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Prostaglandins E_2 and D_2–regulators of host immunity in the model parasite *Diphyllobothrium dendriticum*: An immunocytochemical and biochemical study

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ABSTRACT

The spectrum of immunomodulating molecules produced by tapeworms is not yet well understood. The aims of this study, on the tapeworm *Diphyllobothrium dendriticum*, were: 1) detection and quantification of prostaglandins (PGs) E_2 and D_2 by high performance liquid chromatography; 2) visualization of PGE_2 and PGD_2 in specific cells, using methods of immunocytochemistry and confocal laser scanning microscopy; and 3) investigation of the ultrastructure of the cells potentially producing PGE_2 and PGD_2. The PGE_2 immunoreaction (IR) was found in the apical terminals of the frontal glands and sensory organs in the tegument and in small neurons belonging to the main cords and commissures. PGE_2-IR partly coincided with α-tubulin-IR. PGD_2-IR occurred in the muscle fibers of longitudinal and transverse body muscles and coincided with phalloidin TRITC staining. Both PGE_2 and PGD_2 were found in the flame cells of the excretory system. Ultrastructural study of the tegument revealed two types of structures that potentially produce PGE_2: ciliated and unciliated free nerve endings and frontal gland terminals reinforced with neurotubules. In the main nerve cords, small neurons were identified as potentially exhibiting PGE_2-immunoreactivity. In homogenates of the plerocercoids, the measured content of PGE_2 and PGD_2 was 33.15 ng mg^{-1} and 1.94 ng mg^{-1} of fresh tissue weight, respectively. We found evidence of PGE_2 and PGD_2 in *D. dendriticum* parasitizing *Coregonus autumnalis* (fish) and proved excretion of PGE_2 and PGD_2 in response to *C. autumnalis* blood serum. Prostaglandins produced by *D. dendriticum* probably play a dual role: 1) PGE_2 and PGD_2 potentially modulate the fish antiparasitic immune response; 2) PGE_2 is presumably necessary for proper development and function of the nervous system, and PGD_2 can act as an antagonist against mediators causing muscle contraction.

1. Introduction

Tapeworms of the genus *Diphyllobothrium* are widely distributed throughout the world, with some being agents of human diphyllobothriasis, one of the most important fish-borne zoonoses caused by a cestode parasite. Humans can contract this parasite by eating raw or partially cooked fish containing *Diphyllobothrium* plerocercoids [1]. In 1999, the worldwide prevalence of human diphyllobothriasis was estimated at 9 million cases [2]. The region near Lake Baikal (Russian Federation) is one area with high endemic prevalence of diphyllobothriasis, among other helminth diseases in humans. The main cause of diphyllobothriasis in this region is *Diphyllobothrium dendriticum*.

During the last 50 years, *D. dendriticum* has been studied as a model organism with respect to ecology, life cycle, morphology, cell biology, and neurochemistry (for background information see [3]). In recent years, there has been enormous interest in understanding the molecular basis of helminth immunomodulation [4]. The capacity of helminths to modulate the immune system underpins their

Abbreviations: CLSM, confocal laser scanning microscopy; IR, immunoreaction; MC-RP-HPLC–MS, microcolumn reversed-phase high-performance liquid chromatography with mass-spectrometric detection; MC-RP-HPLC-UV, microcolumn reversed-phase high-performance liquid chromatography with ultraviolet detection; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; ROS, reactive oxygen species.

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longevity in mammalian hosts [5]. The neurons of helminths can synthesize a wide range of molecules that are identical to the ones functioning in the host organism and, thus, helminths can manipulate vital functions of the host – in particular, its immune responses and behavior [6].

Most published articles on immune evasion mechanisms concern mammalian hosts, and interactions of parasites with fish hosts have not been sufficiently investigated [7]. It has been shown that cestodes can regulate the anti-helminthic immune response of fish via excretory/secretory products [8,9]. However, the spectrum of immunomodulating molecules produced by fish parasites is not well understood [10,11]. Prostaglandins (PGs) are possible candidates for substances that cestodes use for host immunoregulation [12,13]. PGs are small lipid molecules that regulate numerous processes in the body, including neurotransmitter release and modulation of immune function [14]. PGs are synthesized in all tissue types, and each type of prostaglandin may play a different role in different tissue types [15]. PGs produced by parasites are known to be immunomodulating molecules in mammalian parasite infections, and molecules of PGE_2 have been detected in parasitic protozoans [16], trematodes [17], nematodes [18], and cestodes [10,12,13]. PGs appear to play a role in penetration, immune suppression, inflammation, and modulation of host haemostasis [19]. The immunomodulating effect of PGE_2 has been demonstrated *in vitro* on leucocytes of fish (*Gasterosteus aculeatus*) infected and not infected with the cestode *Schistocephalus solidus* [9]. Previously, PGD_2 was detected only in trematodes and nematodes [18,20,21]. It has been shown that PGD_2 enables schistosomes to evade the host immune system by inhibiting the TNF-α-triggered migration of epidermal Langerhans cells [20].

The main aims of our study on plerocercoids of the model organism *Diphyllobothrium dendriticum* were: 1) detection and quantification of prostaglandins E_2 and D_2 by means of high performance liquid chromatography (HPLC); 2) visualization of PGE_2 and PGD_2 in cells by immunocytochemical and confocal laser scanning microscopy (CLSM) methods; and 3) investigation of the ultrastructure of potential cellular sources of PGD_2 and PGE_2. We investigated the distribution of PGE_2 and PGD_2 in different tissues of *D. dendriticum* plerocercoids with specific antibodies and fluorescence immunocytochemical methods. Also, we used HPLC techniques to confirm the presence of PGE_2 and PGD_2 and to determine PGE_2 and PGD_2 concentrations in *D. dendriticum*. In addition, we incubated *D. dendriticum* plerocercoids with the blood serum of their host: the Baikal omul (fish) *Coregonus migratorius.* HPLC methods allowed the detection of accumulating PGE_2 and PGD_2 in culture media during incubation.

2. Material and methods

2.1. D. dendriticum *samples*

Plerocercoids of *D. dendriticum* were obtained from the body cavity of the Baikal omul, *Coregonus migratorius* (Salmoniformes), from Lake Baikal, Russia.

2.2. Incubation of plerocercoids

Plerocercoids of *D. dendriticum* were washed with 0.063% physiological solution for cold-blooded animals. The plerocercoids were then incubated in culture media at 4 °C for 3, 6, 12, or 24 h. Culture media consisted of a 1:1 mixture of Hanks' solution (Sigma Aldrich) and blood serum of *C. migratorius*, supplemented with 10^5 IU L^{-1} penicillin and 100 mg L^{-1} streptomycin [9,22]. Each incubation was performed in separate sterile tube. Each incubation tube contained 5 plerocercoids (average weight of a single plerocercoid was 29.6 mg) and 2 ml of culture media. After incubation, culture media was frozen and stored in liquid nitrogen before MC-RP-HPLC-UV investigation (see 2.6).

2.3. Immunocytochemistry

Freshly dissected plerocercoids were embedded in Tissue-Tek (Sakura Finetek, Torrance, California, CA, USA) and stored in liquid nitrogen. Before staining, specimens were cut with a cryotome (Leica CM1850UV, Leica Microsystems GmbH, Wetzlar, Germany). Sections 8–10 μm thick were placed on slides and fixed in acetone at 4 °C for 5 min (according to the method by [23]). Dried sections were treated with 1% Triton X100 in 0.01 M PBS for 1–2 h and pre-incubated in a mixture of 1% Triton X100 + 1% bovine serum albumin (BSA) in 0.01 M PBS for 3 h. To detect the immunomodulator PGE_2, we used antisera directed towards PGE_2. The polyclonal antibody anti-PGE_2 (Abcam, UK; diluted 1:125) was produced in a rabbit against the prostaglandin E_2. It has been used before to label PGE_2-containing structures in the filarial parasite *Onchocerca volvulus* [24]. To detect PGD_2, we used the polyclonal antibody anti-PGD_2 (LifeSpan BioSciences, USA; diluted 1:125). In addition to anti-PGE_2 and anti-PGD_2, we used the monoclonal antibody anti-acetylated (α-) tubulin (SIGMA, USA; diluted 1:1000), produced in a mouse model against α-tubulin. It has been shown to recognize axons, neurons and sensillae [25,26], gland ducts [27], and flames of flame cells [26,27] in cestodes. To identify the occurrence of PGE_2 in the nervous system, sensillae, gland ducts, and flame cells, sections were double-stained with an antibody cocktail of PGE_2 + α-tubulin and PGD_2 + α-tubulin. Sections were incubated in the cocktail in a solution of 0.01 M PBS + 1% Triton X100 + 1% BSA + 0.03% $NaNO_3$ at 4 °C for 24 h. After incubation, sections were washed in 1% Triton X100 in 0.01 M PBS (6 × 15 min). Bound antisera were detected using Alexa 405-, 488-, 532-, and 635-tagged secondary antibodies (Invitrogen, Carlsbad, CA, USA) diluted 1:800 in the same buffer solution. Sections were incubated in a cocktail of anti-rabbit and anti-mouse secondary antibodies Alexa 405, 532, or Alexa 488, 635 at 4 °C for 2 h. In addition, after washing in 0.01 M PBS + 1% Triton X100 (6 × 15 min), sections were stained with phalloidin TRITC (Invitrogen, Carlsbad, CA, USA) diluted 1:1000 in the same buffer, to investigate muscle innervation through visualization of F-actin. After several rinses in 0.1 M PBS, sections were finally embedded in 50% glycerol in 0.1 M PBS. Control samples were processed in 0.01 M PBS + 1% Triton X100 + 1% BSA + 0.03% $NaNO_3$ at 4 °C for 24 h and subsequently incubated in secondary antibodies at the same concentration as the immunostained sections. No staining was detected in the control sections. Stained sections were examined with laser scanning confocal microscopes LSM-510 Meta (Carl Zeiss Microscopy GmbH, Jena, Germany) and Leica TSC SPE (Leica, Germany). Images were processed with the following programs: LAS AF Lite 1.7.0 (Leica Microsystems, Buffalo Grove, IL, USA), Amira 5.2.2 (FEI, Burlington, MA, USA), and Adobe Photoshop CS2 and 4 (Adobe Systems, Inc., San Jose, CA, USA).

2.4. Transmission electron microscopy (TEM)

Worms were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h and post-fixed in 1% OsO_4 in the same buffer. Subsequently, the material was dehydrated in an ascending ethanol series and stained for 12 h in 1% uranyl acetate in 70% ethanol. The material was then dehydrated in acetone and embedded in Araldite resin that was polymerized at 60 °C. Semi-thin and ultra-thin sections were cut with LKB ultramicrotomes. Semi-thin sections were stained with methylene blue (according to [28]) and examined by light microscopy for identification of target components. Ultra-thin sections were cut to a thickness of 60–70 nm with a diamond knife, mounted on grids, stained with Reynolds' lead citrate and examined