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Research Article

Effect of Phycocyanin in Zymosan-Induced Arthritis in Mice—Phycocyanin as an Antiarthritic Compound

Diadelis Remirez,* Addys González, Nelson Merino, Ricardo González, Odelsa Ancheta, Cheyla Romay, and Sandra Rodríguez

Department of Pharmacology, Centro Nacional de Investigaciones Cientificas (CNIC), Ciudad Habana, Cuba

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT The antiinflammatory effect of a phycocyanin extract was studied in zymosan-induced arthritis model in mice. Four days after the intraarticular injection of zymosan, (15 mg/ml), phycocyanin (25, 50, and 100 mg/kg p.o) was administered to animals for 8 days. The mice were then killed and the synovial fluid measured for β-glucuronidase. Each knee joint was totally removed for histopathological and ultrastructural studies. Phycocyanin significantly reduced the levels of β-glucuronidase that had been increased by zymosan. Histopathological and ultrastructural studies showed inhibition in cellular infiltration and reduction of synovial hyperplasia and synovitis. The antiinflammatory activity exerted by phycocyanin may be due, at least in part, to its antioxidative properties, although inhibitory effects on both arachidonic acid metabolism and cytokine production such as tumor necrosis factor (TNF) may also be involved. To our knowledge, this is the first report on the antiinflammatory effect of phycocyanin in an experimental model of arthritis. Drug Dev. Res. 48:70–75, 1999.

Key words: phycocyanin; antiinflammatory; zymosan; mice; arthritis

INTRODUCTION

Rheumatoid arthritis (RA), is a disease of unknown etiology, characterized by joint inflammation and, in its later stages, cartilage destruction [Bondeson, 1997]. Several inflammatory mediators are involved in RA and include prostaglandins and leukotrienes, tumor necrosis factor (TNF), interleukins (IL)-1 and IL-6, as well as reactive oxygen species (ROS) [Szekanecz et al., 1998].

There are many arthritogenic agents, such as zymosan, carrageenan, and dextran, which induce inflammation characterized in the articular joint, and in particular they activate complement by alternative or the classical pathway and, by means of the cleavage product, C3b. Thus, they induce the secretion of macrophage lysosomal enzymes [Schorlemmer, 1977a,b]. Recently, it was discovered that phycocyanin, a biliprotein found in blue-green algae such as *Spirulina (Arthospira maxima)*, exerts a scavenging action against ROS as well as antiinflammatory activity in various in vitro and in vivo experimental models [Romay et al., 1998a,b; González et al., 1999].

Taking into account these findings, we decided to test phycocyanin extract in arthritis induced by zymosan, an animal model that mimics some of the acute inflammatory responses seen in rheumatoid arthritis [Gegout et al., 1994].

MATERIALS AND METHODS

Animals

Female 6-week-old OF1 mice from the National Center for Production of Laboratory Animals (CENPALAB, Havana, Cuba) were used in this study. They were housed under a 12-h light-dark cycle with room temperature maintained at 23°C, humidity 55%, and food and water available ad libitum. The experiments were conducted

*Correspondence to: Diadelis Remirez, MSc, Centro Nacional de Investigaciones Cientificas (CNIC), Ave 25 y 158, Apdo 6880 Playa, Ciudad Habana, Cuba. E-mail: diadelis@quimica.cneuro.edu.cu

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in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research. All reagents not specifically described were purchased from Sigma Chemical (St. Louis, MO).

Preparation of Phycocyanin Extract

Phycocyanin was extracted from microalgae *Spirulina (Arthospira maxima)*, as described in a Cuban patent [Benitez et al., 1997]. The blue powder thus obtained showed a peak in the visible absorption spectrum of 617 nm, which is very close to that reported for c-phycocyanin [Berns et al., 1989].

Zymosan-Induced Arthritis in Mice

The mice (10 per group) were injected intra-articularly with 10 µl of a 15 mg/ml sterile suspension of zymosan [Keystone et al., 1977]. Four days after zymosan injection, a water solution of phycocyanin (25, 50, and 100 mg/kg) or triamcinolone (10 mg/kg), which was used as a reference compound, were administered orally on a daily basis, from days 4 to 12. Mice were then killed by cervical dislocation and the synovial fluid of knee joints was sampled in order to measure the level of β-glucuronidase enzyme. The knee joints were then totally removed for histological and ultrastructural studies. Appropriate controls with mice treated only with phycocyanin and vehicle were used.

Determination of β-Glucuronidase Activity

β-Glucuronidase activity was measured in the synovial fluid of knee joints of mice [Folliard and Terlain, 1992]. The patellar ligament was cut and the synovial cavity incised, the total synovial fluid was then absorbed by means of small pieces of filter paper (No. 1575-Prolabo). The paper tips impregnated with synovial fluid were cut and deposited at the bottom of tubes containing 0.9 ml of 50 mM acetate buffer, pH 4.5. The enzyme was measured in the presence of its substrate (phenolphthalein mono β-glucuronic acid), 20 mM, after incubation for 17 h at 37°C. Then 2.5 vol of 200 mM glycine buffer pH 10.45 was added in order to induce the coloration of the phenolphthalein produced by the enzymatic cleavage of the substrate. Samples were read at 540 nm, the coloration being stable for at least 1 h. Titers were based on comparisons with standard curves obtained with β-glucuronidase (type B-1 from bovine liver). The values are expressed in units of enzymatic activity.

Histological Processing

Knee joints were removed and fixed in phosphate-buffered formalin (10%). The tissues were decalcified with 5% formic acid solution and then processed and embedded in paraffin. Total joints sections (6 µm) were prepared and stained with hematoxylin and eosin. Arthritis was assessed semiquantitatively in a standardized frontal section of the knee joint which included the presence of subsynovial inflammation, destruction of articular cartilage, and general destruction of the joint with pannus formation and bone erosion [Beckman et al., 1998]. These parameters were scored on a scale of 0–4: 0) no abnormalities, normal joint feature; 1) marginal destruction of cartilage only and slight inflammation in the articular tissue; 2) extensive inflamnation and moderate cartilage destruction with slight bone erosion, usually on one side of the joint only; 3) extensive inflammation and significant destruction of both cartilage and bone; and 4) almost complete cartilage destruction with no intact cartilage left; almost complete loss of the general joint architecture with extensive pannus formation and bone erosion; extensive inflammation in the articular tissue, mainly by mononuclear cells (lymphocytes, plasma cells, macrophages) often accompanied by polymorphonuclear granulocytes. The score given to each joint is an average for the three histological sections through that joint.

Transmission Electron Microscopy.

Samples of articular cartilage were fixed in 3.2% glutaraldehyde in cacodilate buffer, postfixed in 2% osmium tetroxide and embedded in Spurr resin [Spurr, 1969]. Sections were stained with lead citrate and uranil acetate [Reynold, 1963], and observed in a JEOL JEM-100s Electron Microscope (Japan).

Statistical Analysis

Data are presented as means ± standard deviation. Mean differences between groups were compared by one-way analysis of variance (ANOVA) with Duncan's test. The level of statistical significance was taken as $P < 0.05$.

RESULTS

Phycocyanin Effect on Zymosan-Induced Arthritis

In zymosan-induced arthritis, complement is activated via alternative pathway and the secretion of lysosomal enzymes into the knee joint synovial fluid is induced. This activity correlates with histomorphological changes observed in the joint, such as vasculitis, synovitis, and, sometimes, pannus formation.

Arthritis induced by zymosan resulted in a significant increase of β-glucuronidase levels, which were decreased by phycocyanin at doses of 25, 50, and 100 mg/kg p.o. This effect was dose-dependent (Table 1). Triamcinolone almost completely abolished enhanced β-glucuronidase activity in the synovial fluid of zymosan-treated animals. Daily doses of 25, 50, 100 mg/kg suppressed the enzymatic activity by 60, 80, and 92.9%, respectively (Table 1).

TABLE 1. Effect of Phycocyanin on β Glucuronidase Activity in Synovial Fluid of Knee Joint in Mice

Kind of treatment	Doses	β glucuronidase (U)	Percent of inhibition
Saline		0.79 ± 0.35	—
Zymosan	15 mg/ml	6.27 ± 1.37	—
Triamcinolone	10mg/kg	1.00 ± 0.31*	96.2%
Phycocyanin + zymosan	25 mg/kg+ 15	3.02 ± 0.88*	60%
Phycocyanin + zymosan	50 mg/kg+ 15	1.93 ± 0.29*	80%
Phycocyanin + zymosan	100 mg/kg+15	1.18 ± 0.32*	92.9%

Mice were killed 24 h after last administration of phycocyanin. It was administered 4 days after zymosan treatment for 12 days. Triamcinolone was used as a positive control drug in this test. Values are means from groups of 10 mice ± SE.
*$P < 0.05$ vs. group treated only with zymosan.

Histopathological Studies

In agreement with these findings, histological evaluation revealed that the group treated with zymosan showed severe destruction of cartilage with loss of the general joint architecture and pannus formation. There was erosion of bone structures accompanied by severe inflammation of articular tissues (Grade 4) (Fig. 1).

Phycocyanin treatment (100 mg/kg) revealed a marked decrease of histology score (Grade 2 and 1), the inflammatory response was less severe, and there was no destruction of general joint architecture or pannus formation. Also, after treatment the reduction of bone erosion was pronounced (Fig. 2).

Ultrastructural Studies

Tibial articular cartilage treated with zymosan shows chondrocytes with ultrastructural changes in the mitochondria, loss of cristae, and swollen matrix. Also observed was separation between collagen fibers packages (Fig. 3).

The cartilage treated with zymosan plus phycocyanin (100 mg/kg p.o) shows well-preserved chondrocytes with normal rough endoplasmic reticulum. The collagen fibers present normal characteristics (Fig. 4).

DISCUSSION

At present, RA remains a pathology for which no complete cure is available. Corticosteroid treatment, which leads to a quick remission of symptoms, must be of short duration because of its serious adverse effects. Other agents, such as nonsteroidal antiinflammatory drugs (NSAIDs), often are not very effective and also present deleterious side effects. It thus remains a valuable therapeutic objective to find a compound with some of the beneficial properties of the steroids and NSAID but without their adverse effects.

It is increasingly recognized that reactive oxygen species (ROS) such as (OH, H_2O_2, HOCL) are involved in rheumatoid arthritis [Miesel and Zuber, 1993]. In accordance with it, some antioxidant compounds such as

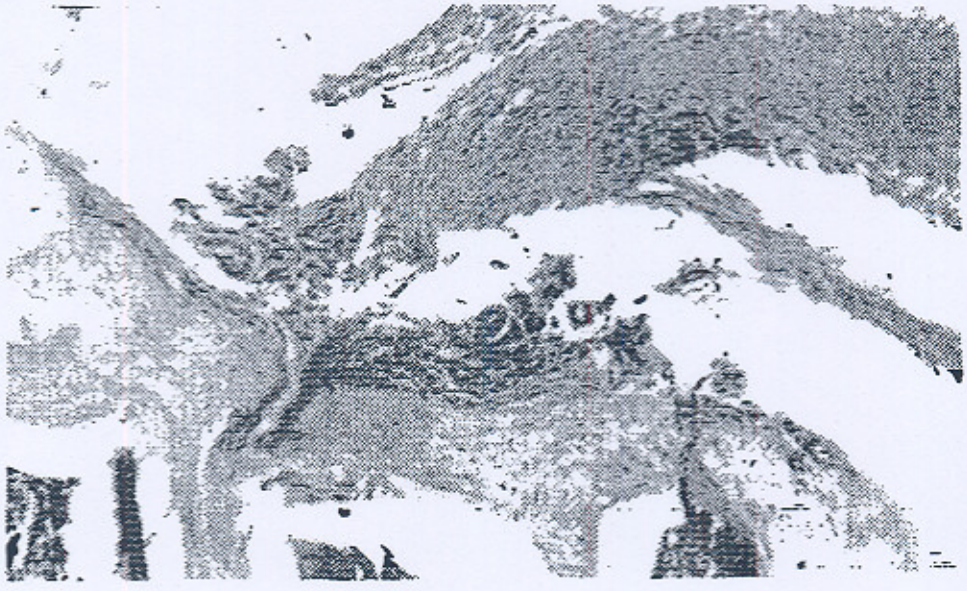

Fig. 1. Histological section of knee joint injected with 15 mg/ml of zymosan. Acute inflammation is present. The surface of the cartilage is eroded and there is pannus formation (Grade 4). Hematoxylin-eosin; magnification 200x.

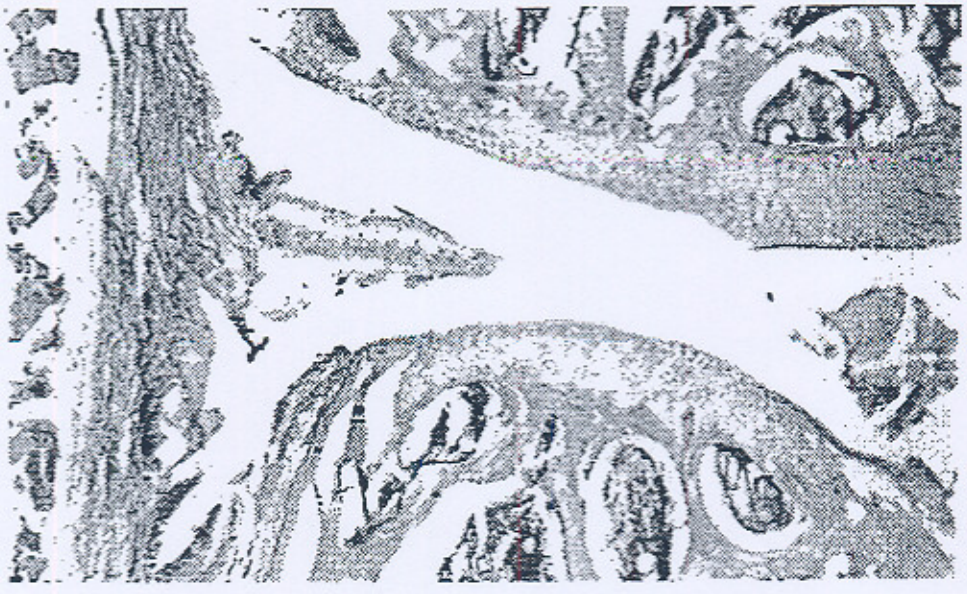

Fig. 2. Animals treated with phycocyanin 100 mg/kg. Histology of arthritic knee joints shows restoration of articular cartilage and absence of pannus formation. Hematoxylin-eosin; magnification 100x.

N-acetyl cysteine [Kroger et al., 1997], ketoprofen [Billany et al., 1995], and CV-3611 [Chikanza et al., 1998], have provided protective effects in some arthritis models and ascribed to its antioxidant properties.

In the present study, we found that phycocyanin, at doses of 25, 50, and 100 mg/kg administered for 8 days after zymosan-induced arthritis, was able to inhibit the levels of β-glucuronidase activity, as well as the histological and ultrastructural lesions produced by zymosan. These effects were dose-dependent.

We recently found that phycocyanin has antioxidant properties [Romay et al., 1998a]. It was able to scavenge alkoxy and hydroxyl radicals. Phycocyanin also inhibited hepatic microsomal lipid peroxidation induced by Fe^{2+}-ascorbic acid, the luminol-amplified chemiluminescent response of PMNLs stimulated with opsonized zymosan, as well as the edema induced by glucose oxidase injection in the mouse paw. This model is suitable for studying the development of arthritic process; it has been shown that injection of a modified form of the H_2O_2-producing enzyme glucose-oxidase into the knee joints of mice causes severe cartilage damage, including the death of chondrocytes [Halliwell and Gutteridge, 1989]. The protective effect of phycocyanin provides evidence in favor of the role of this agent as an ROS scavenger in its antiarthritis effect.

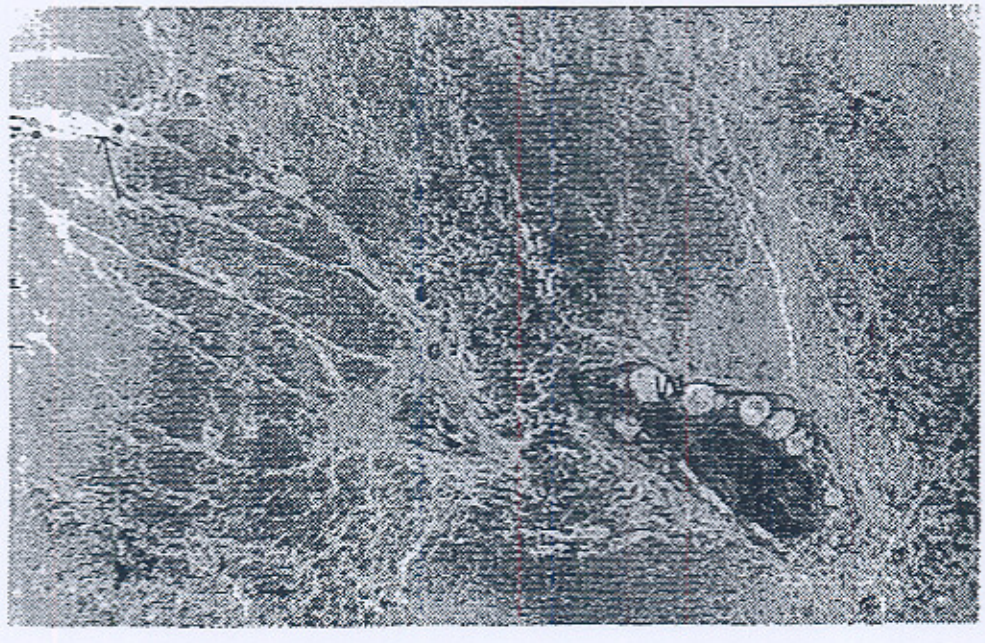

Fig. 3. Transmission electron microscopy of tibial articular cartilage treated with zymosan (15 mg/ml). The chondrocytes show altered mitochondria (M) and edema between the collagen fibrils package (arrow); 5,200x.

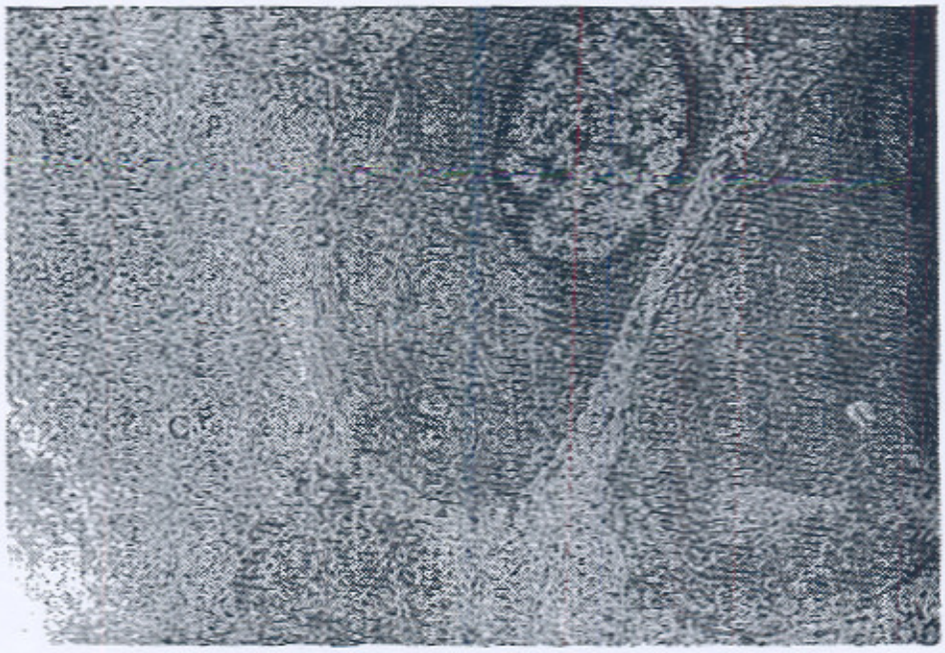

Fig. 4. Transmission electron microscopy of tibial articular cartilage treated with zymosan 15 mg/ml plus phycocyanin 100 mg/kg. Well-preserved chondrocytes with normal rough endoplasmic reticulum (RER) are observed. The collagen fibers present normal characteristics (CF) 6,000x.

For the treatment of arthritis, inhibitors of arachidonic acid metabolism are commonly used [Katz, 1994]. Zymosan is well known as a powerful releaser of arachidonic metabolites that contribute actively to the early phase of inflammatory reaction [Gegout et al., 1995]. In this context, phycocyanin showed antiinflammatory activity at doses of 50–200 mg/kg in the arachidonic acid-induced mouse ear edema model, which is considered a suitable test for the detection of cycloxygenase and/or lipoxygenase inhibitors [Romay et al., 1998b]. Phycocyanin also reduced leukotriene B_4 levels in this animal model of inflammation [Romay et al., 1999]. TNFα and other cytokines are well-known mediators of zymosan-induced arthritis [Fons et al., 1995] and have been found in the synovial fluid [Chikanza, 1996]. Very recently, we found inhibitory effects of phycocyanin on serum TNFα levels in a model of endotoxic shock induced by lipopolysaccharide in mice (D. Remirez, unpublished data), providing additional evidence for an inhibitory effect on TNFα production in the mode of action of phycocyanin as an antiarthritic agent.

In conclusion, oral administration of phycocyanin exerted antiinflammatory effects in arthritis induced by zymosan in mice. Antiarthritic effect of phycocyanin might be ascribed to ROS scavenging properties, as well as inhibition of arachidonic acid metabolism and cytokine production (e.g. TNFα). Currently, additional studies are in progress in our laboratory in order to offer insight into the mode of action of phycocyanin as an antiinflammatory agent.

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