THERAPEUTIC USE OF PIRFENIDONE AGAINST SEPTIC SHOCK

by

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ABSTRACT

Tumor necrosis factor (TNF) is an extremely potent cytokine which is involved in the pathogenesis of a number of diseases. Interruption of its synthesis can result in a reduction of inflammation and subsequent pathology. A new experimental drug, pirfenidone, (5-methyl-1-phenyl-2-(1H)-pyridone, trade name: Deskar) has been reported to have beneficial effects for the treatment of certain fibrotic diseases. The present study describes the use of pirfenidone, which inhibits both the release of TNF *in vitro* as well as circulating TNF *in vivo*. Isolated thioglycollate-induced murine peritoneal macrophages (Mø) were exposed to either lipopolysaccharide (LPS) or another TNF inducer then incubated with 0.1 to 0.9 mg/ml of pirfenidone. This substance inhibited the production of TNF in a dose-dependent manner. The induction of circulating TNF following a single i.v. injection of LPS was also inhibited by an i.p. injection of 100 to 200 mg/kg pirfenidone. Endotoxin shock was induced in mice using injections of galactosamine and LPS. The higher dose of pirfenidone (200 mg/kg) completely inhibited shock and subsequent mortality. Lower doses of pirfenidone or administration either prior to or post challenge only partially inhibited symptoms. These results indicate that pirfenidone is able to inhibit both TNF induction and subsequent endotoxin shock. Additional studies are warranted to establish this drug as a potential treatment for diseases where TNF plays a major role.

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LIST OF ABBREVIATIONS

CO2 -- Carbon Dioxide

DMEM -- Dulbeco's Modified Eagle Medium

ELISA -- Enzyme-Linked Immunosorbent Assay

FBS -- Fetal Bovine Serum

IL -- Interleukin

LPS -- Lipopolysaccharide

mBSA -- Mannosylated Bovine Serum Albumin

Mø -- Macrophage

PBS -- Phosphate Buffered Saline

Pf -- Pirfenidone

TG-Mø -- Thioglycollate Induced Macrophage

TNF -- Tumor Necrosis Factor

CHAPTER I

INTRODUCTION

Background

Inflammation is defined, in Stedman's Medical Dictionary (1982), as a fundamental pathologic process consisting of a dynamic complex of cytologic and histologic reactions that occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation caused by a physical, chemical, or biologic agent. These may include (1) the local reactions and resulting morphologic changes, (2) the destruction or removal of the injurious material, and (3) the responses that lead to repair and healing.

Cells Involved in Inflammation

It is known that there are many cells that play a role in the inflammatory process. It is, however, beyond the scope of this discussion to give a detailed description of each one. Therefore, the focus of this paper will be on the cells termed macrophage (Mø). The Mø stems from progenitor cells in the bone marrow and is called the Mø only when it leaves the blood and enters the tissues. The Mø has two primary functions in the inflammatory process: (1) To phagocytize foreign antigens which are taken into the phagosome and subsequently destroyed by fusion of the phagosome and lysosome and (2) To synthesize a number of cytokines that mediate inflammation. The Mø is central in inflammation and its study is vital to the basic understanding of this dynamic process.

Cytokines Involved in Inflammation

The major cytokines involved in the inflammatory process are tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). Starting with TNF they are produced in series of one another and are often referred to as a "cytokine cascade"

(Blackwell, 1996; Molloy *et al.*, 1993). Tumor necrosis factor-alpha is a 17 kD pleotrophic cytokine that is involved in the modification of both inflammatory and immune reactions. It was originally described as the primary mediator of endotoxin shock and cachexia (Blackwell, 1996; Vilcek, 1991). It was, also, thought that TNF-α was produced by only a few specific cells; however, it is now known to be secreted by an ever widening array of cells. The principal inducer of physiologic TNF-α is lipolysaccharide (LPS) (Beutler *et al.*, 1986; Vilcek, 1991). Some of the major physiologic functions of TNF-α are: (1) stimulate the metabolism of triglycerides and proteins in the liver (Fong *et al.*, 1989) (2) to stimulate the production of prostaglandins from the hypothalamus (Dinarello *et al.*, 1986) and (3) to stimulate the coagulation cascade (Cybulsky *et al.*, 1988). Working in a paracrine, as well as, autocrine fashion, TNF-α functions to stimulate other immune cells for the synthesis of IL-1(Blackwell, 1996; Molloy *et al.*, 1993).

Interleukin-1, described originally as a stimulator of T cells, is now known to mediate the inflammation reaction similar to that of TNF-α (Dinarello, 1994). This cytokine is also a 17kD monomer that follows closely the functions of TNF-α. It increases leukocyte adhesion to the vascular epithelium and actuates coagulation (Dinarello, 1994). Moreover, IL-1 stimulates mononuclear phagocytes and vascular epithelium to secrete chemokines that stimulate leukocytes. However, when released in large quantities IL-1 exerts the same effects on the liver (acute phase response) that TNF does (Dinarello, 1994).

Produced in response to TNF-α and IL-1, IL-6 is a 21 kD glycoprotein produced by a myriad of cells. The function of IL-6 is uncertain in the literature because it has been shown to activate T and B cells (Borden, 1994), yet *in vitro*, is shown to suppress the production of TNF-α (Schindler *et al.*, 1990). Nevertheless, IL-6, also stimulates the acute phase proteins in the liver and actuates the coagulation cascade.

In concert these cytokines, which are termed "pro-inflammatory cytokines," serve as the body's communication link for the modulation of the immune response. Functioning

normally, at physiologic levels, these cytokines help the body engage foreign invaders in a protective manner; however if produced in excess, these cytokines can have deleterious effects on the body as in septic shock.

Purpose of Study

A new low molecular weight experimental drug, 5-methyl-1-phenyl-2-(1H)-pyridone, which is known as pirfenidone (trade name: Deskar) is currently being tested as an antifibrotic drug for the prevention of pulmonary fibrosis. A number of reports have indicated that this drug inhibits the cell cycle and the production of collagen, radicals etc. (Lurton *et al.*, 1996; Vallyathan *et al.*, 1996; Shimizu *et al.*, 1996; Iyer *et al.*, 1996; Gurujeyalakshmi *et al.*, 1997). Since TNF-α represents one of the most predominant pro-inflammatory cytokines and its inhibition reduces inflammation and subsequent disease, the present study was done to determine the effect of pirfenidone on the secretion of TNF-α. In studies described below, murine peritoneal macrophages were stimulated with LPS and the subsequent secretion and production of TNF-α was inhibited by pirfenidone. Further *in vivo* studies using experimental mice indicated that both circulating TNF-α and endotoxin shock were also inhibited.

CHAPTER II

MATERIALS AND METHODS

Animals

C57BL/6 male mice, approximately 22g, were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were maintained using the guidelines established by the federal Animal Care Act.

Materials

Pirfenidone was generously donated by Marnac Inc., Dallas, TX. Lipopolysaccharide (LPS), gentamicin sulfate, and D-galactosamine were purchased from Sigma (St. Louis, MO). Mannosylated bovine serum albumin (mBSA) was purchased from EY Laboratories (San Mateo, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Long Island, NY). HEPES was purchased from Fisher (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY). Thioglycollate broth was obtained and prepared as described from Baltimore Biological Laboratories (Cockeysville, MD) and phosphate buffered saline (PBS) pH 7.2 was prepared as needed.

Macrophage Collection

Mice were injected with 1ml of thioglycollate broth i.p. After 4 days, peritoneal Mø (TG-Mø) were collected as described previously (Lefkowitz, 1986). Mice were sacrificed by cervical dislocation and TG-Mø collected by peritoneal lavage using ice cold PBS. Cells were washed and resuspended in DMEM containing 2% FBS with gentamicin and HEPES. The cell number was adjusted to $1x10^6$ Mø/ml and 100µl of the cell suspension were added to each well of a 96 well tissue culture plate (Costar, Cambridge, MA). Following

incubation at 37°C, 5% CO_2 for 2 hrs, cell monolayers were washed vigorously with DMEM to remove non-adherent cells. The cell monolayers were approximately 99% pure TG-Mø as determined by light microscopy.

Cytokine Induction *in vitro*

In order to ascertain the effect of pirfenidone on the induction of TNF and IL-6, 50 µl of pirfenidone and 50 µl of LPS were sequentially added to wells to give final concentrations of pirfenidone from 0.1 to 0.9 mg/ml. In addition to the pirfenidone treatments, a number of controls were also done without pirfenidone and LPS. The Mø monolayers were incubated at 37°C under 5% CO_2 and supernatants were collected after appropriate intervals and stored at -20°C until use. In order to show that the inhibition in the secretion of TNF by pirfenidone was not LPS specific, Mø were also exposed to mBSA, which is also a TNF inducer, but binds to a different receptor.

In Vivo Model

Initial studies were done to determine if pirfenidone would alter induction of TNF *in vivo*. Mice were injected i.v. with 2.5 µg LPS followed immediately by an i.p. injection of pirfenidone. After 90 min. mice were exsanguinated by cardiac puncture and their sera frozen for future analysis by ELISA. In order to assess the effect of pirfenidone in an endotoxin shock model, one similar to the one described by Sekut *et al.* (Clin. Exp. Immunol., 1995), was used. Briefly, the mice were given a 0.5 ml i.p. injection containing 600 mg/kg D-galactosamine and 0.005 mg/kg of LPS suspended in PBS. This was followed by a 1.0 ml i.p. injection of pirfenidone (200 mg/kg) suspended in PBS also. Injections of pirfenidone were given at various times before, concurrent, and after these injections. Following treatment, mice were observed for 24 hrs. for the effects of the drug induced endotoxin shock. Using a respiration chamber, the metabolic rate of mice was

measured indirectly from their oxygen consumption. At the first signs of shock mice were sacrificed using Halothane. Criteria used for determining signs of shock were as described in Sell, (1996). Another set of mice were given galactosamine and LPS followed by pirfenidone, but were exsanguinated by cardiac puncture after 90 min. Serum samples from the mice were collected, frozen, and assayed for TNF by ELISA.

ELISA

A Mouse TNF-α and IL-6 ELISA kits (Genzyme, Cambridge, MA) were employed as described by the manufacturer.

Western Blot Analysis

In order to ascertain the effects of pirfenidone on the intracellular levels of TNF, Western blot analysis was employed. The procedure used was similar to that described by Towbin *et al.* (Proc. Natl. Acad. Sci., 1979). Briefly, samples were collected and their protein concentration determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were added to each lane, ran and subsequently probed for TNF. Anti-mouse TNF and anti-IgG antibodies were obtained from Peprotech (Rocky Hill, NJ) and Pharmingen (San Diego, CA), respectively.

Statistical Analysis

A one-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison tests were employed in determining the statistical significance of these data.

CHAPTER III

RESULTS

The present study was done to assess possible effects of pirfenidone on TNF production. In the *in vitro* experiments, pirfenidone was inhibitory and reduced the secretion of TNF. It can be seen in Fig. 1 that TNF was inhibited, in a dose-dependent manner, in unstimulated (baseline) Mø exposed to pirfenidone. About a 40% reduction was obtained using the highest concentration of pirfenidone (0.9 mg/ml). TNF from Mø, exposed to from 0.1 to 1.0 ng/ml LPS, was also inhibited, by pirfenidone, in a dose-dependent manner. Figure 2 illustrates the effect of pirfenidone on Mø exposed to 0.1 ng/ml of LPS. It can be seen that the highest dose of pirfenidone reduced TNF from 4500 to 1500 pg/ml. Figure 3 illustrates that TNF inhibition was dose-dependent and not time dependent. Maximum arrest of TNF production was noted after 3 hrs. incubation, whereas TNF production continued to rise in the controls from 6 to 9 hrs. In order to rule out the possibility that inhibition of TNF by pirfenidone was LPS specific, another inducer of TNF, mannosylated bovine serum albumen (mBSA), was used. Various concentrations of mBSA (16-64 µg/ml) induced the production of TNF. As can be seen in Fig. 4 a dose-dependent inhibition of TNF with greater than 80% inhibition, over controls, by 0.9 mg/ml pirfenidone was obtained.

In order to show that the effect of pirfenidone was not limited to TNF, we looked at its effect on the secretion of IL-6. As can be seen from Figure 5, pirfenidone inhibited basal levels of IL-6 in much the same manner as TNF. IL-6 from Mø exposed to 0.1 ng/ml LPS was also inhibited, by pirfenidone, in the same manner as TNF (Fig. 6).

To show that pirfenidone was not inhibiting some post-translational cleavage and, therefore, not allowing for TNF to be secreted, 15 µg of whole cell lysate were subjected to

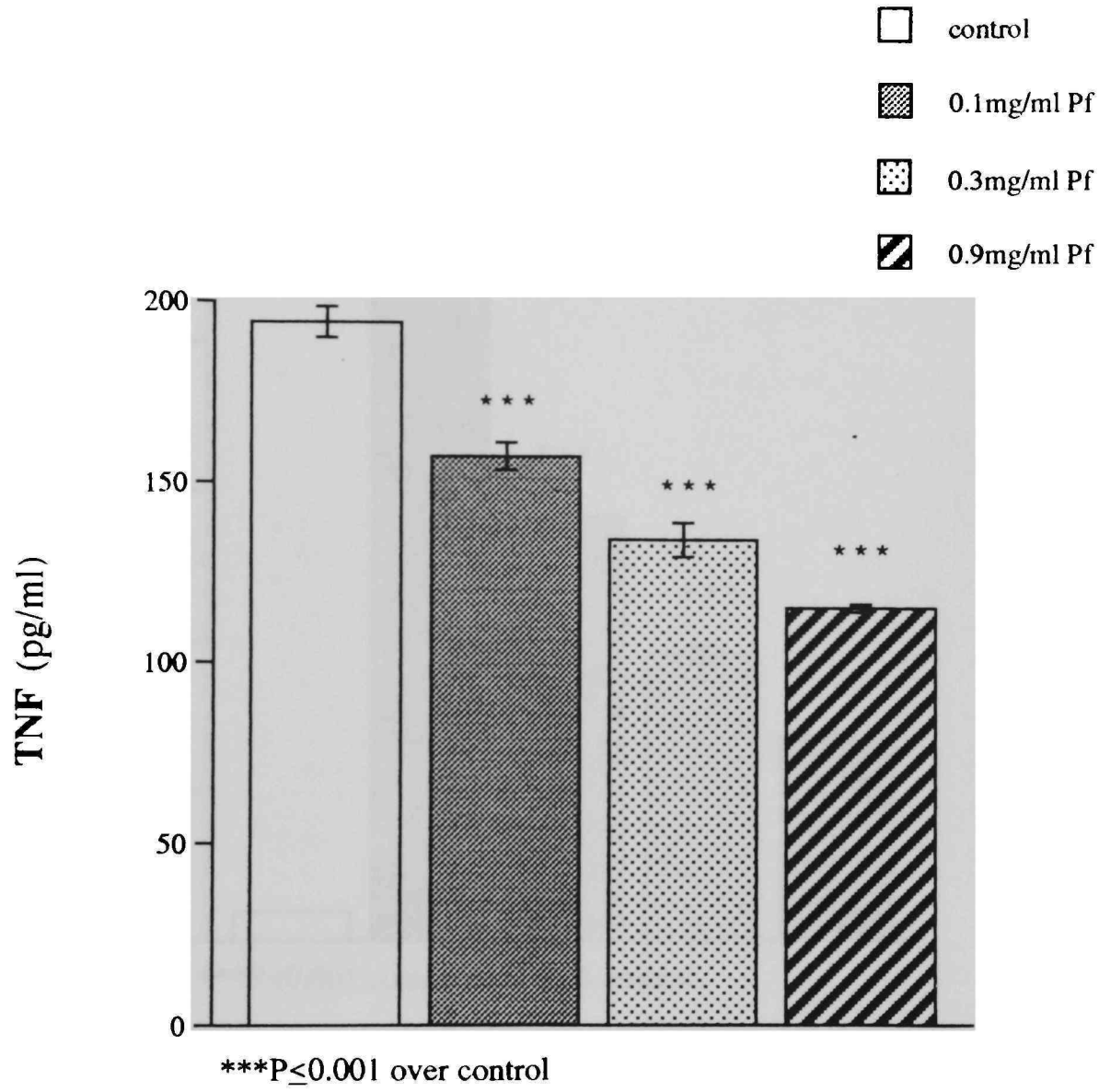

Fig. 1. Thioglycollate-induced peritoneal Mø were exposed to 0.1-0.9 mg/ml pirfenidone to assess the effect on baseline TNF secretion. After 6 hrs. incubation, supernatants were collected and assayed for TNF by ELISA.

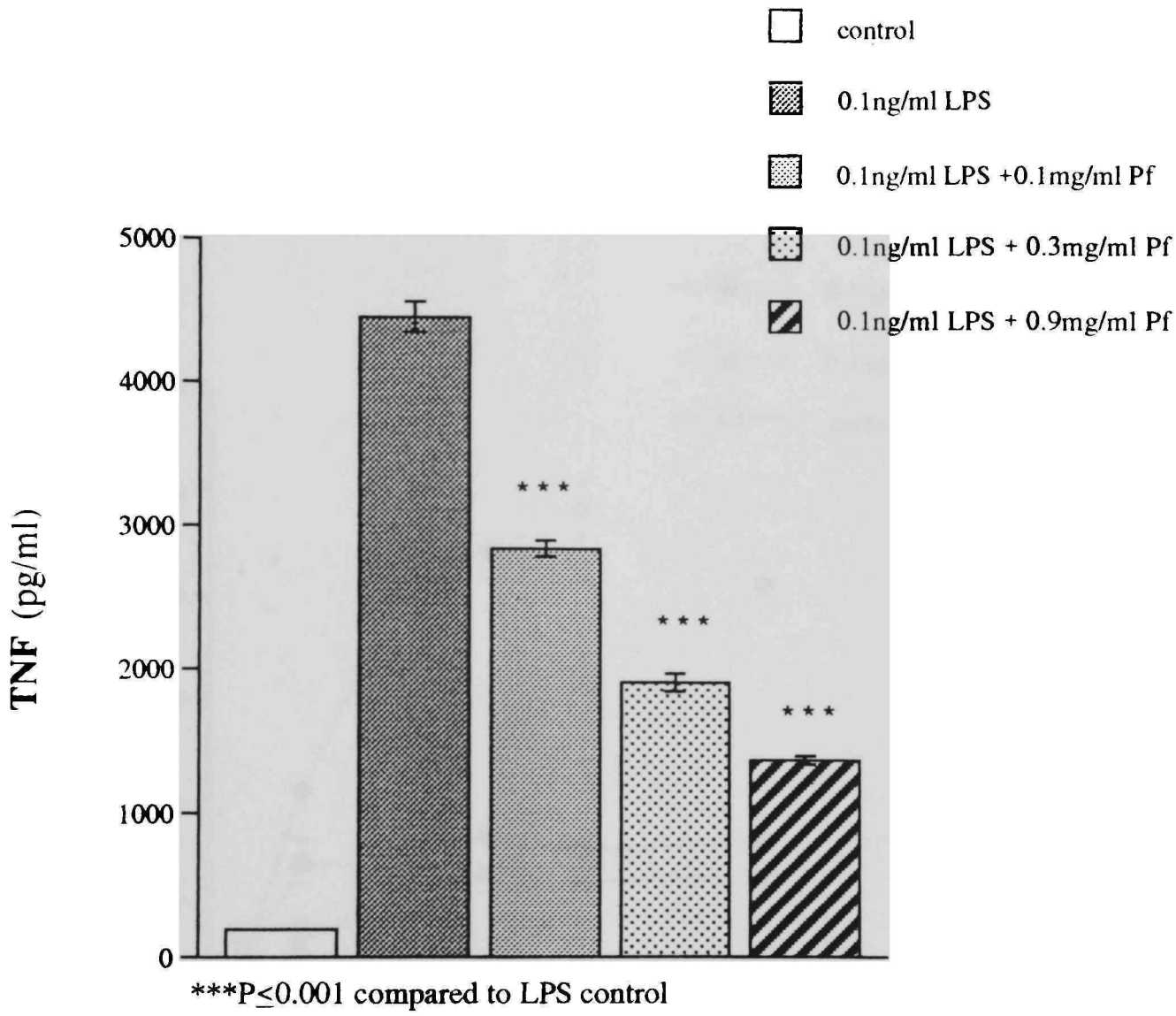

Fig. 2. Thioglycollate induced peritoneal Mø were exposed to 0.1ng/ml LPS and treated with 0.1 to 0.9 mg/kg pirfenidone. After 6 hrs. incubation, supernatants were collected and assayed for TNF by ELISA.

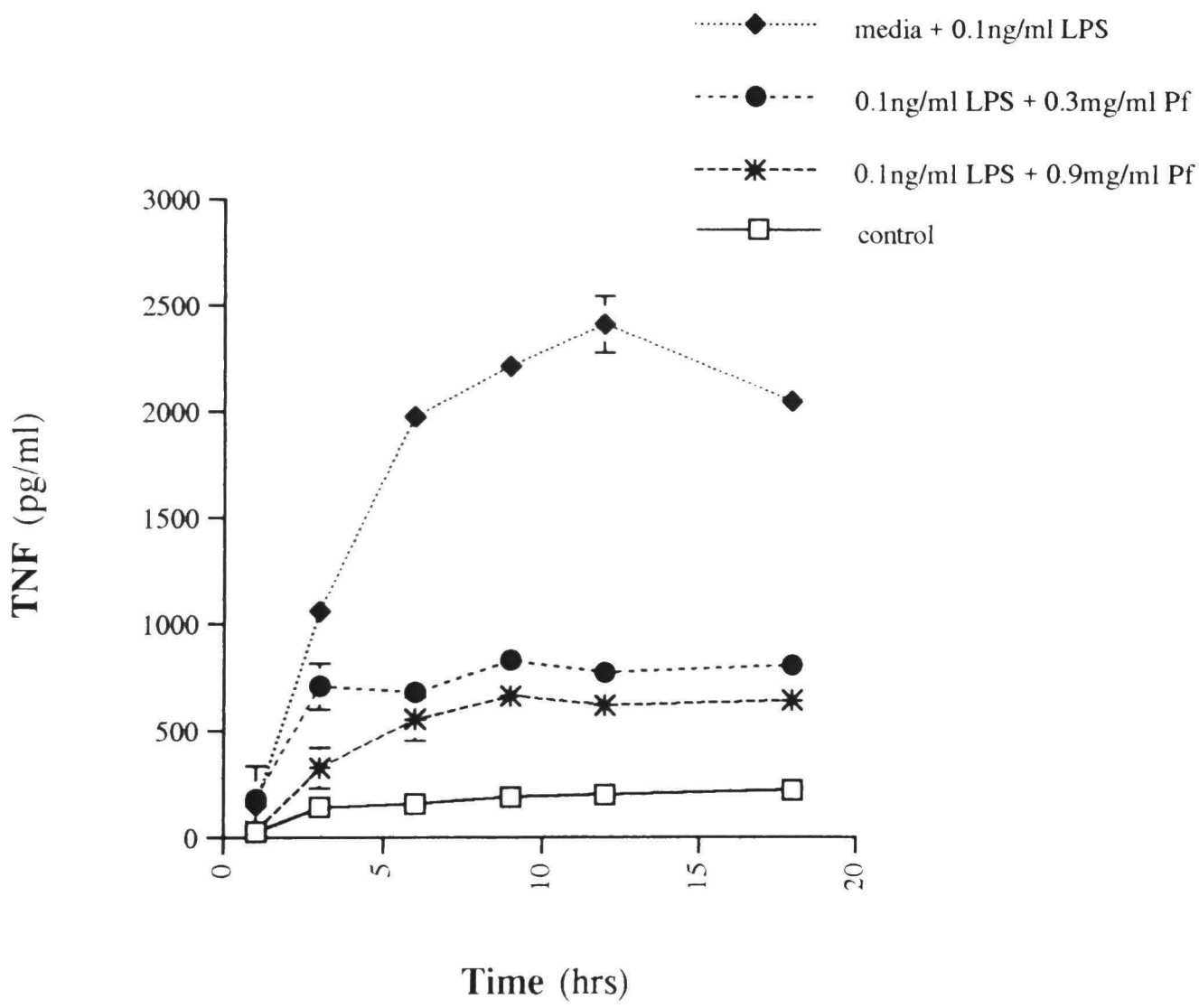

Fig. 3. Thioglycollate-induced peritoneal Mø exposed to 0.1 ng/ml LPS and treated with either 0.3 or 0.9 mg/ml pirfenidone. Supernatants were harvested at various time intervals and assayed for TNF by ELISA.

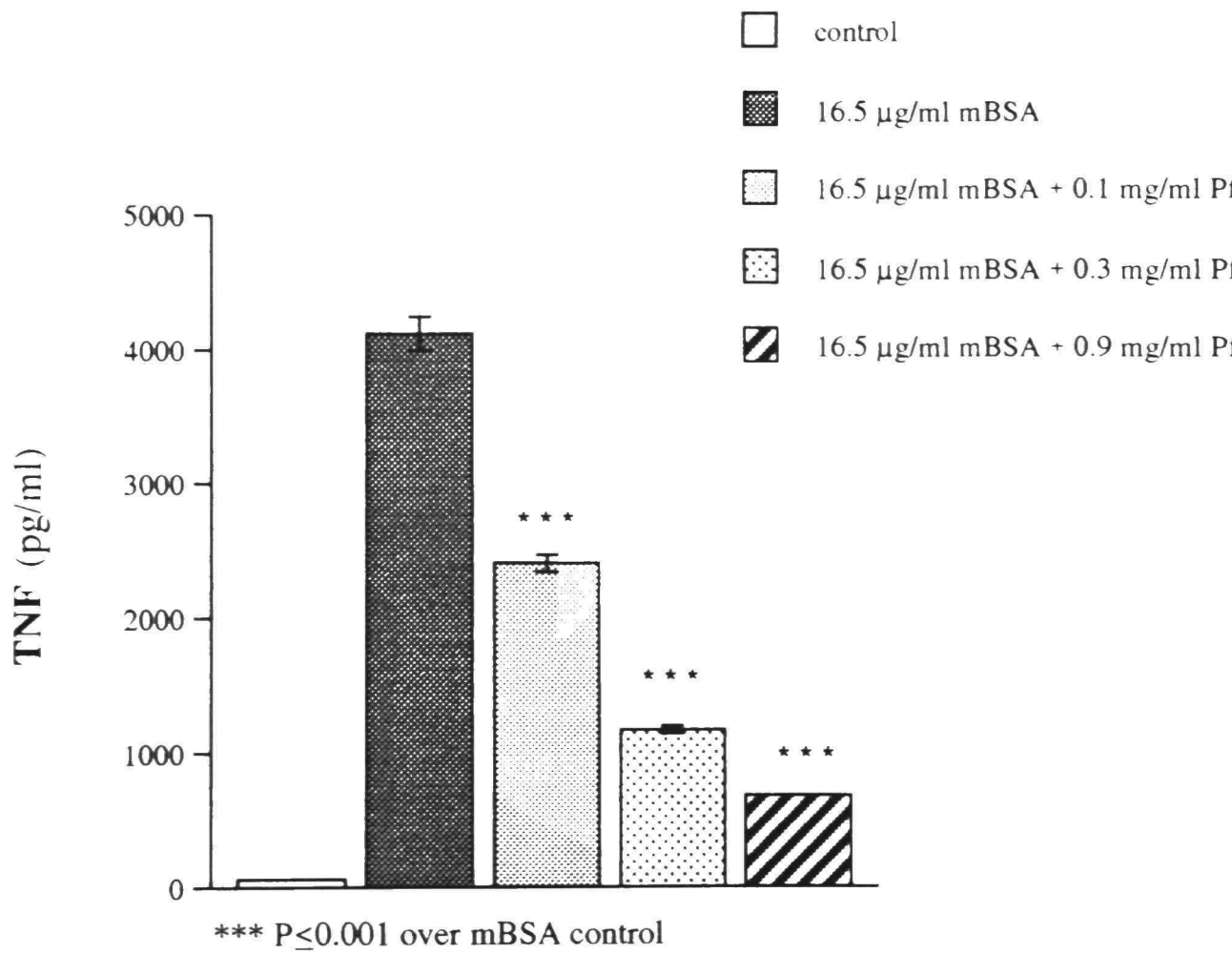

Fig. 4. To ascertain the effects of pirfenidone on TNF secretion by cells stimulated with mannosylated bovine serum albumin (mBSA), thioglycollate peritoneal Mø were exposed to16.5µg/ml mBSA and treated with 0.1 to 0.9mg/ml pirfenidone. After 6 hrs. incubation, supernatants were collected and assayed for TNF by ELISA.

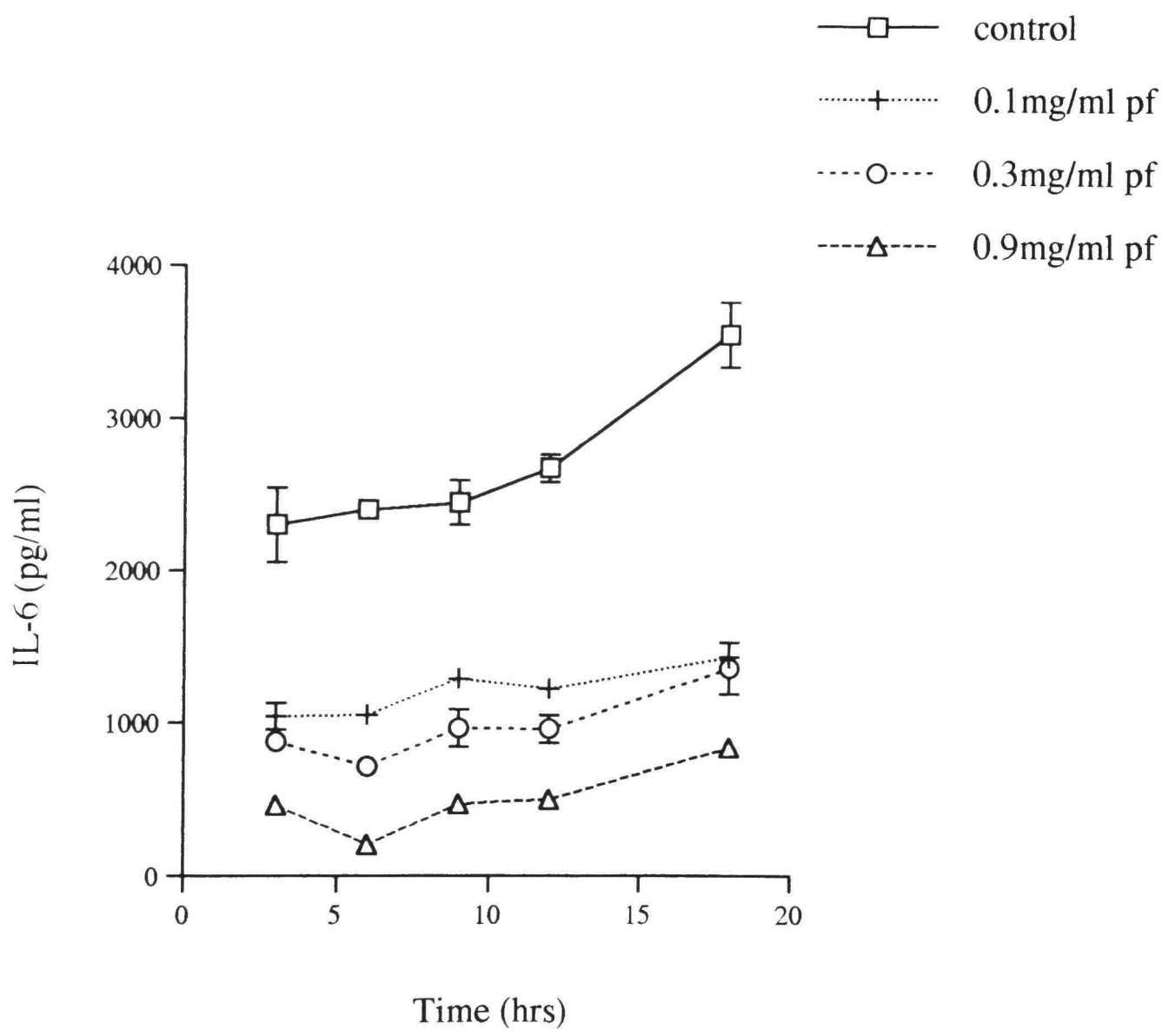

Fig. 5. Thioglycollate-induced peritoneal Mø were exposed to 0.1-0.9 mg/ml pirfenidone to assess the effect on baseline IL-6 secretion. After various times of incubation, supernatants were collected and assayed for IL-6 by ELISA.

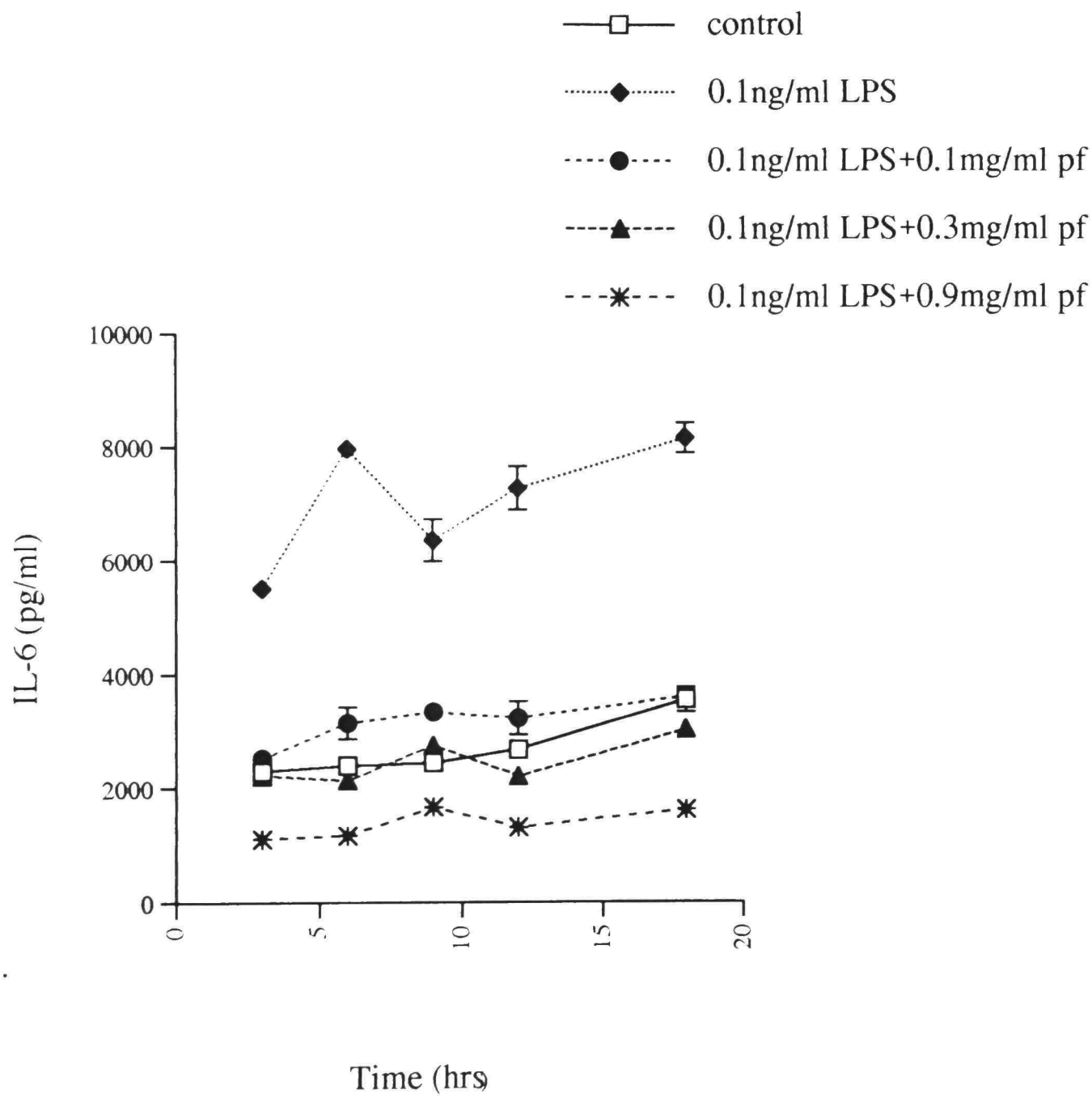

Fig. 6. Thioglycollate-induced peritoneal Mø were exposed to 0.1 ng/ml LPS and treated with 0.1 to 0.9 mg/ml pirfenidone. Supernatants were harvested at various time intervals and assayed for IL-6 by ELISA.

Western blot analysis. In Figure 7, it can be seen that intracellular levels were also inhibited by pirfenidone.

Studies measuring the induction of TNF *in vivo* also indicated an inhibition by pirfenidone. When mice were injected i.v. with 2.5 μg of LPS followed by pirfenidone i.p. and exsanguinated 90 min. later, a dose-dependent inhibition of LPS was obtained (Table 1). Serum titers of TNF from mice receiving 90 mg/kg pirfenidone were significantly lower than control mice ($P \leq 0.05$). Mice receiving 200 mg/kg completely failed to respond to LPS. Administration of pirfenidone prior to or post LPS did not reduce TNF titers significantly (data not shown).

A second *in vivo* model was used to assess the effect of pirfenidone on endotoxin shock. Mice receiving galactosamine and LPS concurrently with pirfenidone also had a reduced level of circulating TNF. It can be seen in Table 1 that a significant reduction ($p \leq 0.001$) in TNF was noted using 200 mg/kg pirfenidone. A significant reduction ($P \leq 0.001$) in endotoxin shock was obtained when mice were injected with pirfenidone. It can be seen in Table 2, 200 mg/kg pirfenidone completely protected mice. Some, but minimal protection, was obtained using lower doses. When pirfenidone was administered prior to the challenge, a greater number of mice (60%) were protected than if administered after (Table 3).

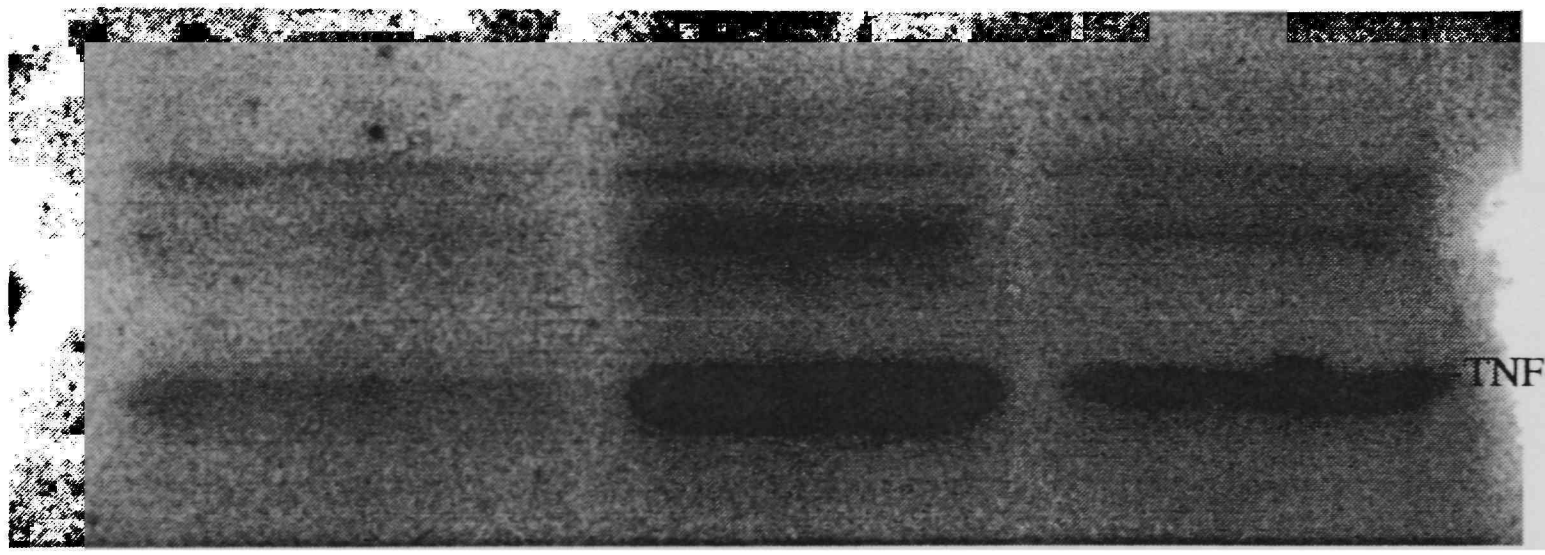

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Fig. 7. Thioglycollate-induced peritoneal Mø were exposed to either media alone (lane 1), 0.1 ng/ml LPS (lane 2), or 0.1 ng/ml LPS and 0.9 mg/ml pirfenidone (lane 3). Cells were lysed after 6 hrs. incubation and whole cell lysates were assayed for TNF via western blot analysis.

Table 1. Reduction in Serum Levels of TNF-α by Pirfenidone

Stimulus	Pirfenidone (mg/kg)				
	0	30	90	200	400
LPS[1]	643±156	1020±213	247±77*	0±0	0±0
LPS + D-gal[2]	5935±1893	ND	ND	1097±286***	ND

* P≤ 0.05

*** P≤ 0.001

[1]Mice were injected i.v. with 2.5 μg LPS followed by i.p. injection with either saline or pirfenidone. Mice were exsanguinated at 90 min., sera collected and assayed for TNF (pg/ml) by ELISA.

[2]Mice were injected i.p. with 600 mg/kg D-galactosamine mixed with 0.1μg LPS followed by i.p. injection with either saline or pirfenidone. Mice were exsanguinated at 90 min., sera collected and assayed for TNF (pg/ml) by ELISA.

Table 2. Protection from Endotoxin Shock by Pirfenidone

Pirfenidone (mg/kg)	Total Number	Number in Shock	% protected
Control	5	5	0
200***	5	0	100
100	5	4	20
50	5	4	20

***P< 0.001

Mice were given simultaneous 0.5ml i.p. injections of 600mg/kg D-galactosamine and 0.005mg/kg LPS. This was followed immediately by a 1ml injection of pirfenidone also i.p. Mice were then monitored 24 hrs. for the signs of endotoxin shock.

Table 3. Timing of Pirfenidone on Induction of Endotoxin Shock

Time of Administration	Total Number	Number in Shock	% Protected
None	5	5	0
60 min. Prior	5	2	60
30 min. Prior	5	2	60
30 min After	5	4	20
60 min After	5	3	40

Mice were given 1ml injections of 200mg/kg pirfenidone at various times prior to or post challenge with 0.005mg/kg LPS and 600mg/kg D-galactosamine. Mice were then monitored 24 hrs. for endotoxin shock.

CHAPTER IV

DISCUSSION

Induction of cytokines and their interactions represents a means to both up regulate and down regulate the immune system. Induction of the pro-inflammatory cytokines usually follows exposure to various microorganisms. The pro-inflammatory cytokines: TNF, interleukin-1 (IL-1), and interleukin-6 (IL-6) have been implicated in a number of diseases including Crohn's disease where they have been found in the colon (Waltraud *et al.*, 1995), in the joints of patients suffering from rheumatoid arthritis (Feldmann, 1996), and in the lungs of patients with pulmonary fibrosis (Lurton *et al.*, 1996). They have also been postulated to play a role in multiple sclerosis (Owens, 1995),and even Alzheimer's (Malek-Ahamadi, 1996). These cytokines have emerged as major participants in the etiology of many diseases.

Reduction of these cytokines may be beneficial to the host and subsequent outcome of disease. It has been suggested that interruption in the synthesis and production of TNF-α and subsequent cytokine cascade can reduce disease expression (Blackwell; Molloy). Anti-inflammatory drugs that target these cytokines are being extensively studied. Nonsteroidal drugs such as indomethacin, ibuprofen, and ketoprofen have been used as a pre-medication to inhibit the effects of administered TNF (Spriggs and Yates, 1992). Other agents that cause an increase in cAMP such as theophyllin and isobutylmethylxanthin have been shown to inhibit production of TNF (Seckinger and Dayer, 1992). Salmeterol, another such agent, has been demonstrated by Sekut *et al.* (Clin. Exp. Immunol., 1995) to suppress the production of TNF *in vivo*.

The studies in this manuscript describe a dose-dependent inhibition of TNF following exposure of Mø to pirfenidone, *in vitro*. This inhibition was not only apparent in LPS exposed Mø, but was also obtained from control cells which were not exposed to

LPS. Further, cells stimulated with mBSA, which enters the Mø through a different receptor than LPS, and treated with pirfenidone exhibited the same inhibition of TNF. This indicates that the inhibition of TNF by pirfenidone is not specific to the stimulant used, rather that it is a general reduction in TNF. However, inhibition was not restricted to TNF; a dose dependent inhibition of IL-6 was also obtained using the same levels of pirfenidone. Together, these data suggest a generalized inhibition in the secretion of not only TNF, but the pro-inflammatory cytokines as a whole. Furthermore, as suggested by Western blot analysis, pirfenidone's action is not limited to inhibition of cytokine secretion, but its production.

The near complete reduction of TNF, *in vivo*, was achieved by simultaneous administration of 200 mg/kg of pirfenidone. It should be noted that at these high concentrations of pirfenidone ($\geq$ 100 mg/kg), soporific effects were apparent. Mice receiving these levels were prostrate for approximately 10 minutes. These data suggests the complete alleviation of symptoms and/or disease caused by TNF, such as endotoxin shock. Simultaneous injection of 200 mg/kg pirfenidone with the induction of endotoxin shock showed a complete inhibition of symptoms and subsequent disease. Attempts at modifying the schedule of administration by injecting pirfenidone either prior to or post challenge failed to significantly alter endotoxin shock. It was apparent that, by inhibiting the amount of circulating TNF, disease expression was prevented.

The reduction of both TNF and IL-6 *in vitro* and TNF *in vivo* suggests that pirfenidone could be a useful drug in the treatment of those illnesses of which pro-inflammatory cytokines are implicated.

CHAPTER V

CONCLUSION

This study indicates that pirfenidone, an experimental anti-fibrotic drug, may be useful for the treatment of diseases which pro-inflammatory cytokines are implicated. This is demonstrated by the conclusions listed.

1. Pirfenidone inhibits the production of TNF, by murine Mø, *in vitro*.
2. Pirfenidone inhibits the production of IL-6, by murine Mø, *in vitro*.
3. Pirfenidone inhibits the production of TNF *in vivo*, as evidenced by a reduction in serum TNF following LPS stimulation.
4. Pirfenidone inhibits the induction of endotoxin shock in mice injected with LPS and D-galactosamine.
5. Pirfenidone may be useful in the treatment of diseases where pro-inflammatory cytokines play a major role.

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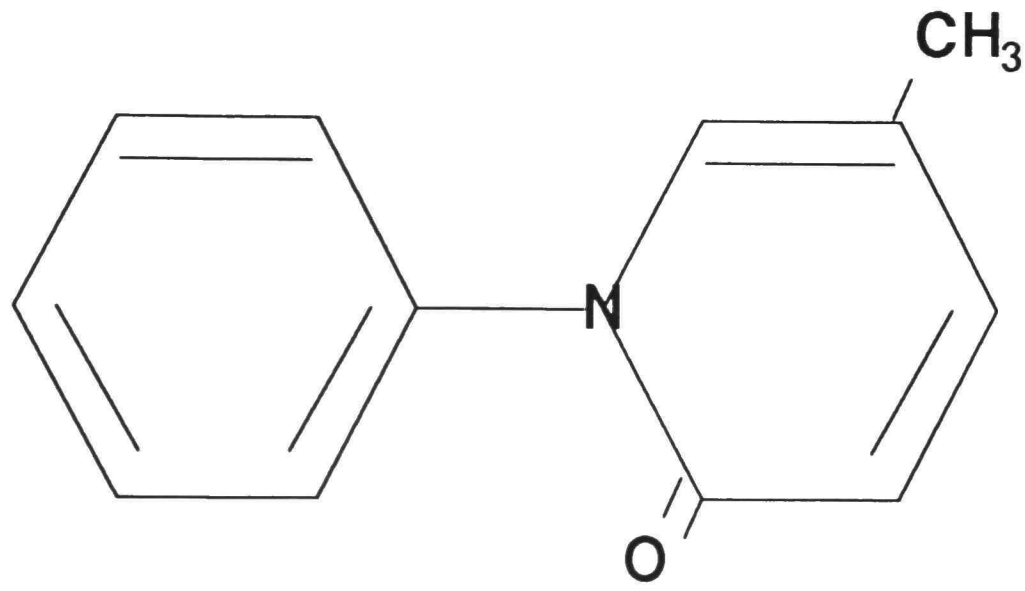
APPENDIX A

PIRFENIDONE

Structural Formula



Physical and Chemical Properties

1. Appearance - It is a white micro crystalline, odorless, solid.

2. Melting point - 104-110°C

3. Solubility - Highly soluble in ethyl alcohol, chloroform, various lipid oils, and other lipid solvents. At 25°C, it is soluble to the extent of 1.5-2.0% in distilled water.

4. Chemical Identity - Carbon, 77.81%; Oxygen, 8.58%; Nitrogen, 7.65%; Hydrogen, 5.96%.

APPENDIX B

CELLULAR TOXICITY

Introduction

The inhibition of cell cycle and *de novo* protein synthesis can lead to cell death. This study was undertaken to ascertain the toxicity and cell cycle inhibition of pirfenidone on different cells.

Materials and Methods

Mø Toxicity Assay

Resident and thioglycollate induced peritoneal Mø were collected, in the same manner as before, and incubated 6 hours with various concentrations of pirfenidone. Cell monolayers were washed and subsequently stained with 0.4% trypan blue. Cells were accessed for viability by light microscopy.

Astrocyte Inhibition Assay

Normal human astrocytes (Clonetics, San Diego, CA) were seeded into 96 well plates, following the protocol provided by Clonetics (3.5×10^3 cells per well), with media containing various concentrations of pirfenidone. Control wells were allowed to reach confluency (approx. 3 days), at which time wells were washed and cells stained with neutral red. Subsequently, cells were lysed with potassium phosphate monobasic and there absorption read at 550 nm.

HL-60 Inhibition Assay

One million HL-60 cells were seeded into T25 flasks and grown with various concentrations of pirfenidone in the media. Aliquots were taken at various times, stained with 0.4% trypan blue, enumerated, and accessed for viability by light microscopy.

Results

At concentrations of 1.8 mg/ml and higher, pirfenidone was significantly toxic to either resident or TG-Mø (data not shown). A dose dependent inhibition of growth was obtained by exposing astrocytes to 0.1 to 0.9 mg/ml pirfenidone (Fig. 8). In figure 9, pirfenidone inhibited HL-60 growth in a dose dependent manner, however, no appreciable cell death was found over controls, as determined by viability staining.

Discussion

The results of the toxicity assay on Mø suggest that the level (0.9 mg/ml) that was used in the in vitro experiments, in this manuscript, was not toxic to the cells. Furthermore, pirfenidone seems to inhibit growth of a variety of cells without causing cell death. This suggests that the inhibition of cytokine production by pirfenidone was due to some other mechanism than cell death.

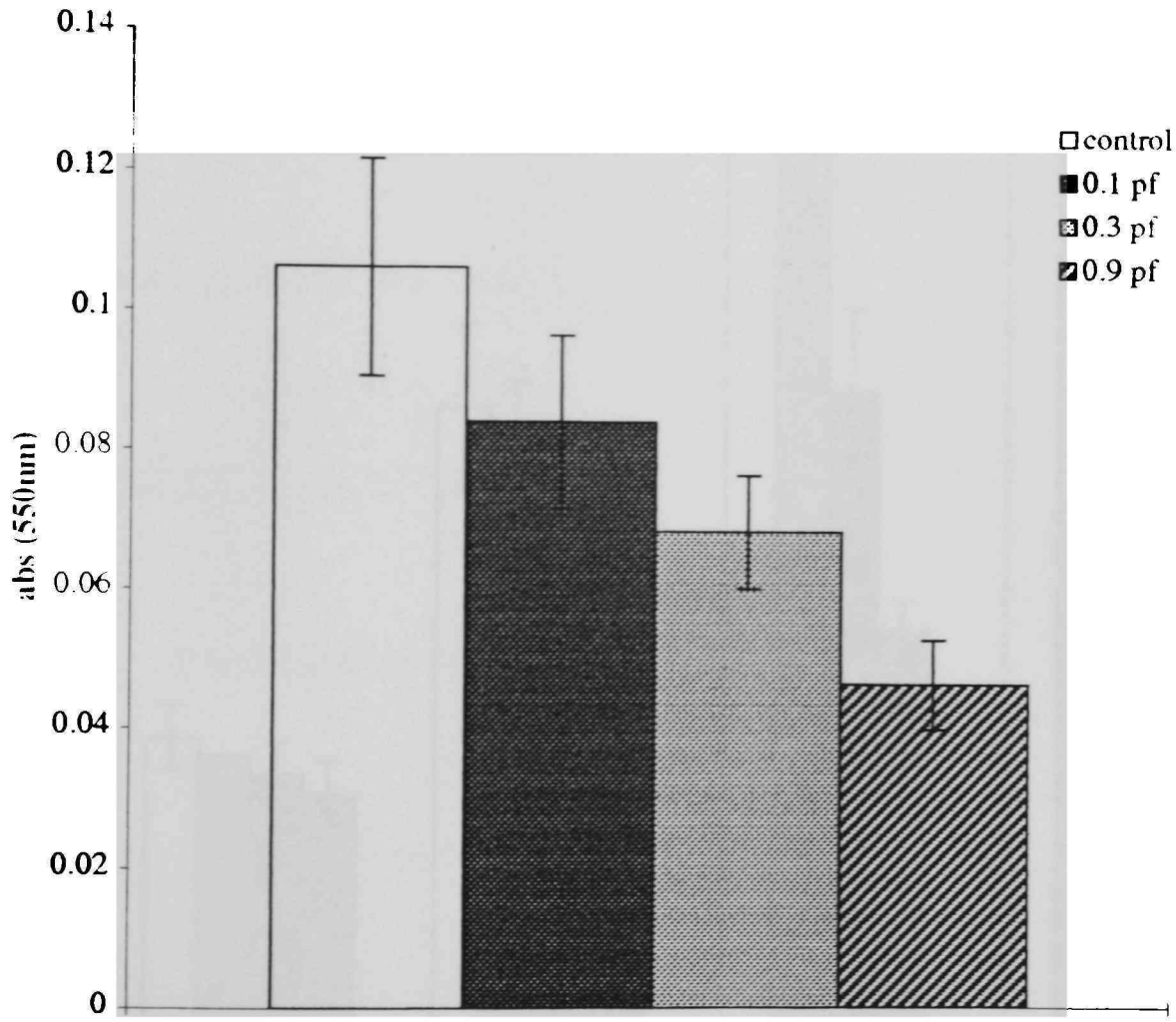


Fig. 8. Astrocytes were seeded into a 96-well plate with 0.1 to 0.9 mg/ml pirfenidone suspended in media and media alone for the controls. Control wells were allowed to reach confluency and cells were stained with neutral red, lysed, and there absorbance read at 550nm.

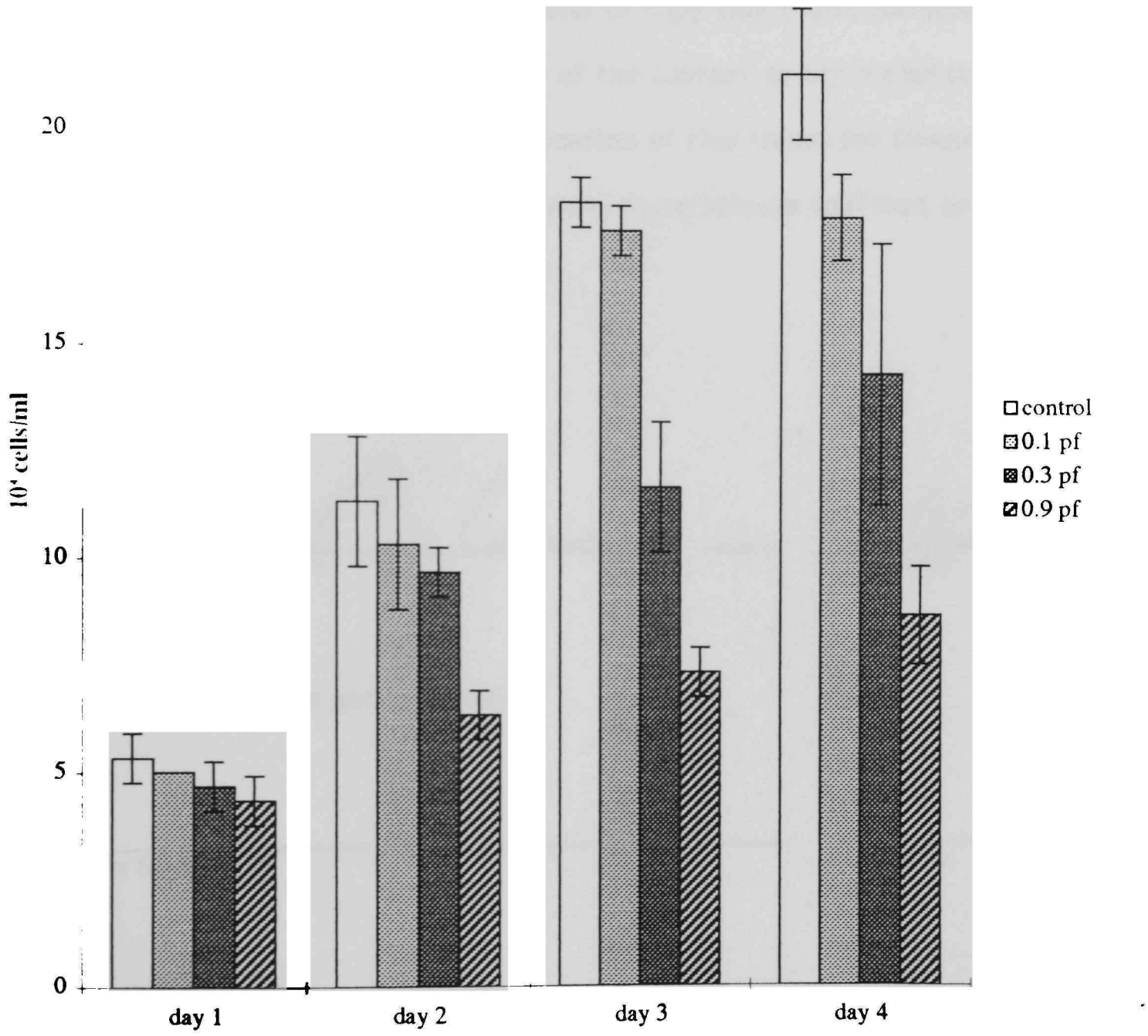


Fig. 9. HL-60 cells were grown in tissue culture flasks with 0.1 to 0.9 mg/ml pirfenidone suspended in media and media alone for controls. Aliquots were removed from each flask stained with trypan blue and enumerated for four days.

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