slides

Sections were examined using an Olympus light microscope (Olympus CX31, model CX31RBSF). Negative staining showed no DAB reaction which was comparable with the negative controls while positive staining appeared as golden-brown to dark brown. The staining reactions were scored as follows: 0 - no reaction, 1+ - mild reaction and 2+ - strong reaction. The scoring was undertaken for the various cross-sectional anatomic sites of the *O. volvulus* sections (figure 1). The examination was carried out together by a biomedical scientist (MAS) and a pathologist (EKW) and both agreed on each score before it was recorded. Micrographs were captured using an Olympus digital camera (model DP20-50) mounted on an Olympus microscope (model BX51TF) and composited using a Microsoft Office power point Program and converted to JPEG images (figure 2).

3. Results

In all, 20 worms were scored in 20 nodules for glucose -6-phosphate dehydrogenase, 33 worms in 30 nodules for lactate dehydrogenase and 32 worms in 30 nodules for pyruvate dehydrogenase kinase 2. Three slides (2 G6PD and 1 PDHK2) containing females were declared non-specific and therefore were not scored. Only two male worms were found and both were in the same nodules (KP1 and KP9). No enzyme was detected in any of them. The enzymes were found mainly in the muscles of most of the worms and to a lesser extent in the walls of the genital tract and some microfilaria in the genital tracts. No enzyme deposits were found in the intestines. The results are elucidated in Figure 2 and shown in Tables 1 and 2

Figure 1. A photomicrograph showing the micro-anatomy of a cross-sectional view of a female Onchocerca volvulus in a paraffin section. H & E stain (x200 magnification) A = Cuticle, B = Intestine, C = Pseudo-coelomic cavity, D = Hypodermis, E= Genital tract with microfilaria, F = Genital tract wall, G = Muscle of worm body.

Figure 2. A photomicrograph of IHC stained of sections of O. volvulus showing LDH positive and negative A/a, PDHK2 positive and negative B/b,G6PD positive and negative C/c.

Table 1. Summary of results on LDH, PDHK2 and G6PD.

ITEM	LDH	PDHK2	G6PD
Total Number of worms	33	33	23
Number of male worms	2	2	2
Number of female worms scored	31	30	18
Enzyme positive female worms	20	17	11
Enzyme positive male worms	0	0	0
% of female worm with enzyme	64.5	56.7	61.1

Table 2. Tests of proportional significance of LDH, PDHK2 and G6PD in O. volvulus (30%, 35% and 40%) at 95% and 99% levels of significance.

	NFW	Positive	%	0.30	0.35	0.40
LDH	31	20	64.5	0.001**	0.002**	0.022*
PDHK2	30	17	56.7	0.001**	0.006**	0.028*
G6PD	18	11	61.1	0.002**	0.010*	0.035*

*Significant at 5%; ** Significant at 1%

Analysis of Results

Although immunohistochemistry has become handy in the analysis of stored paraffin embedded tissues, a lot depends on the preparation that precedes the procedure. In formalin fixed paraffin embedded *O. volvulus* nodules unavoidable negativity could occur in two ways. Firstly, enzymes are usually membrane bound in organelles such as lysosomes and mitochondria. To that effect, anoxia in tissues after removal of the tissues from the body or death of the worm may lead to disintegration of membranes resulting in the discharge and subsequent hydrolysis of the enzymes which can lead to negative results. Immediate fixation of the nodules soon after removal may prevent these losses from live worms but not entirely so and definitely not in dead worms. We are aware that up to 50% of worms in harvested nodules from areas where attempts were made to control the transmission may be dead [20, 21].

These were the reasons why worms from different nodules were used for this study instead of relying on a single nodule with one or two worms under the same preservation protocol and the subsequent statistics of proportions. It means therefore that, whereas positive reactions in this study are considered as confirmations of the presence of the targeted enzymes the negative ones do not necessarily denote the absence of the particular enzymes in those worms.

A test of proportions was performed to determine the proportions of the worms the various enzymes could be found using (30%, 35% and 40%) at the test level of 99% and 95% significance.

The assumption was that we expected to find a minimum of 30% of the worms in a sample with any of the three enzymes. The test shows that at 95% confidence level, a significant number of worms ($\geq$ 40%) will be found with LDH, PDHK2 and G6PD (p-value= 0.022, 0.029, 0.035). It can also be reported that at 99% confidence level, up to 35% of worms will be found with LDH (p-value= 0.002) and 30% will be found with all the three enzymes (p-values 0.001, 0.001, 0.002).

4. Discussion

The aim of this study was to determine the presence and locations of three enzymes (LDH, G6PD and PDHK2), involved in the breakdown of carbohydrate for the provision of energy in adult *O. volvulus.* This is to promote a better understanding of carbohydrate catabolism in the worm. It has long been reported that metabolic requirements in filarial worms range from obligate aerobes to those, which can survive for long periods under anaerobic conditions [22]. It is also known that in some filarial species energy generation is related to muscle activity and overall maintenance of viability [22]. This suggests that no single filarial worm can be used as a model for biological activities in all filarial worms and each worm must be studied in its own right in the development of tools to eradicate it.

The success of suramin as a macrofilaricide, although not entirely safe, is reported to depend on its ability to inhibit the activities of malate and lactate dehydrogenases enzymes [23] suggesting that energy production in filarial worms could be associated with their viability. Allowing for unavoidable false negativity due to sensitivity of primary antibodies and preservation of antigens during fixation and processing, a significant number of worms detected in the nodules had all the three enzymes.

Glucose-6-phosphate dehydrogenase was detected in 61.1% of the worms. This enzyme is essential in the pentose phosphate pathway as well as the Entner-Douhoroff pathway where it aids in the conversion of glucose-6-phosphate into 6-phosphoglucono D- lactone [23]. It is the rate-limiting enzyme of these two pathways that maintains the level of reduced co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the supply of reduced glutathione in the cells that is used to mop up free radicals that cause oxidative damage [24]. In red cells of the only host of *O. volvulus* (humans) the G-6-PD /NADPH pathway is the only source of reduced glutathione. The role of red cells as oxygen carriers puts them at substantial risk of damage from oxidizing free radicals except for the protective effect of G-6-PD, NADPH and glutathione. In all organisms the deficiency causes a reduction of NADPH which is necessary for the formation of Nitric Oxide an important antioxidant [25]. Inhibition of G-6-PD therefore will expose the worm to oxidative stress and death.

In terms of energy production, G-6-PD's contribution through the pentose pathway and Entner-Douhoroff pathways are substantial. The Entner-Douhoroff pathway produces 2-keto-3-deoxy-6-phosphogluconate a key intermediate which is then cleaved to form pyruvate and glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate from this reaction is converted to pyruvate in the second phase of glycolysis. If an organism uses the Entner-Douhoroff pathway to degrade one molecule of glucose to pyruvate, it yields one ATP, one NADPH and one NADH. On the other hand, through the pentose phosphate pathway, three molecules of glucose-6-phosphates are converted into two fructose-6-phosphates and one glyceraldehyde-3-phosphate. These can be converted to glucose-6-phosphate and returned to the pentose phosphate pathway where they can be degraded to CO_2 and NADPH while some glyceraldehyde-3-phosphate molecules can be converted to pyruvate through the glycolytic pathway with subsequent production of energy. The presence of glucose-6-phosphate dehydrogenase in adult *O. volvulus* tissues suggests that the two pathways may be operative and glyceraldehyde-3-phosphate and glucose-6-phosphate may be key intermediates in both pathways. In addition, the two pathways have the capacity to metabolize pyruvate, the main product of the glycolytic pathway.

Lactate dehydrogenase (LDH) was detected in 64.5% of the female worms in the nodules. It has long been accepted that most filarial worms use anaerobic glycolytic breakdown of carbohydrates as their preferred route to supply energy for their requirements [26]. This has been the general opinion of most investigators of *O. volvulus* as well and this opinion is

the basis for one of the most dependable viability test on *O. volvulus* during in-vitro drug assessments [27]. In this test the determination of the amount of lactate produced by a female worm within a stipulated period of incubation is used as the measurement of its level of activity. The viability of male *O. volvulus* that cannot produce enough lactate to be measured also depends on the reduction of 3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a purple formazan due to the release of hydrogen ions from metabolic processes going on in the worm. The hydrogen is believed to be released as a result of LDH activity in the worm that produces energy for its motility [28]. In a similar development 2,3-bis (2-methoxy-4-nitrosul-phenyl)-5-(phenylamino)-carbonyl-2 H-tetrazolium hydroxide (XTT) is reduced to an orange formazan by female worms based on the same principle.

Despite the strong acceptance of anaerobic glycolysis as the preferred energy pathway for filarial worms, investigations have shown that anaerobic glycolysis may not be the sole energy producing pathway for filarial worms. This is because, glutamine has been observed as a major source of energy for some filarial worms as well and that a fully oxidative mitochondrial metabolism can be employed for the utilization of this substrate within the worms [23].

The presence of lactate dehydrogenase in a significant number of *O. volvulus* in this study is a clear indication that glycolysis (aerobic or anaerobic) can occur in *O. volvulus.* Each of these pathways eventually results in the metabolism of pyruvate with key intermediates including glucose-6-phosphate, fructose-1- phosphate, fructose 1,-6-biphosphate, glyceraldehy-3-phosphate and phosphoenol pyruvate [23, 28].

Pyruvate dehydrogenase kinase, isoenzyme 2 (PDHK2) was detected in 56.6% of the total number of female worms. Pyruvate dehydrogenase plays a pivotal role in controlling the balance between glucose and fatty acid oxidation, and its activation state is tightly controlled by the balance between specific PDHK (PDH kinase) and PDP (PDH phosphatases) activities. The presence of PDHK in *O. volvulus* tissues therefore means that the pyruvate dehydrogenase complex which operates under aerobic conditions may be operative. This can increase the potential possibility of the worm extending carbohydrate metabolism to the tri-carboxylic acid (TCA) cycle. The key substrate for PDHK activity is pyruvate, the end product of other pathways.

We were unable to detect any of the enzymes in the two male worms found in the nodules, although both appeared histologically viable. We cannot offer a definite explanation for this finding but we believe that the male worms may have been affected by anoxia, which might have led to the release of enzymes during delays in their preservation since they were found deep in the central parts of the nodules. The presence of these three enzymes in significant numbers of the worms shows that adult *O. volvulus* have all the three enzymes and are thus able to switch from one pathway to another with regards to carbohydrate metabolism. This could allow the worms to diverge from chemotherapeutic inhibition of one

pathway to a less affected pathway during the period of inhibition of the more preferred pathway.

5. Conclusion

This study suggests that the adult *O. volvulus* can operate the glycolytic pathway, the Pentose phosphate and Entner-Doudoroff pathways either independently or concurrently to avoid drug inhibition of energy production and subsequent death. Any candidate drug aimed at inhibiting energy generation from carbohydrate substrate in *O. volvulus* must therefore inhibit all three enzymes, G-6-PD, LDH and PDHK2, in the adult worm to be effective.

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