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Adherence of Mycoplasmas: Phenomena and Possible Role in the Pathogenesis of Disease

Summary: *Mycoplasma pneumoniae* attaches to a variety of surfaces. Adherence to inert surfaces such as glass requires an intact energy metabolism. Interaction with sheep erythrocytes occurs via a binding protein on the mycoplasma surface. The protein reacts with a receptor containing sialic acid. Adherence to other erythrocytes may involve different mechanisms. Different results have been reported on interaction with tissue cells. The various mechanisms probably cooperate and thereby facilitate the colonization of the human respiratory tract.

Zusammenfassung: *Adhärenz von Mycoplasmen: Phänomene und mögliche Rolle in der Pathogenese. Mycoplasma pneumoniae* vermag sich an verschiedenartige Oberflächen anzuheften. Adhärenz an Glas und ähnliche inerte Materialien verlangt einen intakten Energiestoffwechsel. Die Bindung an Schaferythrozyten wird durch ein Oberflächenprotein der Mycoplasma-Membran vermittelt, das mit einem Neuraminsäurehaltigen Rezeptor reagiert. Bei der Anheftung an andere Erythrozyten scheinen noch weitere Mechanismen beteiligt zu sein. Für die Interaktion mit Gewebezellen werden teilweise unterschiedliche Ergebnisse berichtet. Das Zusammenwirken der verschiedenen Mechanismen ermöglicht die erfolgreiche Kolonisierung des menschlichen Respirationstraktes.

Properties of Mycoplasmas

Mycoplasmas are useful as model organisms for several reasons. They not only cause a variety of clinical infections in the human respiratory and genito-urinary tracts (1), but they also have some properties which are interesting in the context of adherence.

Firstly, mycoplasmas are very small. Their average volume is about one tenth that of a staphylococcus. Their minute size enables them to attach very closely to the host cell surface and possibly to hide in crypts and folds of its membrane. The intimate contact is not only protective, but may also facilitate host cell damage by the closely attached microorganisms. A possible reaction of the host cell surface to such an irritation can be expected, although, with the exception of the capping observed on lymphocytes (2), it is not yet known.

Secondly, mycoplasmas have no rigid cell wall (3). Walled bacteria can only change their adherence properties slowly by changing the synthesis of the respective substances. On the other hand, mycoplasmas are the only prokaryotes which, like animal cells, are able to change their surface pattern directly and actively as a result of external stimuli.

Bacteria have apparently developed a wide range of adherence mechanisms, as have the mycoplasmas (Table 1). Some of the species examined so far seem to interact with receptors containing sialic acid, but there are also other species which apparently use different mechanisms.

Mycoplasma pneumoniae, the model organism used in this study, is able to glide along surfaces. When growing on a surface it has a distinct elongated shape with a polar tip structure, a relatively thick body and a long tail-like end (4). The tip was first described by *Biberfeld* and *Biberfeld* (5). It could be involved in motility as well as in adherence, although the latter is not restricted to the tip area.

Methodology

The problem encountered in all *in vitro* adherence studies concerns the different properties of the experimental models and their unknown correlation to the situation *in vivo*. With *M. pneumoniae*, several approaches can be

Table 1: Differences in host receptors for various mycoplasmas.

Mycoplasma species	Erythrocytes or cells	Host cell receptors sensitive to Neuraminidase	Protease
Mycoplasma pneumoniae	Sheep	+	–
	Rabbit	–	–
Mycoplasma gallisepticum	Human	+	–
Mycoplasma dispar	Sheep	+	–
	Rabbit	+	–
Mycoplasma hominis	HeLa	–	+
Mycoplasma salivarium	HeLa	–	+

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tried. Firstly, there is adherence to inert surfaces (6); secondly, there are different kinds of erythrocytes which can be tested in haemadsorption and haemagglutination assays. Cultured cells can be used in a similar way (7, 8). A model on a higher level of structural organization is the organ culture. In the case of *M. pneumoniae,* tracheal rings are often used with success (9). However, tracheal rings cultured in liquid medium provide an experimental environment totally different from the air-filled trachea in which natural infection occurs and in which host defense mechanisms are present. The results therefore have to be interpreted very carefully. The most natural conditions are, of course, provided by infecting an experimental animal, but single factors can hardly be studied in such a complex system.

Attachment of M. pneumoniae to Inert Surfaces

Some recent results are summarized in the following section (6, 7, 8). A characteristic phenomenon of *M. pneumoniae* is its adherence to inert surfaces. In the course of natural infection in the respiratory tract, the agent first comes into contact with a mucus layer and not with cell surfaces. This mucus layer must then be penetrated. In order to study the factors influencing this hypothetical first step of infection, we examined adherence to glass in an environment containing protein. Quantitation was achieved by using mycoplasmas labeled with palmitate. The experiments provided some interesting results. The surface substances of the mycoplasmas involved in this kind of binding seem to be protein in nature. They can be affected by trypsin, and amino acid-blocking substances inhibited adherence to some extent. However, neither sugars nor amino acids had any blocking effect. Antibiotics which influence protein synthesis had only a minor effect. However, the mycoplasmas only attached to the glass surface if their energy metabolism was intact. Sugars which can be metabolized (mannose and glucose) provided a distinct optimum at 0.25 and 0.5 mg/ml, respectively. Inhibitors of energy metabolism or glucose analogues reduced adherence accordingly. The role of energy was further proven by measuring the ATP content under comparable conditions (10). If energized cells were set at 100%, the values with inhibitors such as the ionophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were markedly reduced. With the ATPase inhibitor dicyclohexylcarbodiimide (DCCD), the ATP content accumulated because it could not be utilized. The decreasing effect on adherence, however, was similar with both substances (Table 2).

It can therefore be concluded that this hypothetical first step of attachment is energy-dependent. For which mechanism is this energy required? Perhaps it is necessary to energize the membrane in order to elevate some binding sites above the surface. A lateral movement of the hypothetical binding sites – comparable to the capping of lymphocytes – can also be considered and it may be

Table 2: Effect of m-chlorophenylhydrazone (CCCP) and dicyclohexylcarbodiimide (DCCD) on ATP content and adherence.

Substances tested in BSA buffer	*Mycoplasma pneumoniae* ATP content (%)	Adherence to glass (%)
Glucose 0.5 mg/ml	100	100
Glucose + CCCP 0.01 M	25	6
Glucose + DCCD 0.001 M	347	28

speculated that only patches consisting of a certain number of binding sites are effective. It is also possible that energy is required for the contractile cytoskeleton (11). As mentioned above, *M. pneumoniae* can maintain a certain shape. Observations have been made suggesting that the tip structure has a special role not only in movement, but in the adherence process as well. Perhaps the cell can penetrate the charge barrier more successfully if the tip is more pronounced.

Attachment to Erythrocytes

Penetration of the mucus layer is probably followed by attachment to the host cell surface itself. Models for this step are erythrocyte attachment and adherence to cultured cells.

A quantitative examination of erythrocyte adherence (8) resulted in the following data, confirming in part previous qualitative results (12):

Sheep erythrocytes bind by means of a sialic acid which contains a receptor to mycoplasma binding sites. These sites are protein in nature. The interaction can be reduced by pre-treating red cells with neuraminidase, and can be partially blocked by sialic acid. However, these treatment methods only had a limited effect on interaction with human erythrocytes. Attachment to rabbit erythrocytes was not affected at all (Table 3), suggesting the presence of at least one alternative mechanism.

Banai et al. (13) tried to isolate the binding site by affinity chromatography using glycophorin, a sialic acid which contains glycoprotein, from human red cells. Glycophorin was used as a ligand for the solubilized mycoplasma membranes. Using deoxycholate and subsequently sodium dodecylsulfate (SDS) as detergents, they found a fraction with an increased binding capacity consisting of

Table 3: Effect of neuraminidase pre-treatment of erythrocytes on attachment to *Mycoplasma pneumoniae.*

	% of control
Sheep erythrocytes	10–20
Human erythrocytes	70–80
Rabbit erythrocytes	100

two polypeptides with a molecular weight of approximately 25,000 and 45,000 daltons, respectively. Binding of this fraction to fixed red blood cells was specifically inhibited by glycophorin. At the same time, however, the material was also binding to the hydrophobic protein moiety of glycophorin. Affinity chromatography with sialoglycoproteins is therefore only of limited value for the isolation of binding sites, and other methods will probably be more successful.

Another possible approach is the isolation of adherence-defective mutants. In gel patterns of such mutants, missing bands were detected, mostly in the area of high molecular weight (14). However, not all of the mutants showed the same defect. This also applied to mutants isolated in our own laboatory (unpublished results). When the electrophoretic patterns of the wild type were compared with mutants, defects at 190,000 daltons could be detected in one mutant, possible defects at 90,000 daltons and one missing band at 40,000 daltons in another. The remaining mutants shared the wild type profile. These bands may be important, but their role has not been elucidated as yet.

Table 4: Effect of pre-treatment of *Mycoplasma pneumoniae* and of fibroblasts on attachment.

Pre-treatment of *Mycoplasma pneumoniae*	Effect on attachment
Trypsin (10 μg/ml)	+
EDC (1 mM)[a]	–
TLCK (1 mM)[b]	–
Phenylglyoxal (1 mM)[c]	–
Periodate-borohydrate[d]	–
Pre-treatment of fibroblasts	
Neuraminidases from different sources[e]	–
Anti-fibroblast-antiserum	–
Anti-fibronectin-antiserum	–

[a] 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide blocking carboxyl groups. The substance was present during incubation;
[b] 1-chlor-3-tosylamido-7-amino-2-heptanone, reacting with histidine. Present during incubation;
[c] Reacting with arginine;
[d] $NaIO_4$ 10 mM for 20 min at 0° C, followed by 5 mM Na (BH_4) for 30 min at 0° C, both in tris-maleate buffer pH 7.4;
[e] Neuraminidases from *Vibrio cholerae* (0.1 U/ml), *Clostridium perfringens* type V (2.5 U/ml) and type IV (10 U/ml), 1 h at 37° C in tris-maleate buffer.

Attachment to Tissue Cells

Another type of experimental model employs cultured animal cells. Lung fibroblasts in particular were often used. Sialic acid receptors were also reported to play a role here (15). We found somewhat different results in our own experiments (*Klaus*, thesis in preparation) with fibroblasts from human skin (Table 4). It was not possible to reduce mycoplasma attachment significantly by pre-treating the fibroblast with neuraminidases of various origins. Accordingly, adherence could not be inhibited by N-acetyl-neuraminic acid. Apparently, sialic acid does not necessarily have to be the main functional component. The receptor site on the fibroblasts could be blocked neither by antibodies against whole fibroblasts nor by an antiserum against fibronectin. The latter results suggest that fibronectin does not play an important role. The failure to inhibit attachment by anti-fibroblast-antiserum can be explained in several ways: either (i) the receptor sites are non-immunogenic; (ii) their number is too small to stimulate an immune response; or (iii) the determinants are so widespread in nature that most animals, including the rabbit, are immune-tolerant.

On the other hand, the binding site on the mycoplasma surface was sensitive to trypsin, as expected. The binding site may be a protein, although blocking of the carboxyl groups, of histidine or of arginine by ethyl-dimethyl-aminopropyl-carbodiimide (EDC), chlor-tosylamido-amino-heptanon (TLCK) or phenylglyoxal, had no effect. A further negative result was obtained after periodate treatment with subsequent borohydride reduction; this suggests that sugar groups are not actively involved.

Genetic and immunological methods should also be examined. In contrast to the adherence of *M. pneumoniae* to glass, the attachment to either erythrocytes or to tissue cells is not significantly energy-dependent (unpublished results).

It may be concluded from these results that at least one of the mechanisms by which mycoplasmas attach to cells is mediated by a binding site which is a polypeptide. This protein interacts with host cell receptors which contain sialic acid as a functional component, at least in sheep erythrocytes. There are, however, still many questions to be answered. Why is it that receptors cannot be blocked by antiserum? What are the mechanisms involved in the interaction with erythrocytes of different animal species? Are these interactions really specific? What is the role of these mechanisms *in vivo?*

Biological Role

M. pneumoniae is a microorganism which is totally host-dependent. It has very limited metabolic capacities due to a genom which is four times smaller than that of *Escherichia coli.* It is extremely sensitive to host factors such as antibodies or even complement alone, and yet it is a successful and rather persistent parasite. We may assume that it is very well equipped to rapidly penetrate the flowing mucus layer covering the host cells, to find the host cell surface (probably by chemotactic attraction), and to attach to it by one or perhaps several mechanisms. However, this alone would not be sufficient for colonization and multiplication because the host defence mechanisms would easily destroy such an agent. We must assume that *M. pneumoniae* is somehow able to colonize sites which are less accessible to the host's humoral and cellular factors. Some clinical data suggest that *M. pneumoniae* can persist for a long time, despite adequate

antibiotic therapy. It could be shown in experimental models that some of the mycoplasmas which had attached to macrophages could not be reached by an otherwise killing concentration of complement (16). It may be speculated that the same mechanisms may also protect them to some extent from host defence, as well as from the effects of antibiotics.

In the case of infections with *M. pneumoniae,* it seems that attachment is the first and most important step for the parasite in a sequence of events which finally leads to disease. The clinical symptoms, caused by mechanisms which are as yet unknown, follow much later in this sequence of events after an incubation period of about three weeks.

What possibilites are there to interfere with this first and perhaps crucial step of infection?

Antibiotics have little specific effect. They neither prevent nor terminate attachment at minimal inhibiting concentrations, but they do prevent multiplication altogether. Interference by an excess of either binding sites or receptor substances seems theoretically possible, but is unlikely to have any clinical value. This leaves prophylaxis. Here, local immunization against the binding substance seems possible, although even this approach may be questionable if the theory of disease due to secondary infection (17) is correct.

"Most cells will stick to most surfaces . . ." (18). This sentence does not apply to adherence in host-parasite interactions. Mycoplasmas only stick to surfaces under specific conditions, but so far our knowledge of these conditions is still quite limited. Even if this knowledge has no immediate clinical implications, we may expect to acquire a better understanding of pathogenesis, and this in turn may influence our management of disease.

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