Preparation of Lipopolysaccharide Derived from *Pantoea agglomerans* Labeled with Fluorescence as a Tracer for Kinetics Analysis

TAKESHI KADOWAKI[1], HIROYUKI INAGAWA[2,3,4], CHIE KOHCHI[1,3,4,5], MITSUOMI HIRASHIMA[1] and GEN-ICHIRO SOMA[1,3,4,5]

[1]Department of Faculty of Medicine, Kagawa University, Miki-cho, Kida-gun, Kagawa-ken, 761-0793, Japan;
[2]Department of Applied Aquabiology, National Fisheries University, Nagatahon-machi, Shimonoseki-shi, Yamaguchi-ken 759-6595, Japan;
[3]Macrophi Inc., Hayashi-cho, Takamatsu-shi, Kagawa-ken, 761-0301, Japan;
[4]Institute for Drug Delivery Systems, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba-ken 278-8510, Japan;
[5]Institute for Health and Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima-shi, Tokushima-ken 770-8514, Japan

Abstract. *Background: Intradermal and/or oral administration of lipopolysaccharides derived from Pantoea agglomerans (IP-PA1) have shown multiple positive effects such as phylactic, anti-allergic and anti-tumor effects. It has been reported that the effects of IP-PA1 were derived from the induction of activated macrophages. However, it has not been actually clarified whether or not the orally administered IP-PA1 absorbed in the intestine reached and activated tissue macrophages. The aim of this study was to prepare and evaluate IP-PA1 labeled with fluorescence as a tracer, which could be used for IP-PA1 functional studies (administration, distribution, metabolism and excretion). Materials and Methods: IP-PA1 was labeled with fluorescein isothiocyanate (FITC). IP-PA1 was converted to the monomeric form using triethylamine solvent. Borate buffer (pH 10.5) containing FITC was added to the IP-PA1 solution, and then a sodium deoxycholate solution was added and the mixture incubated for 18 h. The conjugate in the supernatant was dialysed against phosphate-buffered saline and then the purified FITC-IP-PA1 was analyzed on thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) gel filtration. The biological activity of FITC-IP-PA1 was tested with a Limulus assay using a commercial endospecy kit and with a nitric oxide (NO) assay in the RAW 264.7 cell line using Griess reagent. The binding of FITC-IP-PA1 on RAW 264.7 cells was measured by flow cytometry. Results: FITC-IP-PA1 and free FITC molecules had different Rf values on TLC. The peak of FITC-IP-PA1 and unlabeled IP-PA1 had the same retention time by HPLC. The Limulus activity of FITC-IP-PA1 was 101% compared with unlabeled IP-PA1 (±18.4%). NO production by FITC-IP-PA1 induced dose dependency. In the flow cytometric analysis, RAW 264.7 cells exhibited high fluorescence intensity (99.4%) when cells were incubated with FITC-IP-PA1 (1 μg/ml). The binding of FITC-IP-PA1 to RAW 264.7 cells was inhibited by adding unlabeled IP-PA1. Conclusion: These results demonstrate that both FITC-IP-PA1 and unlabeled IP-PA1 are biologically active. The described FITC-IP-PA1 could be utilized in a variety of IP-PA1 functional studies such as biochemistry, immunohistochemistry, and molecular cell biology.*

Correspondence to: Gen-Ichiro Soma, Institute for Health Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushimashi, 770-8514, Japan. Tel: +81 886028103, Fax: +81 886028103, e-mail: sma5628@tokushima.bunri-u.ac.jp

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Bacterial lipopolysaccharide (LPS), which is generally considered to be an endotoxin, is the major constituent of the outer surface of Gram-negative bacteria (1). At very low concentrations (100 pg/ml), LPS is involved in the important phenomenon of activating monocytes/macrophages (2). It is well known that the activation of macrophages can be helpful for various diseases. When LPS behaves in this fashion, it helps provide protection from these diseases. Nevertheless, LPS has been little used clinically because at high concentrations it behaves as a Gram-negative bacterial toxin and is responsible for the induction of septic shock. Major events in the pathogenesis of sepsis include neutrophil, monocyte, and macrophage inflammatory responses, intravascular coagulopathy resulting from activation of plasma complements and clotting cascades, endothelial cell

damage, and hypotension. Thus, LPS exerts pleiotropic effects on many tissues and organs, and can result in multiple organ damage, circulatory collapse, and death.

Intradermal and/or oral administration of LPS derived from *Pantoea agglomerans* (IP-PA1) did not cause serious side-effects and also provided an antitumor effect in mouse experimental models and in a clinical study (3, 4). Moreover, oral administration of IP-PA1 had an analgesic, and preventative effect on diabetes, and on infectious diseases caused by bacteria and viruses (5). We have previously reported about the molecular and cellular mechanism of orally administered IP-PA1 in a teleost fish model (6). Briefly, orally administered IP-PA1 augmented the mRNA expression levels of interleukin (IL)-1β, tumor necrosis factor-α and IL-8, and reduced levels of IL-6. As a result, the functions involved in eliminating foreign substances in macrophages (phagocytosis, bactericidal activity, lysozyme activity) were enhanced after IP-PA1 was orally administered. However, it is still not clear whether or not the orally administered IP-PA1 that was absorbed in the intestine reached tissue macrophages. From the drug delivery point of view, it is important to analyze residual properties, administration, distribution, metabolism and excretion (ADME) in the body, and to identify the IP-PA1-binding cells after oral administration. In order to accomplish these objectives, it is essential to develop methods for detecting IP-PA1 *in vitro* and/or *in vivo*.

Worldwide, the *Limulus amebocyte* lysate (LAL) test is perhaps the most widespread *in vitro* assay for detection of LPS (7, 8). The LAL reagent, a crude extract of the hemocyte cells (amebocytes) of the horseshoe crab and consists of a mixture of clotting enzymes. The assay as originally described was primarily a quantitative endpoint type of endotoxin determination and was referred to as the gel-clot test. A more recent modification of the assay incorporates a spectrophotometer and measures the turbidity or color when a synthetic peptide substrate that contains a chromophore is added to the LAL reagent. The LAL assay is an enzymatic cascade and the consequent formation of a gel (gel-clot assay) and/or increases of turbidity (turbidimetric assay), or color (chromogenic assay) can be quantitatively measured as a function of the amount of endotoxin added. Although all these assay variations have been used successfully to measure endotoxin, it is difficult to analyze the differences among LPSs which have different structures. In addition, it has been reported that LAL inhibitors exist in human blood that can mask the ability of the LAL assay to detect the endotoxin (9, 10). This means that administered IP-PA1 cannot be reliably detected with a LAL assay, as it cannot be distinguished from other types of LPS. To resolve these problems, we prepared labeled IP-PA1 that can potentially be detected directly and specifically. Methods that have been used in the past involve labeling the LPS itself either with radiochemical (11, 12) or with fluorescent probes (13-16). The use of fluorescent probes has an advantage in that it allows an analysis of binding to different cell types at the same time on a per cell basis; these preparations can be studied using either flow cytometry or with a microscope. Labeling LPS with fluorescence has been one of the most common methods of studying LPS binding characteristics (14, 15, 17-21). Hence, we hypothesized that IP-PA1 labeled with fluorescence might be detected specifically as IP-PA1 not only *in vitro* but also *in vivo*. The aim of this study was to produce and evaluate IP-PA1 labeled with fluorescence for functional studies.

Materials and Methods

Preparation of FITC-IP-PA1. LPS from *Pantoea agglomerans* (IP-PA1) was purchased from Macrophi, Inc (Kagawa, Japan). IP-PA1 was labeled with fluorescein isothiocyanate (FITC) according to the modified method of Weersink *et al*. (see 14, 16). IP-PA1 (4 mg) was converted to the monomeric form by treatment with 2 ml of 0.5% triethylamine (Sigma-Aldrich, Tokyo, Japan) and by sonication for 15 min on ice. After the sonication, 200 μl of 100 mM ethylenediaminetetraacetic acid (Dojindo, Kumamoto, Japan) were added. The pH was then adjusted to 5 by adding 10 μl of 1 N HCl. A total of 800 μl of 0.25 M borate buffer (pH 10.5) containing 20 mg of fluorescein-5-isothiocyanate (Invitrogen, Tokyo, Japan) was added to the IP-PA1 solution. The mixture was sonicated again for 1 min. After the addition of 1 ml of 1.6% sodium deoxycholate (Sigma-Aldrich), the mixture was incubated for 18 h at 37˚C while rotating. Aggregates were pelleted by centrifuging the mixture at 10,000×g. The supernatant was dialysed against phosphate-buffered saline (PBS) using seamless cellulose tubing (Sanko-Junyaku, Tokyo, Japan) and passed through a PD-10 column (GE Healthcare, Tokyo, Japan) in order to separate the FITC-IP-PA1 from the free FITC molecules. The fractions containing FITC-IP-PA1 were pooled, concentrated, and dialyzed against PBS. The purification of FITC-IP-PA1 was analyzed on thin layer chromatography (TLC). TLC analysis was performed on 20×20 cm glass plates, Kieselgel 60˚F 254 (Merck, Darmstadt, Germany). Samples were applied with 5-μl ringcaps (As-one, Osaka, Japan). Samples of 5 μl were applied as 5-mm bands with a 1-mm distance between the centers of bands. Plates were developed for 20 min in a presaturated vertical glass chamber (Camag, Tokyo, Japan) with ethyl acetate (Wako, Osaka, Japan) as a mobile phase. Migration distance of the mobile phase was 7 cm. After development, the plates were dried and visualized using a UV trans-illuminator (ATTO, Tokyo, Japan)

Gel filtration analysis of FITC-IP-PA1. FITC-IP-PA1 (20 μg) was injected onto a TSK-gel G3000SW column (TOSOH, Tokyo, Japan). The column was pre-equilibrated with 100 mM ammonium acetate buffer (pH 5.0) at a flow rate of 1 ml/min. The fractions eluting from the column (monitored at 210 nm) were collected. Fluorescence intensity of the fractions was detected with a fluorescence microplate reader (SPECTRA max GEMINI XPS; Molecular Devices, Tokyo, Japan) equipped for excitation at 490 nm and emission at 520 nm.

Biological activity of FITC-IP-PA1. The biological activity of the FITC-IP-PA1 preparation was tested with a LAL assay (Endospecy; Seikagaku Co, Tokyo, Japan) according to the manufacturer's

Table I. *Limulus test of FITC-IP-PA1*.

Preparation	Comparison with unlabeled IP-PA1	Free FITC content
	LAL activity (%)	(%)
FITC-IP-PA1	101±18.4	<0.5

The biological activity of FITC-IP-PA1 was determined by the *Limulus* test. The tests were performed essentially as recommended by the manufacturer as described in the Materials and Methods.

instructions and calibrated with unlabeled IP-PA1. Biological activity was further assayed as the induction of nitric oxide production in a RAW 264.7 cell line, as described elsewhere (22, 23)

FITC-IP-PA1 Flow cytometric binding assay. Murine macrophages, RAW 264.7 cells (1×10^6 cells) were incubated with FITC-IP-PA1 in RPMI-1640 containing 10% fetal calf serum (FCS) for 30 min at 37°C. After incubation, the cells were washed twice with PBS and fixed with IntraPrep reagent 1 (Beckman Coulter, Tokyo, Japan). To analyze FITC-IP-PA1 binding to subpopulations of RAW 264.7 cells, the cell suspensions were analyzed by using a flow cytometer (Cytomics FC 500; Beckman Coulter) equipped with an argon-ion laser operating at 488 nm. Fluorescence intensity of each cell was recorded on a logarithm scale and data were analyzed with WinMDI 2.8 software (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). For the binding specificity of FITC-IP-PA1, RAW 264.7 cells were incubated with unlabeled IP-PA1 (100 ng/ml) and FITC-IP-PA1 (100 ng/ml). The results were expressed as the mean fluorescence of 5000 cells. The same cell suspensions were poured onto glass slides and analyzed using a fluorescence microscope (ECLIPSE Ti; Nikon, Tokyo, Japan.)

Results

Preparation of FITC-IP-PA1. To analyze the purified FITC-IP-PA1, the experiment was performed on TLC plates. As shown in Figure 1, FITC-IP-PA1 exhibited different Rf values from that of the free FITC molecules and the mixture, which contained FITC molecules and IP-PA1. This means that FITC-IP-PA1 does not contain free FITC molecules. In the HPLC gel-filtration analysis, the peak of FITC-IP-PA1 and unlabeled IP-PA1 detected by UV 210 nm and fluorescence showed the same retention time (Figure 2). This means that detecting fluorescence of FITC-IP-PA1 is equivalent to detecting IP-PA1.

FITC-IP-PA1 retains biological activity. In order to investigate the biological activity of this conjugate, RAW 264.7 cells were tested with both a LAL assay and for NO production. In the LAL assay, FITC-IP-PA1 retained LAL activity equivalent to that of unlabeled IP-PA1 (Table I). In the induction of NO production by RAW 264.7 cells, the amount of NO produced increased with increasing concentrations of FITC-IP-PA1 (Figure 3). This shows that FITC-IP-PA1 has biological activity.

Binding to RAW 264.7 cell. The binding of FITC-IP-PA1 to RAW 264.7 cells was tested by flow cytometry. RAW 264.7 cells were incubated with FITC-IP-PA1 (0, 10, 100, 1000 ng/ml) in RMPI-1640 containing 10% FCS. As shown in Figure 4, RAW 264.7 cells incubated with 1000 ng/ml of FITC-IP-PA1 exhibited strong fluorescence intensity (>98%) when compared with a negative control (medium, FITC, FITC and unlabeled IP-PA1 mixture). The fluorescence intensity increased with increasing concentrations of FITC-IP-PA1. In order to test the specificity of the binding of FITC-IP-PA1, RAW 264.7 cells were incubated with 100 ng/ml of unlabeled IP-PA1 and with the FITC-IP-PA1 mixture. The fluorescence intensity of FITC-IP-PA1 was inhibited by unlabeled IP-PA1. This demonstrated that the FITC-IP-PA1 binding was specific. The same cell suspension was poured onto glass slides and analyzed using fluorescence microscopy (Figure 5).

Discussion

In the present investigation, we produced fluorescent-labeled IP-PA1 (FITC-IP-PA1), and evaluated its physical and biological properties for utilization in IP-PA1 functional studies. The *Limulus* activity of FITC-IP-PA1 was equivalent to that of unlabeled IP-PA1 (Table I). The induction of NO production by FITC-IP-PA1 was enhanced with dose dependency (Figure 3). It is known that these assays depend on lipid A, which plays the central role of binding to target cells and in exerting a biological activity (24-30). Due to its amphipathic nature, LPS has strong tendency to form micelles in solution (31). The micelle behavior depends on the concentration and the nature of the LPS molecule. Smooth-type LPS is believed to self-assemble into micelle structures that have molecular masses of greater than 1 M Da (32). This self-aggregation behavior is derived from the characteristics of the lipid A component of the LPS molecule. In the gel-filtration analysis, the peaks of FITC-IP-PA1 and unlabeled IP-PA1 were detected at the high molecular-weight position for the same retention time (Figure 2). The present study supports the idea that the lipid A of FITC-IP-PA1 retains essentially the same functions as in unlabeled IP-PA1. This means that FITC-IP-PA1 should be a good tracer for IP-PA1 in functional studies.

In the flow cytometric analysis, RAW 264.7 cells incubated with FITC-IP-PA1 exhibited strong fluorescence intensity (Figure 4). The fluorescence intensity of FITC-IP-PA1 was also inhibited by unlabeled IP-PA1. These results suggest that both FITC-IP-PA1 and unlabeled IP-PA1 share the same receptors in RAW 264.7 cells. Therefore, it should also be possible to use FITC-IP-PA1 for receptor analyses of IP-PA1. It has been well elucidated that bacteria and/or LPS activates transcription of genes encoding inducible enzymes and cytokines through toll-like receptor 4 (TLR4) (26, 33-37). In

Figure 1. *Thin layer chromatogram of FITC-IP-PA1. Samples were applied as 5 μl spots, and developed in ethyl acetate. After development, the plates were dried and visualized using UV trans-illuminator (see Materials and Method). (1) IP-PA1, (2) FITC, (3) FITC and IP-PA1 mixture, (4) FITC-IP-PA1.*

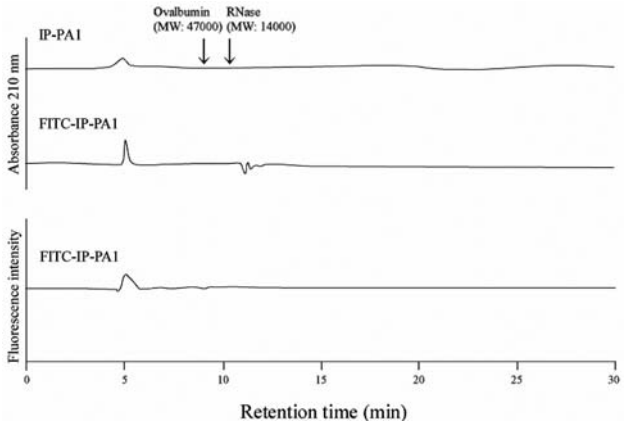

Figure 2. *Elution profile of FITC-IP-PA1 gel filtration chromatography using HPLC. The column was eluted by 100 mM ammonium acetate at 25˚C at a flow rate of 1 ml/min.*

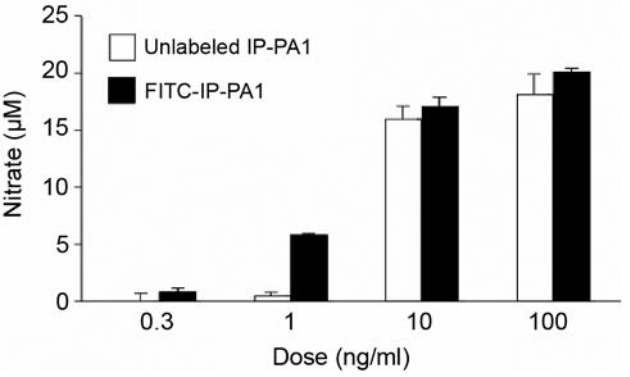

Figure 3. *Nitric oxide (NO) production by RAW 264.7 cells incubated with IP-PA1 and FITC-IP-PA1. RAW 264.7 cells were stimulated with 0.3-100 ng/ml of FITC-IP-PA1. The concentrations of NO released in the supernatant were measured as nitrite using Griess reagent. Data are represented as means±SD (n=3).*

addition, it has been noted that phagocytic receptors (such as scavenger receptors and complement receptor 3 (CR3, CD11b/CD18)) bind LPS on bacterial surfaces and also recognize microbes (38-41). Owing to this, there are two distinct routes of LPS trafficking with two distinct biological outcomes. LPS aggregates are clearly delivered to lysosomes (42-44). Delivery to lysosomes results in degradation of LPS, and the action of one particular lysosomal enzyme (45) results in the transformation of LPS to a form that antagonizes the cell-stimulating capacity of complete LPS (46). It has been reported that monomeric LPS is delivered to the Golgi apparatus, a process associated with cell stimulation (18). However, little is known about the interaction of receptor and intracellular dynamics of IP-PA1. To clarify the intracellular dynamics of IP-PA1, further analysis is needed.

We have already reported that IP-PA1 showed preventative or therapeutic effects after oral or percutaneous administration for various diseases including infectious diseases, and it did not cause serious side-effects. It is notable that lipid A of IP-PA1 has a structure that is similar to LPS derived from *Escherichia coli* (LPSe) (47). In other words, the difference between IP-PA1 and LPSe is in the structure of the polysaccharides (especially the O antigen). Therefore, we hypothesize that there are unknown receptors on the cell membrane which recognize the structure of IP-PA1 polysaccharides. If this is the case, FITC-IP-PA1 would be useful for identifying this receptor.

In conclusion, we prepared IP-PA1 with fluorescence labeling, which resulted in a biologically active conjugate. This preparation enabled us to visualize IP-PA1, which means it could be used for IP-PA1 functional studies such as ADME and for identifying new receptors.

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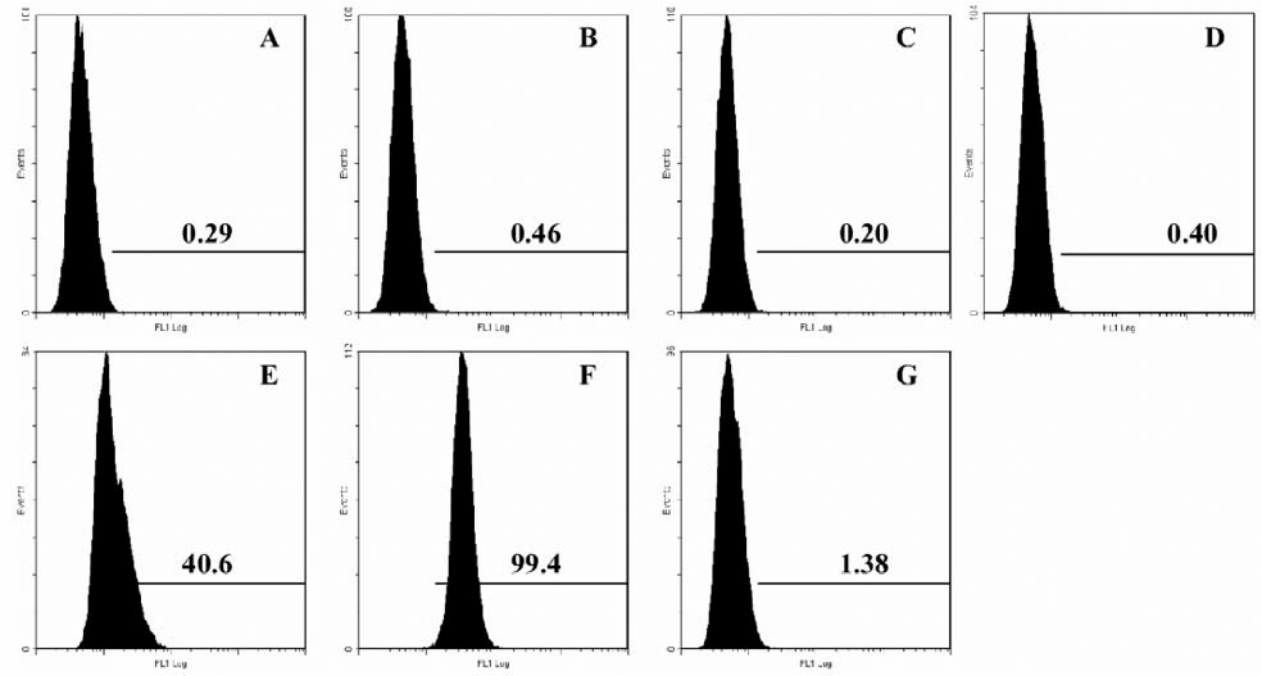

Figure 4. *Flow cytometric analysis of the binding of FITC-IP-PA1 to RAW 264.7 cells. RAW 264.7 cells were untreated (A) or treated with FITC (B), an FITC and IP-PA1 (1 μg/ml) mixture (C), FITC-IP-PA1 (10 ng/ml) (D), FITC-IP-PA1 (100 ng/ml) (E), FITC-IP-PA1 (1 μg/ml) (F), or IP-PA1 (100 ng/ml) and a FITC-IP-PA1 (100 ng/ml) mixture (G) for 30 min.*

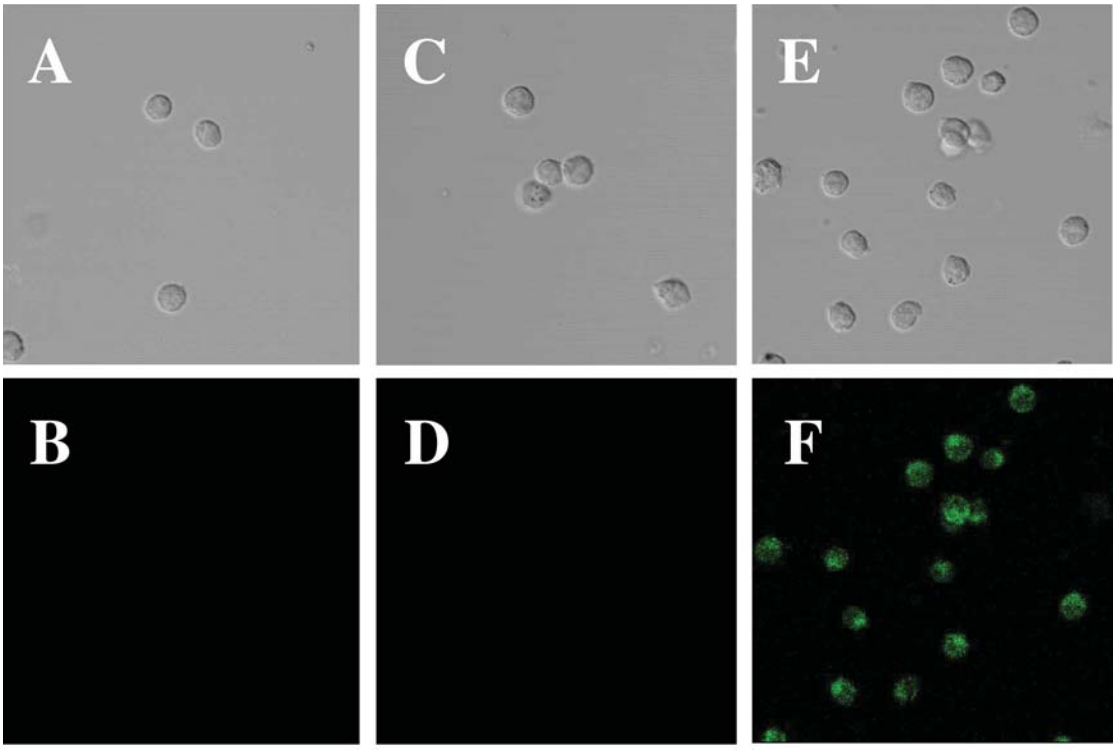

Figure 5. *Fluorescence microscopy images of the binding of FITC-IP-PA1 to RAW 264.7 cells. A, B: Not treated; C, D: FITC; E, F: FITC-IP-PA1 (1 μg/ml). The top panel shows images taken using a light microscope. The bottom panel shows images taken using a filter with a wavelength of 490 nm.*

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