Airway Biofilm Disease: Clinical Manifestations and Therapeutic Possibilities Using Macrolides

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INTRODUCTION

In 1969, Yamanaka and coworkers[1] introduced the concept of diffuse panbronchiolitis, based on postmortem investigation of lung tissue.

The morphologic findings in the lung consist of chronic inflammation associated with lymphocyte and plasma cell infiltration, often with follicule formation, observed around respiratory bronchioles and the terminal bronchioles. Since their detailed report, a total of 511 cases who met these criteria have been diagnosed across Japan. In 1983, the clinical features and pathologic findings of diffuse panbronchiolitis (DPB) were described by Homma et al.[2] The clinical symptoms of infected DPB are nonspecific. Persistence of cough, increased production of purulent sputum, wheezing and exercise intolerance are often noted. Approximately 80% of the patients present with concomitant chronic sinusitis. Multiple small nodular shadows disseminated throughout the middle and lower fields of both lungs are characteristic in chest x-ray findings (Fig. 1).

However, these shadows may transform into a complicated one indicating extensive ectasia, fibrosis and subsegmental atelectasis. Although *Hemophilus influenzae* can initially infect the airways, the presence of *Pseudomonas aeruginosa*, particularly mucoid strains are usually found in 70% of the patients. Mucoid *Pseudomonas aeruginosa* with alginate biofilms persist in the small airways (Fig. 2), and exacerbation of infection often occurs when bacteria are released from the biofilms,[3] or when other pathogens invade the airways. The prognosis is influenced by repeated episodes of exacerbation of the infection and/or by persistent colonization of mucoid *Pseudomonas aeruginosa*. The accumulated data from the Japanese Investigation Committee showed in 1983 that colonized *Pseudomonas aeruginosa* is found in only 8% of patients at the 5-year survival point.[2]

The ethnological evidence that DPB is prevalent among Japanese and Mongolians has often been discussed on the gene level, and compared with cystic fibrosis which is more frequently seen in the Caucasian population. The symptoms, bacteriological and pathological findings in DPB are also closely related to those of cystic fibrosis with regard to lung infection.

CLINICAL EFFICACY OF MACROLIDES IN DPB PATIENTS

In 1987, the 10-year survival rate of DPB patients was only 12.4% for patients colonized with *Pseudomonas aeruginosa* and 73.1% for those without *Pseudomonas aeruginosa*.[4] At that time, broad spectrum β-lactams were the main compounds used to treat infected DPB.
In the recent ten years, however, long-term administration of erythromycin (EM) at a dose of 600 mg per day as the basic therapy, together with the occasional use of new quinolones when the infection is exacerbated have become the treatment of choice. The prognosis of patients including that of patients colonized with Pseudomonas remarkably improved and their 10-year survival increased to 94%.[4]

The clinical efficacy of EM in DPB was first noted in 1982 by Dr. Miyazawa. This was later confirmed by Kudoh et al.,[5] and Sawaki et al.[6] They employed a 600 – 1,000 mg daily dose of EM for DPB patients for more than three months, and reported an efficacy rate of 100% in 18 patients[5] and 86% in 14 patients.[6] Retrospective analysis of the results obtained in DPB patients treated with EM compared to new quinolones was conducted by Yamamoto's committee.[7] Improvement was observed in 67% of patients treated with EM and in 22% of those treated with new quinolone group. Deterioration was reported in 9% of patients treated with EM and 27% of those treated with the new quinolones. Furthermore, a double-blind trial of EM and placebo for DPB patients was performed by a study group sponsored by the Ministry of Health and Welfare of Japan in 1991.[8] After three months of continuous administration of EM, improvement was observed in 60% of the cases. In contrast, improvement in the placebo group was only 15% ($p < 0.05$). Furthermore, deterioration was observed in only 6% of the patients in the EM group compared to 41% in the placebo group. The results of the above studies indicate that long-term administration of EM is useful for the treatment of diffuse panbronchiolitis.

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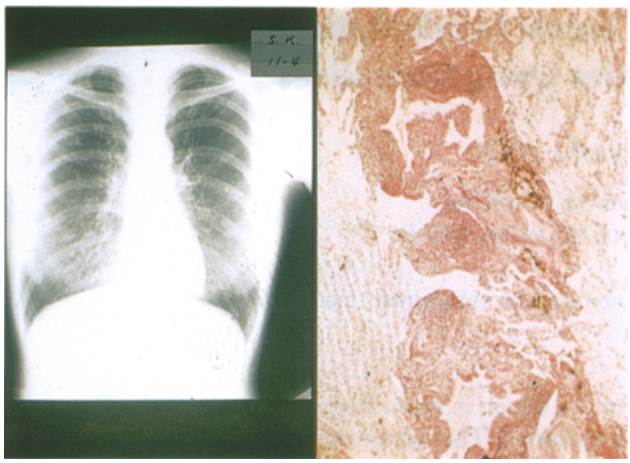

Fig. 1: *Typical findings in patients with DPB. Multiple small nodular shadows disseminate through middle and lower fields of both lungs, and lung volume is hyperlucently expanded by emphysematous changes. Lymphocyte infiltration associated with granulomatous changes is seen around the terminal bronchioles. PMNs influx is also seen in airway lumen.*

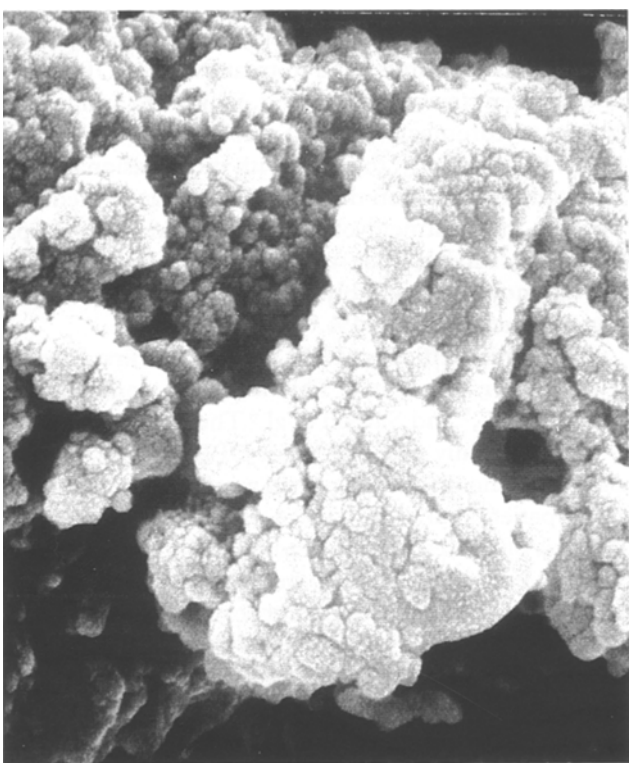

Fig. 2: *High power view of a single strain of* P. aeruginosa *inside biofilm on airway epithelium (x60000). Mucoid exopolysaccharide (=alginate or glycocalyx) is produced around the bacterium. Each bacterium is aggregated by mucoid material.*

Clarithromycin (CAM) was found to be also useful in DPB. We have evaluated the efficacy of long-term use of CAM in patients with diffuse panbronchiolitis.[9] Sixteen patients were given CAM at a dose of 300 mg per day for 14 to 26 months. A satisfactory clinical improvement inferred from a reduction of purulent secretion and increase in vital capacity, was obtained in 14 cases. The efficacy ratio was 87%. A single strain of *Moraxella catarrhalis* and six strains of *Hemophilus influenzae* were all eradicated within two weeks. Seven of eight strains of *Pseudomonas aeruginosa* were eradicated between three and eighteen months after the commencement of treatment. The mechanism of elimination of *Pseudomonas aeruginosa* remains obscure. Furthermore, CAM was also effective in patients in whom EM was either ineffective or showed little effect after long-term administration.[10]

Azithromycin (AZM), a 15-membered macrolide, was also used in 52 patients with infected DPB. AZM, at a dose of 250 mg, was administered twice a week for three months and its efficacy was 84.6% (44/52).[11] This indicates that a long-term intermittent administration of AZM can be expected to have the same effect in the treatment of diffuse panbronchiolitis as 14-membered macrolides.

The clinical efficacy of macrolides in DPB patients has only been observed in 14- or 15-membered macrolides, but not in 16-membered macrolides. However, while our clinical experience using these compounds has increased the basic mechanism of action remains obscure. The basic problem on this subject will be reviewed.

SIDE ACTION OF MACROLIDES

Several studies discussed the side action of macrolides. Most Japanese studies focusing on the clinical efficacy of macrolides in DPB, divided their action into three different categories, that is, their action on airway cells, anti-inflammatory effects and their anti-bacterial effects (Table 1).

Table 1. *Side action of macrolides (1993).*

On airway cells
Chloride ion channel block inhibitory effect on mucus secretion
On inflammatory cells
activating effect on phagocyte action, inhibitory effect on chemotaxis (PMN)
On bacteria
inhibitory effect on production of virulent factor, anti-biofilm effect

1. Macrolide action on airway cells

Goswami et al.[12] demonstrated that EM reduces respiratory glycoconjugate secretion by human airway cells and Ishikawa's adenocarcinoma cells in a dose-dependent manner. It was noted that EM reduces glycoconjugates secretion and secretion stimulated by histamine and methacholins. In contrast, other antibiotics such as peni-

cillin, ampicillin, cephalosporins and tetracycline did not have any effect on glycoconjugate secretion from either airways or adenocarcinoma cells. EM selectively inhibits the secretion of chloride across the airway mucosa and presumably decreases the subsequent movement of water toward the airway lumen by blocking chloride ion transport, as demonstrated in canine cultured tracheal epithelium.[13] These effects indicate that EM can reduce airway mucus secretion, particularly hypersecretion through mechanisms other than those controlling infection. The inhibitory effect of EM on bronchial hyperresponsiveness in asthmatic patients has also been reported.[14] These effects resemble those of corticosteroid, thus indicating that EM may have anti-inflammatory effects.

Tracheobronchial mucins are the preferential site for adherence and colonization by *Pseudomonas aeruginosa*. Furthermore, Ramphal et al.[15] demonstrated that bacterial adhesins, which were previously shown to mediate the binding of *Pseudomonas aeruginosa* to cells, also mediate bacterial binding to mucins, and those of mucoid exopolysaccharide bound to mucins enhance the adherence of mucoid strains to this site. If the inhibition of mucus hyperseretion by EM observed in the above in vitro experiments actually occurred in airway infection, the effect of EM would also be found in adhesive receptors on the bronchial epithelium.

2. *Macrolides action on inflammatory cells*

Several possible effects of macrolide on inflammatory cells have been investigated. Alveolar macrophages are known to take up all macrolide antibiotics.[16,17] Interleukin (IL)-1 produced by BDF mice peritoneal macrophages was inhibited by CAM and EM[18] in a dose-dependent manner. On the other hand, Katahira et al.[19] demonstrated that the production of IL-1 and TNF from peripheral mononuclear cells and alveolar macrophages was enhanced by EM, CAM and josamycin in a dose-dependent manner. These drugs also had a priming effect, producing IL-1α. These results support the view that the metabolic changes in Na^+, K^+, -ATPase, protein kinase C and/or calmodulin induced by such macrolides are important factors in IL-1 production.

Several other studies have demonstrated that IL-1 production by monocytes in patients treated with EM for a long period of time was enhanced and so was IL-4 production by mice splenocytes that were treated with EM over a long period.[20] IL-4 can activate macrophages, probably by increasing α_1-antitrypsin production by alveolar macrophages, which neutralize proteolytic elastase. It is well known that polymorphonuclear leukocyte (PMN) uptake[21–28] and chemiluminescence response to bacteria is enhanced. EM in concentrations, which can be achieved in humans, did not affect the granulocyte migration[29,30] except at a level 200 times that of serum levels.[31]

PMNs influx into the bronchoalveolar lavage fluid (BALF) is significantly reduced in DPB patients who have been treated with EM over a long period.[32] This is due to the inhibition of IL-8 production by 14-membered macrolides in vivo. The same phenomenon also occurred in chronic bronchitis as described by Kadota et al.[33,34] The chemotactic activity of PMNs from peripheral blood of normal volunteers and patients with DPB treated with EM for more than two weeks is also inhibited when incubated with EM.[35] These results show that 14-membered macrolides may activate the chemiluminescence response when PMNs take up bacteria, but suppress the chemotactic activity of PMNs.

The level of immunogloblins and complement components are not affected by treatment with therapeutic doses of EM in healthy volunteers.[36,37] However, lymphocyte proliferation and chemotaxis are inhibited when these cells are incubated with EM.[38] Furthermore, lymphocyte influx in antigen antibody reaction through alginate was inhibited by pretreatment of mice with AZM and EM.[39] Although it is generally accepted that lymphocyte activity is enhanced in infected DPB, the analysis of each cell subclass is necessary to clarify the role of lymphocytes in relation to the action of macrolides.

3. *Action of macrolides on mucoid* Pseudomonas aeruginosa

Since EM was introduced for clinical use in 1952, the major target of macrolides has been considered to be the infecting bacteria. EM is active against gram positive cocci, certain gram negative bacteria, anerobic bacteria and especially intracellular pathogens, but not against *Pseudomonas aeruginosa*. Recently, other actions of macrolides, that is, apart from their bactericidal effect on pathogens, have been investigated.

Kita et al.[36] described that the production of elastase and leucocidin by *Pseudomonas aeruginosa* was suppressed when incubated with 0.1 to 0.5 μg of EM. However, this effect was not observed in *Pseudomonas aeruginosa* isolated from patients who did not respond to long-term EM therapy. Such inhibitory effect of EM on Pseudomonas elastase has been observed in 80% of the strains without any effect on their proliferation, but it was not observed in the 16-membered ring macrolide midecamycin (MDM), or ampicillin.[40] Hirakata et al.[41] demonstrated that the survival rate of *Pseudomonas aeruginosa* bacteremia in immune suppressive mice induced by preadministration of cyclophosphamide was significantly enhanced by the use of the minimum inhibitory concentration (MIC) dose of EM. The authors concluded that this phenomenon was due to the dose dependency of EM by reducing the production of exotoxin A, total protease, elastase and phospholipase C from *Pseudomonas aeruginosa*.[42] AZM at sub MIC doses also suppressed the synthesis of elastase, protease lecithinase and DNAase by *Pseudomonas aeruginosa*, as

was the case with EM, CAM and roxithromycin (RXM).[43,44] Furthermore, Ras and coworkers[45] showed that the proinflammatory interaction of pyocyanin and 1-hydroxyphenazine with PMNs was antagonized by EM, RXM as well as by clindamycin. They concluded that macrolides with antioxidant properties may have useful modulatory effects on PMN-mediated tissue damage in diseases, such as infected bronchiectasis.

Pseudomonas aeruginosa, especially the mucoid strains, easily form a bacterial biofilm by producing glycocalyx or mucoid alginate around their bodies when they persistently adhere to mucosa or medical instruments.[46,47] Typical biofilm diseases are catheter-induced infections,[48,49] medical instrument-induced infections,[50,51] subacute endocarditis caused by Streptococcus,[52] chronic osteomyelitis caused by *Streptococcus aureus*,[53]chronic airway infection by *Pseudomonas aeruginosa*, including DPB,[3] and cystic fibrosis.[54] These diseases are characterized by the difficulty in eradicating the pathogens, even if concomitant treatment with bacterial agents is used. Such difficulty results from the resistance of encapsulated biofilm material against antibacterial agents[55–60] and reduced interaction with phagocyte cells[3,61,62] and complement.[63] PMNs elastase cleaves C3bi on opsonized Pseudomonas as well as CR1 on PMNs to create a functionally important opsonin-receptor mismatch. Thus, PMN elastase may be the primary mediator by which this response is more injurious than beneficial.[64] These findings observed in alginate biofilms are lacking in DPB and other biofilm diseases which promote the persistence of bacterial colonies.

Veringa et al.[65] reported that glycocalyx (alginate) production was inhibited in *Pseudomonas aeruginosa* upon contact with clindamycin and consequently the bacteria were easily eliminated by the phagocytic activity of PMNs. The bacteriocidal effect on *Pseudomonas aeruginosa* inside the biofilm was expressed by the combined use of ciprofloxacin (CPFX) and CAM[47,66] or AZM[67] compared with CPFX alone. The evidence was observed in EM, but not in josamycin.[68] A possible interpretation is that 14- and 15-membered macrolides facilitate the penetration of the bacterial biofilm by CPFX which would eliminate the bacteria inside the biofilm. That is, it depends on the combination effect of the anti-pseudomonas action of CPFX and the anti-alginate action of such macrolides. An experimental Pseudomonas biofilm was destroyed after incubation with 10 μg/ml of CAM for five days, and their forms changed into free bacteria (Fig. 3). A similar result was observed with EM and AZM, but not with josamycin and MDM. The anti-biofilm effects of only 14- and 15-membered macrolides are very attractive in light of their clinical efficacy in patients with DPB.

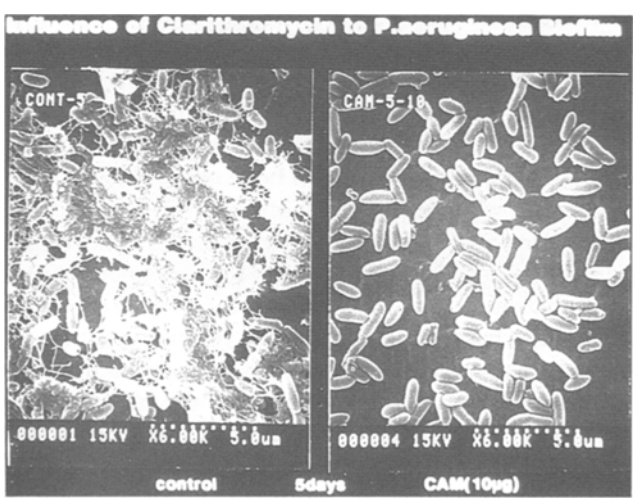

Fig. 3: *Effect of CAM on* P. aeruginosa*—Biofilm. In the* in vitro *experiment, Pseudomonas biofilm persists (left). Adding 10 μg/ml of CAM to this medium, Pseudomonas biofilm was completely destroyed 5 days after incubation.*

4. Conclusive remarks

Most of the Japanese studies presented in this review were probably performed while focusing on the clinical efficacy of macrolides on DPB patients. The obtained results are very important to elucidate the side action of these compounds. It can be said, however, that the results do not explain directly the pathophysiologic features of infected DPB since the etiology of DPB is still unclear and an experimental model is hard to design. A more detailed systematic observation focusing directly on DPB is necessary.

With this viewpoint as a background, our recent results will be introduced and discussed in the following section.

ANALYSIS OF INFECTED DPB

1. Clinical analysis

Although the primary etiology of DPB remains obscure, in clinical practice patients with DPB often present with a concomitant chronic persistent infection. Individual findings in such patients were subjected to factor analysis. Important factors finally remained after the elimination approach, including mucoid *Pseudomonas aeruginosa* bacteriologically, lymphocyte infiltration surrounding small airways morphologically, and airway secretion with increased PMNs symptomatologically. These factors were also observed in lung infection associated with cystic fibrosis patients.[60] The three factors are the hallmark of infected DPB (Fig. 4), but the individual factors seem to have no direct relationship with each other. These factors, however, are observed in infected DPB patients in the same airway site at the same time. To detect and solve the mutual relationship between these factors may lead not only to a better understanding of the pathophysiologic state of infected DPB, but also to elucidate the mechanism of the efficacy of macrolide on this disease.

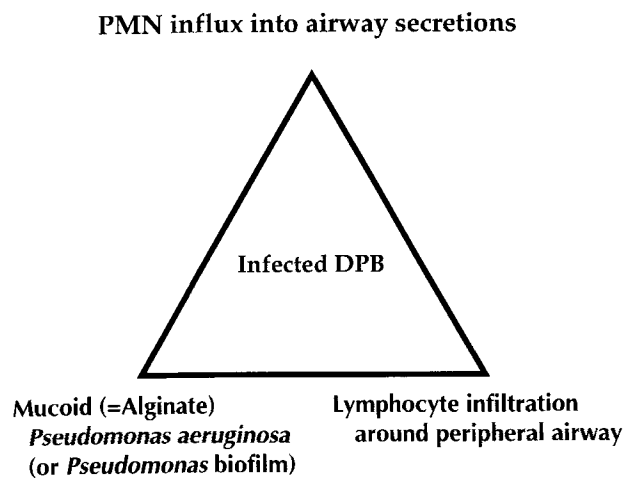

Fig. 4: Important factors observed in infected DPB.

2. *Relationship between mucoid* Pseudomonas aeruginosa *and PMN influx*

Nonmucoid *Pseudomonas aeruginosa* initially invade the airways and subsequently change their form into mucoid strains producing alginate, a major component of the mucoid material. The process is similar to that observed in lung infections in patients with cystic fibrosis.[69,70] The mechanisms of morphologic switching from nonmucoid strains to mucoid types have not been clarified. Kilbourne et al.[71] demonstrated that mucoid switching occurred in 2.9% of nonmucoid strains when incubated with a high concentration of NaCl solution in vitro. But this observation was not confirmed in our followup examination, even if mucoid production was observed. To avoid confusion, a mucoid strain refers to the strains that produce mucoid even after several passages in culture medium. Furthermore, mucoid switching is often observed in DPB patients, even though their airway secretion is not salty enough. Terry et al.[72] pointed out the importance of environmental conditions rather than the phage-regulated appearance of mucoid variants reported by Martin[73] and Miller and Robero.[74] An interesting observation made by Terry and coworkers was that the growth of PAO-1 strain supplied with phosphorylcholine, a product of phospholipase C activity on the major lung surfactant phosphatidylcholine, resulted in isolation of mucoid variants.

Mucoid *Pseudomonas aeruginosa*, including both alginate-producing strains and biofilm-forming strains, exhibits less virulent factors than the nonmucoid strains. Clinically, the septic syndrome induced by mucoid strains is extremely rare, even if persistent colonies exist in lung tissues.[75] Serum sensitivity of mucoid strains is usually enhanced,[76] but when they form a bacterial biofilm, their resistance against phagocytic activity in whole blood is enhanced.[77] Under these conditions, a survival rate of 100% in an experimental mouse bacteremia was observed without any infectious manifestation.[78] The production of virulent factors is less marked in proteases,[79] exoenzymes, inexotoxin A, phospholipase C,[80] and in DNAase.[81] PMN chemotactic ability was reduced in mucoid strains[61] and superoxide production was also inhibited (Fig. 5). Alginate suppression of the expression of C3 receptors in PMNs resulted from converting the calcium ion channel in PMNs (unpublished observation). The direct relationship be-

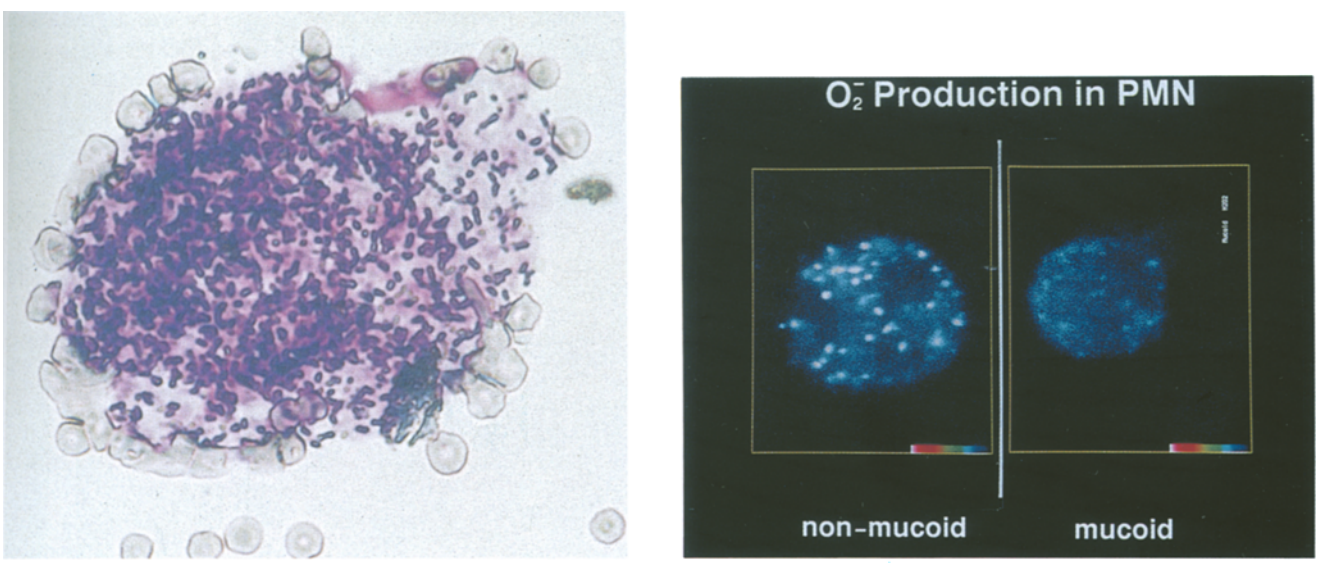

Fig. 5: Interaction with mucoid alginate-produced P. aeruginosa *and PMN. A piece of Pseudomonas biofilm in BALF (a). Only erythrocyte attachment around the biofilm is seen, and super oxide production from PMNs is inhibited in incubation with the mucoid strain. The pictures indicate that mucoid alginate-produced strains show a weak interaction with PMNs both in chemotaxis and in activation.*

tween mucoid *Pseudomonas aeruginosa* and PMN influx into airway lumen shown in Fig. 4 will be negligible. However, a possible relationship remains to be investigated. The floating-type bacteria released from the biofilm produce no glycocalyx, thus, this bacteria can adhere directly to other areas of the airway surface. Such a direct binding constitutes a strong stimulus for tissue cells and a new infectious lesion with PMN influx is formed. It explains the cause of recurrent infection often seen in airway biofilm diseases.[3,82]

3. *Relationship between mucoid* Pseudomonas aeruginosa *and lymphocyte infiltration of small airways*

Although the initial adherence is by nonmucoid *Pseudomonas aeruginosa* in DPB patients, alginate-producing mucoid strains commonly emerge soon after and remain at that site. The pathogenic role of mucoid alginate in chronic airway infection has been discussed from the viewpoint of bacterial adherence[83–87] and immune reaction.[88–90] Bryan et al.[91] detected low levels of antibody to alginate in sera of cystic fibrosis patients. High antibody levels in immunized mice by alginate prepared from *Pseudomonas aeruginosa* were also detected. The protective role of anti-alginate antibodies in sera from patients with cystic fibrosis and experimental animals was discussed by Woods and Bryan.[92] The polymers of alginate with the largest molecular size safely elicit opsonin antibodies in proportions large enough to vaccinate to cystic fibrosis patients against mucoid *Pseudomonas aeruginosa*, if the progression of the infection is due to the lack of alginate-specific opsonic antibodies.[93]

Serum titers of anti-alginate antibody IgG (Fig. 6), IgM and IgA in a group of Pseudomonas-positive DPB were higher compared with a group of patients who had

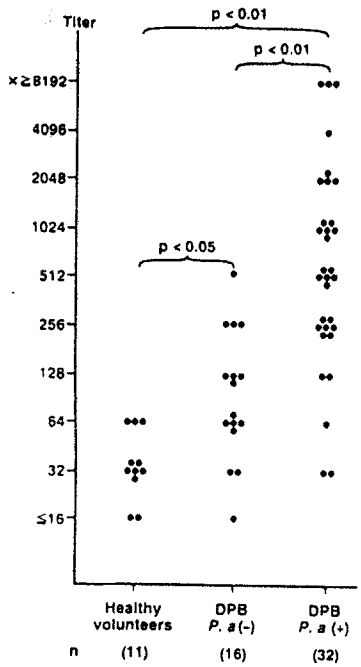

Fig. 6: Serum titer of anti-alginate antibody in DPB patients.

Pseudomonas-negative DPB in whom the majority were patients whose mucoid strains were already eliminated by long-term administration of CAM. On the other hand, the positive cases showing higher titers of anti-alginate antibody IgG, generally had active symptoms of airway infection. This finding suggests that anti-alginate antibody may act harmfully compared with opsonin. Høiby[94] demonstrated in his series of experiments that the most remarkable host response to lung infection in cystic fibrosis was the pronounced antibody response that continued to increase over several years. Such an increase in antibody response was an indicator of poor prognosis, especially if the IgG_3 subclass had

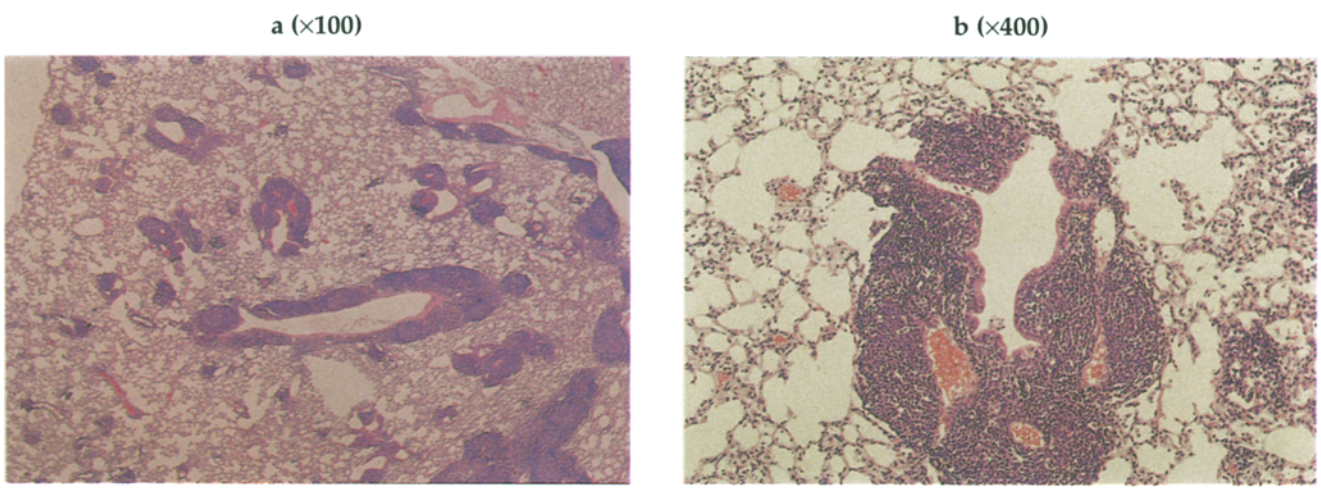

Fig. 7: Morphologic finding on the immunized mouse lung three days after P. aeruginosa PT1252 strain infection by inhalation. The immunized mouse was prepared by four intra-abdominal injections with 40 mg alginate extracted from P. aeruginosa *PT1252 strain. Lymphocyte infiltration appeared surrounding small airways and small vessels. This was noted on the first day after inhalation and continued for 3–4 weeks. It may be thought that the antigen-antibody reaction occurred between alginate and antialginate antibody on the level of the small airways.*

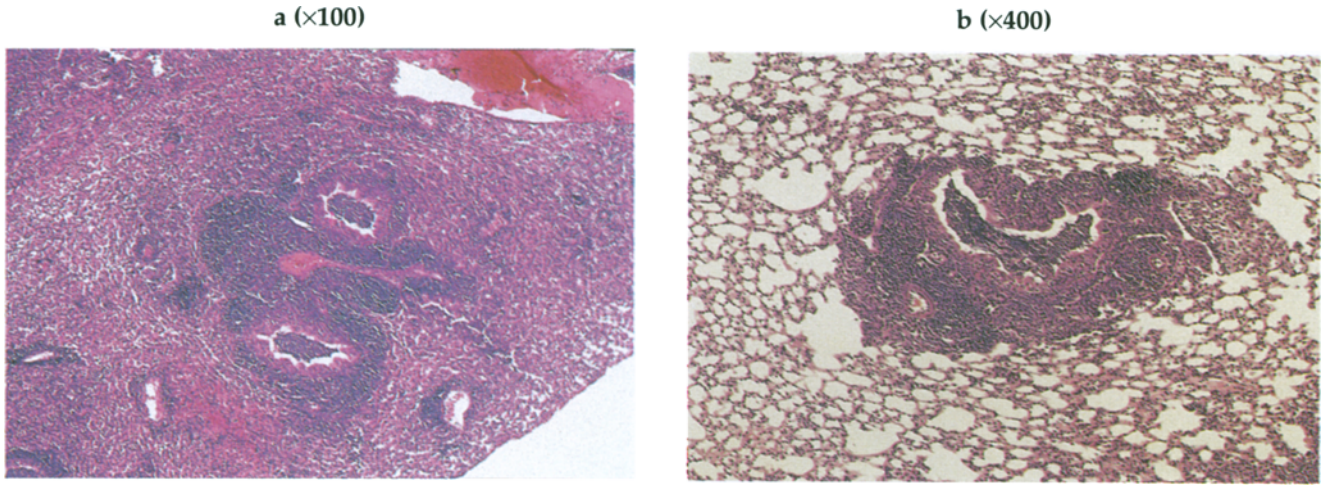

Fig. 8: Morphologic findings in the immunized mouse lung reinfected by P. aeruginosa *PT1252 strain. The lymphocyte cell infiltration stabilized and lymphocyte granuloma including plasma cells was formed. Neutrophil infiltration in airway secretions was also seen in the airway narrowed by the granuloma. The appearance is similar to that of the findings in human diffuse panbronchiolitis.*

the strongest complement activity. However, so far no relationship between serum IgG_3 and symptomatic activity of DPB patients has been seen. It is possible that a positive relationship between IgG_3 and complement may be found in bronchial secretions in DPB patients, though no such finding has been described in the blood. To clarify the pathogenic role of anti-alginate antibody, lungs of previously immunized mice, induced by intraperitoneal infection with free alginate were studied (Fig. 7) after they were infected with mucoid *Pseudomonas aeruginosa* through inhalation. A remarkable lymphocyte infiltration around the peripheral airways appears just after inhalation, but neither pneumonic lesions nor PMN infiltration is observed.[95] The majority of lymphocytes infiltrated around the peripheral airways would be defined homing-receptor positive L_3T_3-CD_{44} cells activated by alginate from our observation in BALF.[39] The antigen antibody reaction through alginate around the airway is explained. The finding is persistently observed over a long period after repeated infection of mucoid *Pseudomonas aeruginosa* with final formation of follicules, including plasma cells. In addition, the airway lumen is pressed and deformed by the infiltration, showing excessive secretion of organized materials accompanying PMNs (Fig. 8). This finding is similar to that of human DPB. At this stage, alginate must act as an antigen in these immunized mice. The antigen-antibody reaction occurs in the area of the peripheral airways, showing a sequential process of lymphocyte infiltration as a result of antigen stimulation due to continued reaction and PMN infiltration into the airway.

It is well known that antigen excess results in the formation of an immune complex (IC). ICs may form with Pseudomonas antigens in cystic fibrosis patients on the high number of corresponding specific precipitation antibodies in serum and sputum from the patient with a lung infection. Lipopolysaccharide (LPS) was initially thought as the primary candidate for IC formation.[96] Lately, LPS was determined to be the major antigen component of IC,[97–99] coexisting with alkaline protease antigen and elastase antigen.[100,101] Woods and Bryan[92] demonstrated that IC formation should be considered as a possible consequence of immunization with alginate in their animal studies on protective immunogen against *Pseudomonas aeruginosa*. They concluded that IC may not be a good candidate as a protective immunogen. IC in cystic fibrosis patients has been found not only in lung tissue, but also in other organs and body fluids, and a good correlation between a high level of IC and

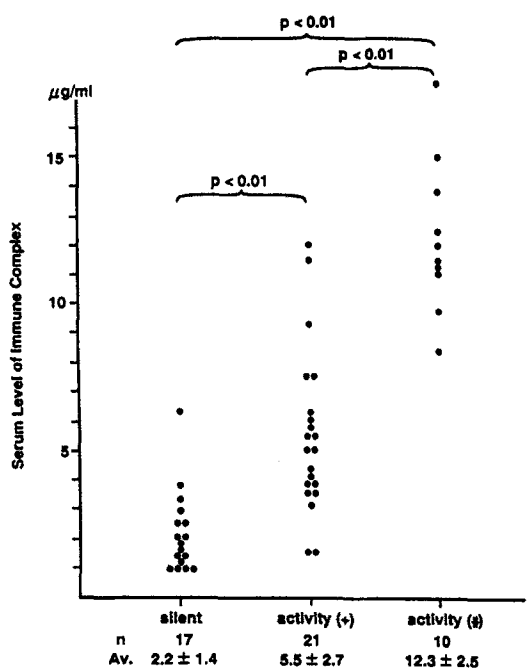

Fig. 9: Relation between serum-immune complex and symptom activity in DPB.

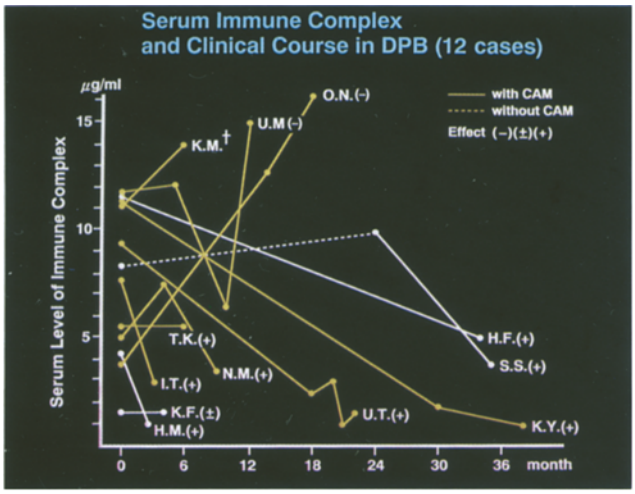

Fig. 10: *Serum levels of IC and clinical prognosis during long-term administration of CAM. (+) patient outcome was favorable. (–) patient outcome was poor. In the cases where treatment was effective, serum levels of IC gradually decreased as treatment progressed, but this did not occur in cases where treatment was not effective.*

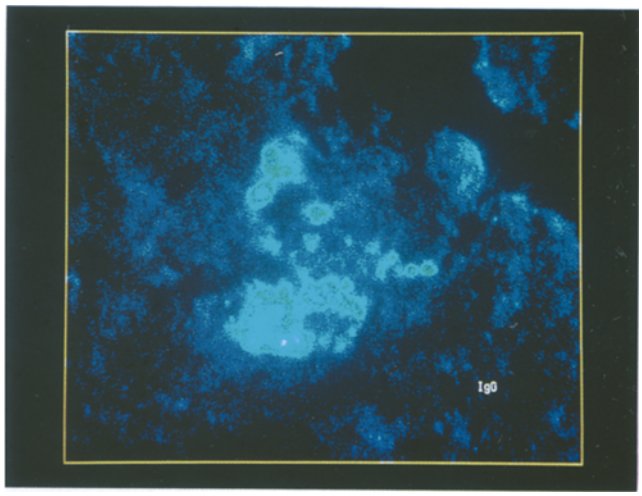

Fig. 11: *Deposition of anti-alginate antibody IgG on lung tissue. Anti-alginate antibody IgG deposited on affected parts of lung tissue. The picture indicates that anti-alginate antibody IgG may have some destructive effect on lung tissue in chronic* P. aeruginosa*-infected mice.*

poor prognosis has been demonstrated.[102] In fact, the serum level of IC in Pseudomonas-positive DPB patients was higher than that of Pseudomonas-negative patients and that of healthy volunteers; its level was well correlated with clinical manifestations with higher values in patients whose symptoms were obvious (Fig. 9). This suggests modified clinical symptoms by these ICs. In practice, clinical prognosis improved in patients with decreased IC after CAM treatment, while IC showed no change or increased in aggravated cases (Fig. 10). The evidence mentioned above indicates that the IC plays an important role in clinical manifestations in DPB patients.

4. Relationship between IC and PMN influx

Repeated inhalation of mucoid *Pseudomonas aeruginosa* by alginate-immunized mice introduces PMN infiltration into the airway lumen (Fig. 8). On the other hand, a high level of circulating IC is observed in patients with active DPB infected with *Pseudomonas aeruginosa*. It is not difficult to connect these two events. IC has been detected in sputum from the patients with both cystic fibrosis[103] and DPB,[95] and may stimulate the chemotactic mediators IL-1β and TNF or the direct chemotaxin IL-8; the persistence of mucoid *Pseudomonas aeruginosa* in the airway establishes a suitable condition for IC formation.[104] IC present in conditions characterized by PMNs enfolding the airway. Høiby et al.[102] described in their excellent review on IC that with the deposition of IC the lung tissue stimulates PMN chemotaxis and function by activated complement resulting from local deposition of IC. The influent PMN bind to the Fc portion of IC. Complement activity is usually enhanced in the sputum of cystic fibrosis patients[105] and DPB patients (unpublished observation). Also, the deposition of antibody in tissue can result from deposition of circulating ICs from the serum or from the development of autoantibodies. Anti-alginate antibody IgG was characteristically deposited on the affected part of lung tissue (Fig. 11), as well as IC deposition.[58,106] Our immunologic observations both in in-

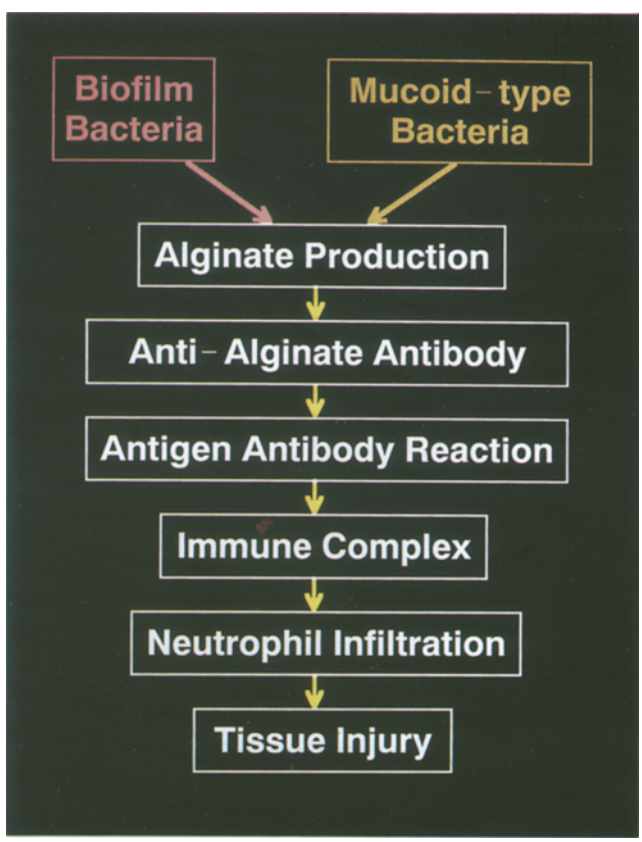

Fig. 12: *Pathogenic role of mucoid alginate on infected DPB.*

fected DPB patients and in the experimental models strongly support the theory advocated by Høiby and coworkers[102] demonstrating that PMN influx into the airway lumen originated from IC deposited on airway tissue. However, another possible mechanism is that PMN influx results from exacerbation of infection caused by floating bacteria released from bacterial biofilm, as described above. Based on the above discussion, two mechanisms could explain the influx of PMNs into the airway, i.e., the immunologic process and the infectious invasion by floating bacteria. However, the former is characteristic rather than the latter, when the pathogenic process of infected DPB is considered.

5. Conclusive remarks

Persistent colonization by mucoid *Pseudomonas aeruginosa*, lymphocyte infiltration around peripheral airways and neutrophil influx in airway secretion represent the hallmark of infected DPB. The relatioship between these factors can be explained by the following process (Fig. 12). Alginate produced by mucoid *Pseudomonas aeruginosa* acts as an antigen to produce anti-alginate antibody in the host. It also exhibits an antigen-antibody reaction locally in the small airways following infection, with the resultant infiltration of lymphocytes. Furthermore, continued or repeated infection promotes these changes, leading to a narrowed or deformed airway due to lymphocyte follicules or granuloma-like infiltrations. Excessive antigen following persistent colonization of alginate-producing mucoid *Pseudomonas aeruginosa*, on the other hand, forms circulating ICs. When the ICs deposit on the airway tissue, the complement-activated neutrophils bind the Fc portion of ICs, resulting in the appearance of PMN influx and tissue destruction due to these PMNs. Based on this mechanism, the clinical manifestation of infected DPB seems to be clarified to some extent. The effect of macrolides on DPB patients will be discussed along these points in the following section. Such manifestations in infected DPB would be closely related to those of cystic fibrosis in lung infection, with respect to symptoms, bacteriological and pathological findings.

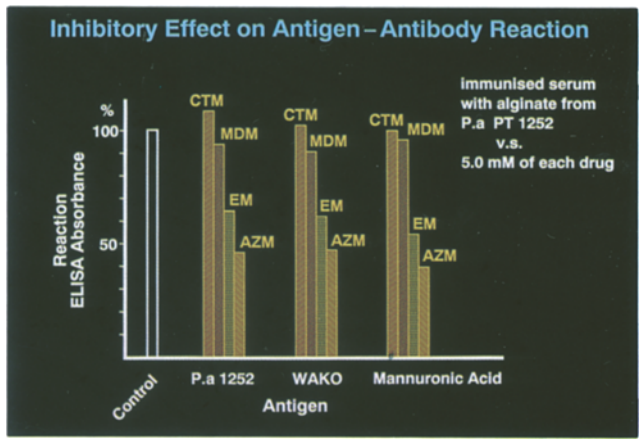

Fig. 13: Inhibitory effect of some drugs on antigen-antibody reaction through alginate in vitro. Inhibitory effect is seen only in 14-membered macrolide, EM and 15-membered macrolide, AZM.

EFFECTS OF MACROLIDES ON INFECTED DPB

It must be emphasized that the clinical efficacy of macrolides on DPB can be observed only with 14- and 15-membered macrolides but not with 16-membered ones. Thus this point should always be kept in mind throughout the discussion.

1. Inhibitory effect on the immune reaction

In our in vitro experiment, alginate extracted from *Pseudomonas aeruginosa* PT 1252 as an antigen reacted with mouse serum immunized with this alginate as an antibody. Although these reactions were not inhibited by cefotiam (CTM) and midecamycin (MDM), a 16-membered macrolide, a significant inhibitory effect on this

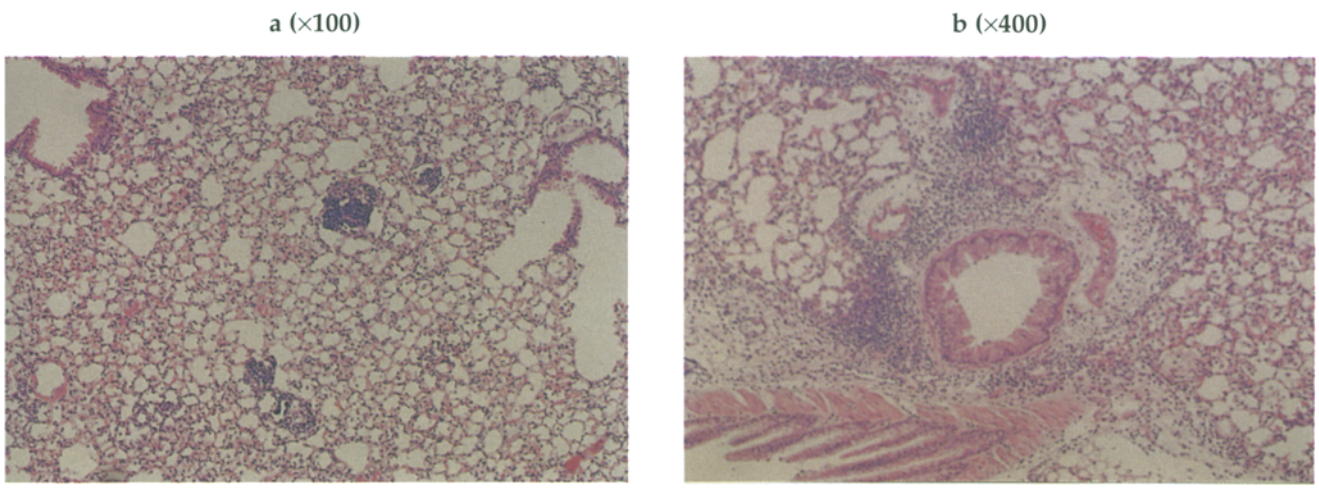

Fig. 14: Morphologic findings of the immunized mouse lung that was pretreated with 10 mg of azithromycin (AZM) for 4 days before P. aeruginosa *PT1252 strain inhalation. The lymphocyte infiltration of peribronchiolar or perivascular areas seemed to be inhibited compared with nontreated animals (Fig. 7).*

immune reaction could be observed with EM and AZM (Fig. 13). In this case, a similar result was obtained when the antigen was replaced by a commercially available alginate (Wako Pure Chemical Industries, Ltd.) or by mannuronic acid. In addition, since this inhibitory effect observed with EM and AZM was attainable only when these drugs and antibody had been incubated previously, the drug competing with the antibody may contribute to this effect. FK 506,[107] a 23-membered macrolide, has already been used as an immune suppressant. Although an immune suppressive action of 14- and 15-membered macrolides has not been investigated fully, it is possible that one or more parts of the macrolide ring may show such suppressive action. The changes in the alginate-immunized mouse lung tissue were also observed after a pretreatment with 10 mg of AZM for 4 days followed by infection via *Pseudomonas aeruginosa* inhalation. The degree and density of lympho-cyte infiltration on peripheral airway or perivascular areas seemed to be less than that of nontreated animals (Fig. 14). The in vivo inhibitory effect of AZM on lymphocyte infiltration was further examined by the BALF method. The analysis revealed that lymphocyte count increased markedly in immunized mice, while a small increase in neutrophil count was observed on the first day after infection in nonimmunized mice. In addition, this increase in lymphocytes was inhibited in the AZM pretreated group and returned to the pretreatment level two days after infection.[95] Likewise, the same phenomena were also observed in both CAM and EM. From these morphologic findings observed in the lung tissue and in BALF, it is feasible that the in vivo immune reaction was inhibited by AZM, a 15-membered macrolide as well as by CAM and EM, 14-membered macrolides.

2. Influence on alginate production

We also examined the influence of macrolides on alginate production, serving as an antigen, and correlating clinically to poor prognosis in cystic fibrosis[108] and DPB.[95] When various drugs were examined using mucoid *Pseudomonas aeruginosa*, 14-membered EM and CAM as well as 15-membered AZM inhibited alginate production in a dose-dependent manner, though no inhibitory effect was observed in the case of 16-membered MDM (Fig. 15). Thus the influence of 16-membered macrolide was revealed to be different from that of 14- and 15-membered macrolides. These changes were also observed for other mucoid strains with similar results. As a clinical problem, alginate increases sputum viscosity, probably leading to difficult discharge of sputum. Thus a long-term culture of mucoid *Pseudomonas aeruginosa* PT 1252 was attempted in an AP medium. Fourteen days after incubation, the viscosity of the medium increased markedly by alginate production (Fig. 16a). The average concentration of the mucoid exopolysaccharide (alginate) in sputum from cystic fi-

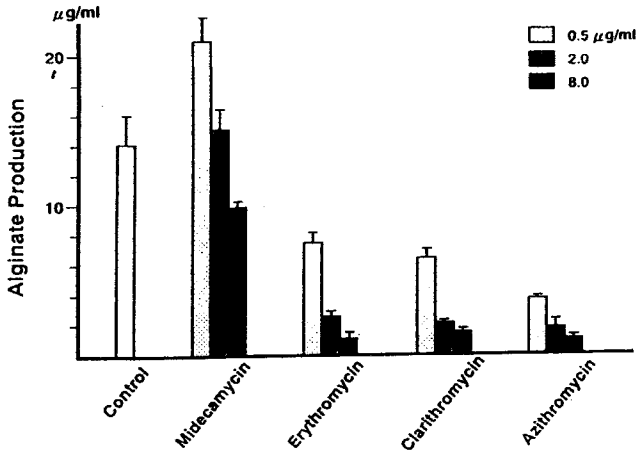

Fig. 15: *Influence of macrolides on alginate production from mucoid* P. aeruginosa *354 (10^9/ml). Inhibitory effect of alginate production was seen in 14-membered macrolides, EM, CAM and 15-membered macrolide, AZM, but not in 16-membered MDM.*

(a) (b)

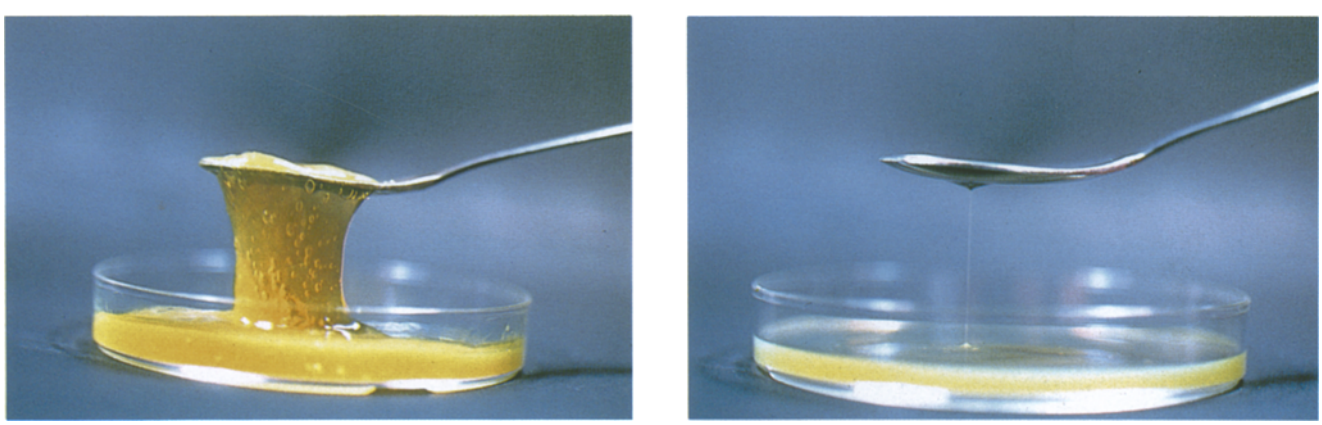

Fig. 16: *A long-term culture of mucoid* P. aeruginosa *PT1252 in AP medium. Fourteen days later, the viscosity of the medium was markedly increased by alginate production (a). By adding 10 μg/ml of azithromicin (AZM), in contrast, no increase in viscosity was observed due to the inhibitory effect of alginate by AZM (b). Good clinical outcomes more easily obtained when airway secretions are thinner and therefore more easily eliminated.*

brosis patients (35.5 μg/ml)[109] was significantly higher than that of chronic bronchitis patients. The highly viscous secretion in the respiratory tract, leading to a thick inspissated mucus is considered to be the reason cystic fibrosis patients suffer from recurrent and chronic infection. This type of secretion has an inhibitory effect on the mucociliary system and promotes bacterial colonization.[15] By adding 10 μg of AZM, the increased viscosity was inhibited through alginate reduction by AZM (Fig. 16b). By enhancing the discharge of airway secretion, one is more likely to obtain a good clinical effect. As for alginate production by *P. aeruginosa*, it is known to be synthesized through a process beginning first with fructose-6-phosphate production in the mycelium, followed by enzyme systems, including PMI, PMM, GMP and

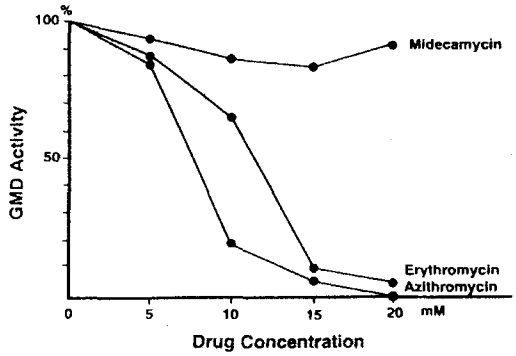

Fig. 17: *Influence of macrolides on GMD activity. Enzymatic activity of guanosine diphospho-mannose dehydrogenase (GMD) was decreased in incubation with EM and AZM, but not in MDM.*

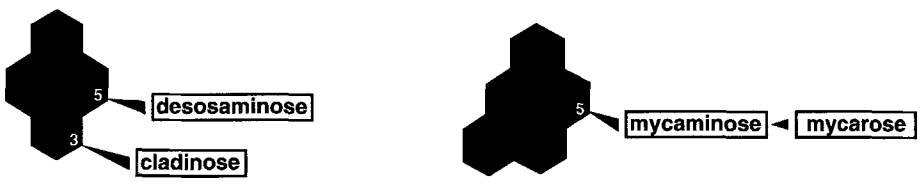

Fig. 18: *Structural difference between 14- or 15-membered macrolide (left) and 16-membered macrolide (right). The difference is in the arrangement of the side sugar.*

GMG[110,111] that were not present in extracts of nonmucoid strains.[112] When these enzyme activities were examined, EM and AZM were found to inhibit guanosine diphosphomannose dehydrogenase (GMD) activity in the final stage of alginate production and to suppress the alginate production. However, 16-membered MDM and RKM (rokitamycin) showed no such inhibitory effect on enzyme activities. Thus, this inhibitory effect could be recognized as a specific action observed only in 14- and 15-membered macrolides (Fig. 17).

3. *Specificity of macrolide structural activity*

When the structural formulae of individual macrolides are observed at this time, 14- and 15-membered macrolides are found to be different from 16-membered macrolides in the arrangement of the side sugar chain moiety. Namely, another mycarose is linked to the end of mycaminose, so that two linked glucose chains are present at the Position 5 side chain as in the case of 16-membered macrolides. In the case of 14- and 15-membered macrolides, the glucose corresponding to mycaminose (desosaminose) is present at position 5 (Fig. 18).

Secondly, an experiment using a compound eliminating mycarose moiety, a terminal glucose chain of 16-membered macrolides, was performed. Namely, CP 4305, obtained by cutting mycarose from MDM, and also M-leuconoside, obtained by cutting mycarose from RKM were tested for their influences on alginate production by *Pseudomonas aeruginosa*. As a consequence, the inhibitory effect on GMD activity could be observed when the terminal glucose was cut with exposed mycaminose even in the case of the 16-membered macrolide (Fig. 19). Furthermore, in the case of 14-membered macrolide CAM with established inhibitory effect, the effect was maintained when the glucose at Position 5 was preserved even after the side chain at Position 3 was cut.

These results indicate that the inhibitory effect on alginate production, observed for 14- and 15-membered macrolides, is not based on the structural specificity of the macrolide ring per se, but depends on the glucose chain connected with Position 5. Furthermore, an adequate inhibitory effect can be obtained if the end glucose chain is removed even in the case of the 16-membered macrolide. In addition, these compounds also

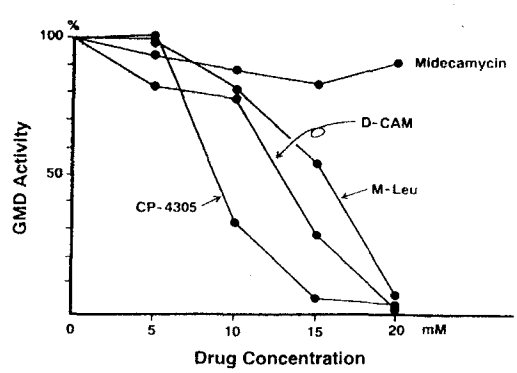

Fig. 19: *Influence of macrolide derivatives on GMD activity. CP4305 obtained by cutting out mycarose from MDM, and M-leuconoside obtained by cutting out mycarose from RKM both expressed an inhibitory effect on GMD enzymatic activity, in spite of 16-membered rings. The effect may be dependent on the glucose chain connected with Position 5 of the macrolide ring.*

exhibit a destructive effect on biofilms (unpublished observation). Thus the efficacy of 14- and 15-membered macrolides on DPB seems to be clarified from the structural activities.

SUMMARY

From the investigation of DPB as it manifests in chronic persistent biofilm disease, it has been found that the most important factors are the excess antigen-antibody reaction (where alginate acts as the antigen) and the resultant formation of immune complex. The factors which specifically "link the disease state of infected DPB to" the effects of 14- and 15-membered macrolides are the inhibition of immune reaction induced by alginate and their inhibitory effect on alginate production, acting as antigens at the GMD level. Furthermore, the specificity of macrolides on these actions was also evident from the standpoint of structural activity.

The present serial processes represent an approach quite dissimilar to those of conventional reports, and the results thus obtained provide an entirely new facet to current knowledge. The pathologic category of DPB was first presented in Japan and its therapeutic approaches by 14- or 15-membered macrolides have likewise been developed in this country. In my opinion, "macrolide therapy", i.e., long-term administration of 14- or 15-membered ring macrolides should be tried in patients with infected cystic fibrosis in Europe and North America due to the apparent therapeutic success of utilizing these macrolides in patients with Pseudomonas biofilm disease in Japan.

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