FEBS Letters 355 (1994) 183–186

FEBS 14816

Mycotin: a lectin involved in the adherence of *Mycobacteria* to macrophages**

Swapnanjan Goswami, Saurav Sarkar, Joyoti Basu, Manikuntala Kundu, Parul Chakrabarti*

Department of Chemistry, Bose Institute, 93/1, APC Road, Calcutta 700 009, India

Received 14 October 1994

Abstract Pathogenic *Mycobacteria* colonize host macrophages. Attachement of these organisms to macrophages is the preliminary step prior to invasion of the macrophages by the bacteria. Western blot confirmed that walls of *Mycobacterium avium* and *Mycobacterium tuberculosis* contain molecules which are immunologically related to mycotin, a lectin found in *Mycobacterium smegmatis*. We have demonstrated that the adherence of *Mycobacteria* to macrophages is significantly inhibited by anti-mycotin antibody or the mycotin-specific sugar, mannan. These observations suggest that prevention of the interaction of mycotin-related molecules on the surfaces of *Mycobacteria* with mannose-specific receptors on macrophages, offers an important approach for blocking attachment of pathogenic *Mycobacteria* to macrophages, thereby preventing infection.

Key words: Mycotin; Mycobacteria; Macrophage; Mannan; Adhesion; Lectin

1. Introduction

Mycobacterial infections (leprosy, tuberculosis and AIDS-associated *Mycobacterium avium* infection) pose an ever-increasing threat due to the emergence of multi-drug resistant strains and the susceptibility of AIDS patients to *M. tuberculosis* and *M. avium* infections. The need to develop new strategies for the prevention of infection by all these mycobacterial pathogens that proliferate within the host macrophages [1] persists. Since adhesion of pathogens to the host cell surface is the first and most crucial step in the entry of the pathogen inside the host cell, this represents one of the targets for designing better chemotherapeutic approaches towards combatting such infections. The importance of lectins in mediating adherence to surfaces colonized by microorganisms, has been amply documented in recent times [2]. We have previously reported the characterization of an extracellular lectin from the cultivable strain *M. smegmatis* [3].

The lectin was of M_r 12–14 kDa and was specific for mannan. The present report demonstrates for the first time the involvement of this lectin in *Mycobacteria*–macrophage interaction.

2. Materials and methods

2.1. Strains and media

All *Mycobacteria* strains except *M. leprae* were grown in Middlebrook 7H9 broth containing ADC supplement. *M. leprae* was obtained from tissues of patients at the School of Tropical Medicine, Calcutta.

2.2. Purification of lectin

M. smegmatis lectin, hereinafter called mycotin, was purified as described before [3].

2.3. Isolation of cell walls of M. smegmatis

Cells of *M. smegmatis* were sonicated for 15 min at 250 W using a sonicator (Labsonic 2000, B. Braun, Germany). Unbroken cells were pelleted by centrifugation at $1,000 \times g$ for 10 min twice, and at $2,500 \times g$ for 40 min. Supernatants were further centrifuged at $5,000 \times g$ for 40 min and cell walls were prepared from pellet as described by Nikaido et al. [4].

2.4. Immunochemical detection of mycotin-related molecules on the cell surface of Mycobacteria

Microtitre wells were coated with bacteria (10^6) overnight at 4°C. The load of adherent bacteria in the microtitre wells was measured as described earlier [5]. Anti-mycotin antibody, raised in rabbit, was then used to detect the presence of mycotin-like molecules by standard ELISA techniques. Cells of *Escherichia coli* and *Shigella dysenteriae* were used as negative controls.

2.5. Adherence of mycotin to mouse peritoneal macrophages

Monolayers of macrophages (2.5×10^4) were prepared in each well of the microtitre plate by incubating them for 1 h at 37°C in a CO_2 incubator. Non-adherent cells were removed by washing with RPMI 1640. Adherence of mycotin to macrophages was studied by incubating the macrophages with varying doses of mycotin either in the presence or absence of mannan (or other sugars) for 1 h either at 4°C or at 37°C. Monolayers were then fixed with methanol. Mycotin molecules bound to macrophages were detected by incubating with rabbit anti-mycotin antibody in 5% BSA containing 10 μg of human IgG (Pierce) per ml (to block the F_c receptors of the macrophages) by standard ELISA techniques using peroxidase-conjugated second antibody and *o*-phenylenediamine as substrate. Controls consisted of wells without mycotin, and anti-mycotin antibody or conjugated goat antibody each added directly to macrophages alongwith human IgG as described above.

2.6. Adherence of bacteria to mouse peritoneal macrophages

Monolayers of macrophages (2.5×10^4 per well) in microtitre plates were incubated with bacteria (2×10^7 in 100 μl) for 30 min at 4°C. The monolayers were then washed with RPMI 1640 to remove unattached bacteria and fixed with methanol [6], followed by ELISA as described above, using anti-*M. smegmatis* cell wall antibody as the first antibody. To study the effect of anti-mycotin antibody or mannan, bacteria were preincubated for 30 min at 37°C with anti-mycotin antibody or mannan, washed thoroughly and then added to wells containing murine macrophages. Treatment of bacteria with anti-mycotin antibody was also carried out in the presence of varying concentrations of mycotin, in order to study the ability of mycotin to inhibit the binding of anti-mycotin antibody to bacteria. The procedure followed to study the attachment was the same as described above. Negative controls were performed by incubating bacteria with pre-immune sera or non-inhibitory sugars like glucose, galactose, dextran and fucose.

*Corresponding author. Fax: (91) (33) 350 6790.

**A portion of this work was presented at the 15th International Lectin Meeting in Szeged, Hungary, 1993.

Abbreviations: ADC, albumin-dextrose-catalase; IFA, incomplete Freund's adjuvant; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; ELISA, enzyme-linked immusorbent assay; OPD, *O*-phenylenediamine.

SSDI 0014-5793(94)01203-2

2.7. Western blotting

Cell wall components, solubilized with 2% sarkosyl, were run on 10% SDS gels. Western blotting was performed using anti-mycotin antibody, according to the method of Towbin et al. [7] with peroxidase-conjugated second antibody and 4-chloro-1-napthol as the colour development reagent.

3. Results

The presence of molecules immunologically related to mycotin was studied by ELISA using anti-mycotin antibody following attachment of bacteria to microtitre wells. All five species, *M. smegmatis*, *M. tuberculosis*, *M. leprae*, *M. kansasii* and *M. avium*, were found to contain molecules immunologically related to mycotin on the cell surface (Fig. 1). In contrast, non-mycobacterial species e.g. *Shigella dysenteria* and *Escherichia coli* did not show the presence of any mycotin-related molecules.

As a preliminary step towards determining whether mycotin was in any way involved in the binding of *Mycobacteria* to macrophages, the attachment of mycotin to murine macrophages was studied by ELISA. Mycotin was found to bind to macrophages in a dose-dependent manner (Fig. 2). Binding of mycotin to macrophages at 4°C, were similar to those obtained at 37°C (Fig. 2a,b), suggesting that there is no internalization of mycotin by macrophages at 37°C. Binding was completely inhibited by mannan, at a concentration of 80 $\mu g \cdot ml^{-1}$ (Fig. 3). This obviously suggested that macrophages probably contain glycoconjugate receptor(s) capable of binding mycotin, and opened up the possibility that mycotin is at least one of the determinants of attachment of *Mycobacteria* to macrophages. The binding of mycotin to macrophages however, could not be inhibited when mycotin was preincubated with non-specific

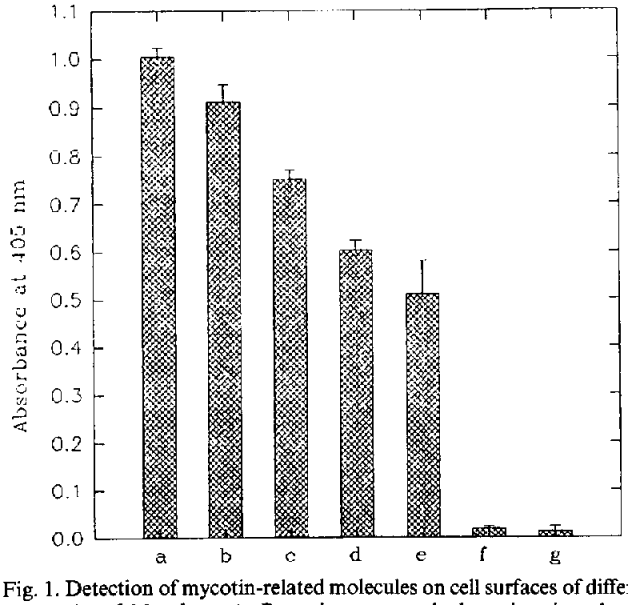

Fig. 1. Detection of mycotin-related molecules on cell surfaces of different species of *Mycobacteria*. Bacteria were attached to microtitre plates and the presence of mycotin-related molecules on the cell surface was measured using anti-mycotin antibody and conventional ELISA. Results represent the mean of three separate determinations with separate samples of bacteria. All assays were performed in triplicate. Error bars indicate standard deviations from the mean. a, b, c, d, e, f and g represent data obtained for *M. smegmatis*, *M. tuberculosis*, *M. leprae*, *M. kansasii*, *M. avium*, *E. coli* and *S. dysenteriae*, respectively.

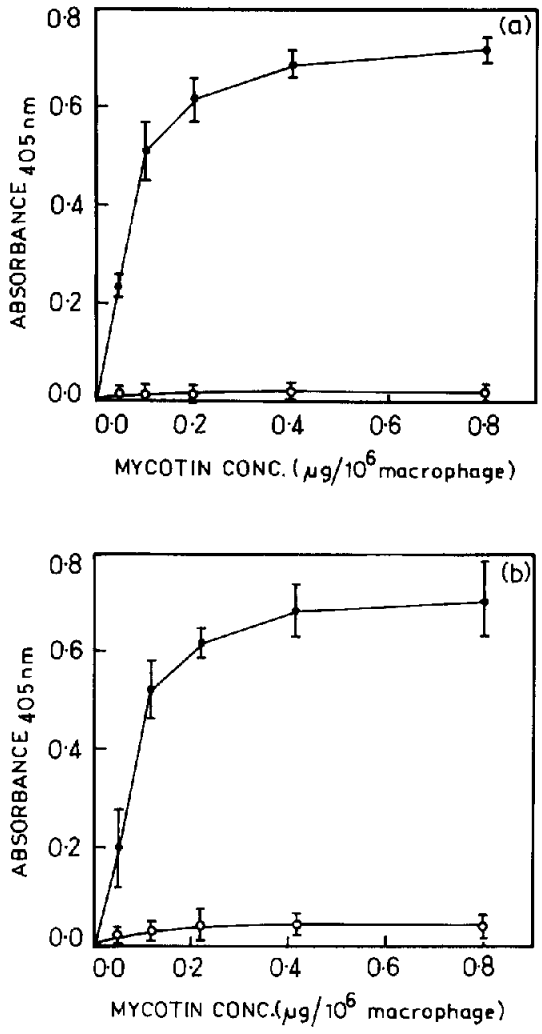

Fig. 2. Dose dependence of binding of mycotin to macrophages. Macrophages were attached to microtitre plates. Mycotin at different concentrations was added to the wells and incubated in presence (○–○) or absence (●–●) of mannan either at 37°C (a) or at 4°C (b) for 1 h. Unbound mycotin was washed, and the monolayers were fixed with methanol. Bound mycotin was assayed by ELISA. Binding of anti-mycotin antibody directly to macrophages was < 5% of the value obtained in the presence of mycotin. Results represent the mean of three separate determinations using separate samples of macrophage. All the assays were performed in triplicate. Error bars indicate standard deviations from the mean.

sugars such as dextran, glucose, galactose and fucose (data not shown).

Antibody against the cell wall of *M. smegmatis* was found to cross-react with all species of *Mycobacteria* tested and was subsequently used for detection of adherence of these species to macrophages. All four species of *Mycobacteria* were found to adhere to macrophages (Fig. 4), and the adherence was inhibited in all the cases with mannan and anti-mycotin antibody. In contrast, no inhibition was found when the bacteria were preincubated with pre-immune sera or other soluble sugars like dextran and glucose. The inhibition with mannan which is specific for mycotin, was 68%, 88%, 89% and 98% in the case of *M. smegmatis*, *M. tuberculosis*, *M. avium* and *M. kansasii* respectively (Fig. 5A). A similar experiment, performed only

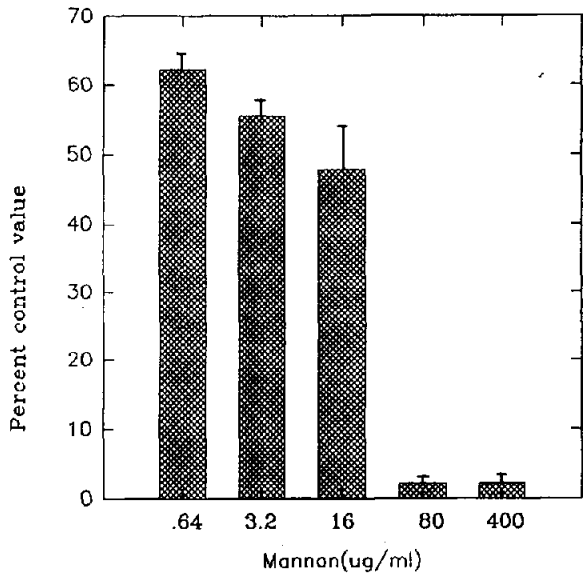

Fig. 3. Inhibition of the attachment of mycotin to macrophages by mannan. The attachment of mycotin to macrophages as described in Fig. 2, was carried out in the absence or presence of different concentrations of mannan. 0.6 μg of mycotin was used in each assay. The result obtained in the absence of mannan was taken to be the control value. Results represent the mean of three separate determinations with three separate samples of macrophage. All the assays were performed in triplicate. Errors bars indicate standard deviations from the mean.

once with *M. leprae*, showed 42% inhibition. The data confirmed that the interaction of mycotin with a mannose-containing glycoconjugate receptor is probably one of the determinants of adhesion of the mycobacterial species to macrophages. The dose-dependent inhibition by mannan (Fig. 5B) showed 70% inhibition at a concentration of 0.2 mg·ml^{-1}. No further inhibition was obtained by increasing the concentration of mannan. Anti-mycotin antibody inhibited adherence to macrophages to the extent of 50%, 66%, 87% and 67% in the case of *M. smegmatis*, *M. tuberculosis*, *M. avium* and *M. kansasii* respectively (Fig. 4).

In the single experiment performed with *M. leprae*, the inhibition was 21%. Dose-dependence studies of the inhibition of binding of *M. smegmatis* to macrophages, by anti-mycotin antibody showed that a maximum inhibition of 80% was obtained when the antibody was used at a dilution of 1 : 75 (Fig. 5A). The ability of anti-mycotin antibody to inhibit the binding of *M. smegmatis* to macrophages was completely abolished when mycotin (80 μg·ml^{-1}) was added in the incubation mixture containing bacteria and anti-mycotin antibody. In contrast no inhibition was obtained when the bacteria were incubated with non-inhibitory sugars like dextran, glucose, or with pre-immune sera. It may be assumed that mycotin acts competitively with cell surface mycotin-like molecules for binding to anti-mycotin antibody, thereby abolishing the effect of anti-mycotin antibody of inhibiting the adherence of bacteria to macrophage.

Anti-mycotin antibody gave only one positive band in Western blots using cell wall protein of *M. avium* (67 kDa) and *M. tuberculosis* (35 kDa) (Fig. 6). Negative controls were performed with cell walls of *E. coli* and *S. dysenteriae*, in which cases no bands developed (data not shown). This experiment was not performed with *M. leprae* due to paucity of material. In the case of *M. kansasii*, sufficient cell wall protein could not be obtained due to poor efficiency of cell breakage. The disparity in the size of the positive bands obtained in Western blots suggests that mycotin carrying the carbohydrate-binding epitope, possibly represents a protein, imunologically related to larger cell wall proteins.

4. Discussion

One of the steps which merits consideration as a target for developing new approaches for combatting *Mycobacteria*-caused diseases, is the primary step of attachment of the bacteria to the host cell surface. Interest in this approach was enhanced by our finding that the fast growing *Mycobacterium*, *M. smegmatis* secretes an extracellular lectin [3]. Existing reports have shown that the integrin receptor $\alpha_v\beta_3$ on monocytes and monocyte-derived macrophages binds to *M. avium-M. intracellulare* in the absence of complement [8,9]. Evidence has also been provided for binding of *M. tuberculosis* to three types of receptors on human macrophages: complement receptor 3, fibronectin receptors and mannoside receptors [10–12]. A fibronectin receptor has been purified from *M. vaccae* [13]. Although lectins have been implicated in the attachment of several bacterial species to macrophages [2,14] our studies demonstrate for the first time, the novel involvement of lectin-like proteins on the cell surface of *Mycobacteria* and identify lectin–sugar

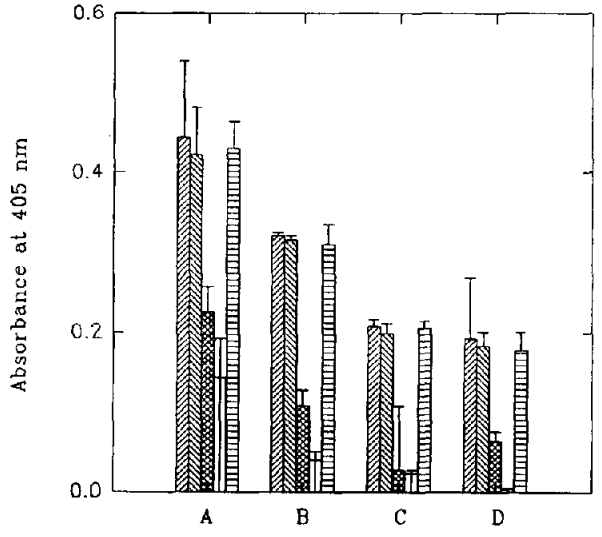

Fig. 4. Adherence of *Mycobacteria* to macrophages and inhibition of adherence by mannan or anti-mycotin antibody. Macrophages were attached to microtitre plates. Bacteria were bound to macrophages at 4°C for 30 min, followed by washing and fixing of the monolayers with methanol. Bound bacteria were detected using anti-*M. smegmatis* cell wall antibody. Treatment of bacteria with mannan (0.2 mg·ml^{-1}) or anti-mycotin antibody (dilution of 1 : 250) prior to binding to macrophages was carried out as stated under methods. Results represent the mean of three separate determinations with separate samples of bacteria and macrophages. All assays were performed in triplicate. Error bars represent standard deviations from the mean. Groups A, B, C and D represent data obtained for *M. smegmatis*, *M. tuberculosis*, *M. avium* and *M. kansasii*, respectively. ▨, untreated bacteria; ▧, bacteria treated with preimmune sera; ▩, anti-mycotin; □, mannan and ▤, glucose. Binding of anti-cell wall antibody or conjugated goat antibody directly to macrophages was < 3% of the value obtained when bacteria were added to macrophages.

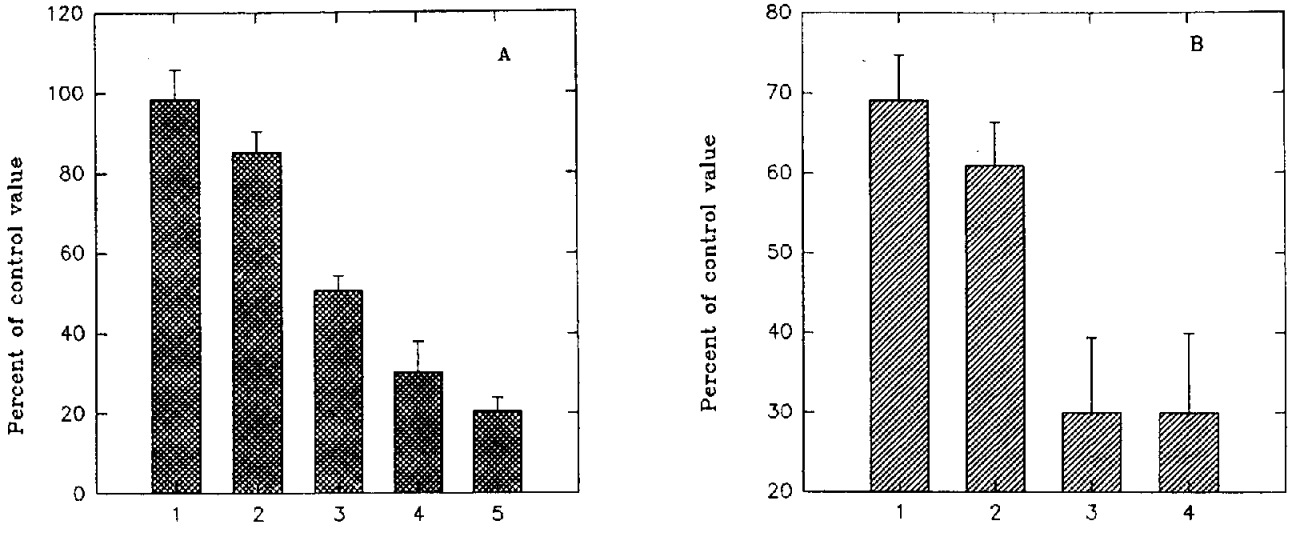

Fig. 5. (A) Dose dependence of inhibition of binding of *M. smegmatis* to macrophages by anti-mycotin antibody. Inhibtion was studied as described in Fig. 4. Results represent the mean of three separate determinations with separate samples of bacteria and macrophage. Assays were performed in triplicate. Error bars represent standard deviations from the mean. 1, 2, 3, 4, and 5 represent anti-mycotin antibody at dilutions of 1 : 10,000, 1 : 1,000, 1 : 250, 1 : 150 and 1 : 75, respectively. Results obtained with untreated bacteria were taken as control values. (B) Dose dependence of inhibition of binding of *M. smegmatis* to macrophages by mannan. Binding of bacteria to macrophages was studied as described in Fig. 4. Results represent the mean of three separate determinations with separate samples of bacteria and macrophage. All the assays were performed in triplicate. Error bars represent standard deviations from the mean. 1, 2, 3, and 4 represent mannan used at concentrations of 0.002, 0.02, 0.2 and 2 mg · ml^{-1}, respectively. Results obtained with untreated bacteria were taken as control values.

interactions as a means of attachment of pathogenic *Mycobacteria* to macrophages. Western blots confirmed the presence of two mycotin-related proteins of M_r 67,000 and 35,000 in *M. avium* and *M. tuberculosis*, respectively. The specificity of lectin-mediated attachment was established by observations that preincubation of the bacteria with mannan or anti-mycotin antibody considerably inhibited binding of the bacteria to the macrophages. The findings support the view that mycotin or related-proteins play a potential role in mycobacterial infections. Intervention of the mycotin–macrophage interaction may open avenues for combating mycobacterial diseases. This is particularly important in view of the ever increasing problem of drug resistance in mycobacterial infections.

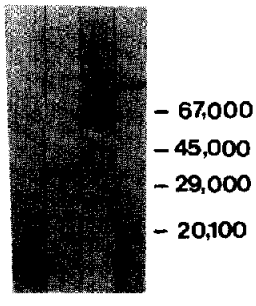

Fig. 6. Lane A represents a Western blot of purified mycotin using anti-mycotin antibody . Lanes B, C and D represent similar Western blots of sarkosyl-solubilised cell wall proteins of *M. tuberculosis*, *M. avium*, and *M. smegmatis*, respectively.

Acknowledgements: This work was supported in part by grants from the United Nations Development Programme executed through the World Health Organization and the Department of Science and Technology, Government of India. Saurav Sarkar was a post-doctoral fellow under the Department of Biotechnology-sponsored Post-doctoral Training Programme in Bose Institute.

References

[1] Crowle, A.J. and Poche P. (1989) Infect. Immun. 57, 1332–1335.
[2] Ofek, I. and Sharon N. (1988) Infect. Immun. 56, 539–547.
[3] Kundu, M., Basu, J. and Chakrabarti, P. (1989) FEBS Lett. 256, 207–210.
[4] Nikaido, H., Kim, S.-H. and Rosenberg, E.Y. (1993) Mol. Microbiol. 8, 1025–1030.
[5] Athamna, A and Ofek, I. (1988) J. Clin. Microbiol. 26, 62–66.
[6] Athamna, A., Ofek, I., Keisari, Y., Markowitz, S., Dutton, G.G.S. and Sharon, N. (1991) Infect. Immun. 59, 1673–1682.
[7] Towbin, H., Stachlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[8] Bermudez, L.E., Young, L.S. and Enkel, H. (1991) Infect. Immun. 59, 1697–1702.
[9] Catanzaro, A. and Wright, S.D. (1990) Infect. Immun. 58, 2951–2956.
[10] Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R. and Horwitz, M.A. (1990) J. Immunol. 144, 2774–2780.
[11] Schlesinger, L.S. and Horwitz, M.A. (1990) J. Clin. Invest. 85, 1304–1314.
[12] Swartz, R.D., Naai, D., Vogel, C.W. and Yeager, H., Jr. (1988) Infect. Immun. 56, 2223–2227.
[13] Ratliff, T.L., McCarthy, R., Telle, W.B. and Brown, E.J. (1993) Infect. Immun. 61, 1889–1894.
[14] Kitz, D.J., Stahl, P.D. and Little, J.R. (1992) Cell. Mol. Biol. 38, 407–412.