Adsorption of Mycobacteriophage on Cell-wall Components

BY T. IMAEDA AND F. SAN BLAS

Department of Microbiology, Instituto Venezolano de Investigaciones Cientificas
Apartado 1827, Caracas, Venezuela

(Accepted 3 July 1969)

SUMMARY

Heat-killed whole cells, purified cell walls, and the three main polymers isolated from cell walls of Mycobacterium sp. ATCC 607, M. smegmatis ATCC 14468 and M. phlei ATCC 11758, were examined to find the receptor sites for phage GS-7. Among heat-killed whole cells of insusceptible species, M. smegmatis adsorbed the phage at a higher rate than the susceptible Mycobacterium sp. ATCC 607, whereas M. phlei did not adsorb the phage at all, suggesting a distinction between phage-resistance (absence of receptor) and phage-immunity (presence of both receptor and repressor).

Each of the three polymers of the cell wall (the lipopolysaccharide of the outer layer, the lipopolysaccharide-lipoprotein complex of the middle layer and the lipopolysaccharide-mucopeptide or mucopeptide complex of the inner layer) contain receptor substances. The lipopolysaccharide moiety isolated from the lipopolysaccharide-lipoprotein complex adsorbed phage at the same rate as the whole complex; implying that the possible receptor substances were the lipopolysaccharide moieties of the three polymers. Delipidation of both cell wall and the lipopolysaccharide-mucopeptide complex resulted in a loss of phage-adsorbing capacity, suggesting that the lipid portion of the lipopolysaccharides may constitute the phage receptors.

INTRODUCTION

Studies on the cell walls of mycobacteria revealed a three layered structure consisting of an outer lipopolysaccharide, a middle lipopolysaccharide-lipoprotein complex, and an inner lipopolysaccharide-mucopeptide complex (Imaeda, Kanetsuna & Galindo, 1968). These lipopolysaccharides are characterized by glucose, arabinose and galactose in the outer layer, arabinose, galactose, glucose and mannose in the middle, and arabinose and galactose in the inner (Imaeda et al. 1968; Kanetsuna, 1968). We have now studied the adsorption of mycobacteriophages to these cell wall components to identify the specific receptor sites. The relationship between phage-adsorption and phage-insusceptibility has also been investigated.

METHODS

Phage. Phage GS-7 lysates (supplied by W. B. Redmond, Veterans Administration Hospital, Atlanta, Ga. U.S.A.) were prepared with Mycobacterium sp. ATCC 607, filtered through Millipore filter HA 0.45 μm. and stored at 5°C.

Preparation of test materials. Susceptible species, Mycobacterium sp. ATCC 607, and insusceptible species, M. phlei ATCC 11758 and M. smegmatis ATCC 14468, in
exponential growth in Penassay broth (Difco Laboratories, Detroit, Mich.) containing 0.04% Tween 80 were harvested for preparation of the test materials. Heat-killed cells were prepared by autoclaving a suspension in 0.7% saline. Cell walls and cell-wall fractions were obtained from living cells as described previously (Imaeda et al. 1968). Defatted cell walls were prepared by saponification with 2.5% (w/v) KOH in methanol benzene (1:1) for 6 hr with reflux.

The cell-wall fractions were further purified as follows. Crude preparations of lipopolysaccharide, obtained from an aqueous phase after 45% (w/v) phenol treatment of cell walls, treated several times with 45% (w/v) phenol at 68°C were being precipitated each time from the aqueous phase by dialysis against acetone. This removed free lipids, low molecular weight carbohydrate-lipid compounds, and lipoprotein. The partially purified lipopolysaccharide fraction thus obtained was further treated with 10% (v/v) trichloracetic acid at 10°C in order to sediment protein moieties derived as contaminants from the middle layer. The lipopolysaccharide fraction, obtained from the trichloracetic acid supernatant portion by adding 6 volumes of acetone, was washed with ether, resuspended in distilled water and finally lyophilized. The lipopolysaccharide fraction contained less than 1% amino acids, indicating minimal contamination with the middle layer protein. The absence of glucosamine, muramic acid and diaminopimelic acid indicated no cross-contamination with the inner lipopolysaccharide-mucopeptide polymer.

The lipopolysaccharide-lipoprotein fraction obtained from the phenol phase after treatment of cell walls with 45% (w/v) phenol was purified by repeated extractions with 45% (w/v) phenol at 68°C. Each phenol phase was dialysed against acetone and the precipitated material was further treated with phenol. The partially purified lipopolysaccharide-lipoprotein fraction was suspended in 10% (v/v) trichloracetic acid at 10°C in order to remove the lipopolysaccharide contaminants soluble in trichloracetic acid, as described above. The sedimented fraction was washed several times with distilled water followed by acetone and ether extractions. The lipopolysaccharide-lipoprotein fraction thus obtained was suspended in distilled water and lyophilized. Chemical analyses showed the absence of muramic acid and diaminopimelic acid, indicating no cross-contamination with the inner lipopolysaccharide-mucopeptide polymer.

The inner lipopolysaccharide-mucopeptide complex, obtained as an insoluble fraction after phenol treatment was purified by repeated extractions with 45% phenol at 68°C, followed by washing with acetone and ether, and finally digestion with pronase. The purified lipopolysaccharide-mucopeptide fraction did not contain any amino acids other than diaminopimelic acid, alanine and glutamic acid, indicating no cross-contamination with the middle lipopolysaccharide-lipoprotein fraction. The absence of glucose, contained in both the outer lipopolysaccharide and the middle lipopolysaccharide-lipoprotein complex, also indicated the purity of this lipopolysaccharide-mucopeptide fraction.

The lipopolysaccharide portion of the lipopolysaccharide-lipoprotein complex was prepared by pronase treatment (1 mg/ml in 0.05 M-tris HCl buffer, pH 7.2, at 37°C for 5 hr), followed by 45% phenol at 68°C. After phenol extractions, the lipopolysaccharide moiety was recovered from an aqueous phase by dialysis against acetone. The lipopolysaccharide moiety was purified by the same method described for the purification of the lipopolysaccharide.
Adsorption of mycobacteriophage

Owing to the difficulty of separating the lipopolysaccharide portion from its mucopeptide complex (Kanetsuna, 1968), the polysaccharide (arabinogalactan)-mucopeptide complex was used as a subfraction of the lipopolysaccharide-mucopeptide polymer. For this purpose, the lipopolysaccharide-mucopeptide was saponified in the same way as described in the preparation of the defatted cell walls.

Adsorption test. Adsorption experiments were performed by a modification of Fischetti & Zabriskie's (1968) method. Four and one-half ml. of heat-killed cells (3 x 10⁶ cell/ml.), cell walls (1 mg./ml.), or cell-wall fractions (100 μg./ml.) suspended in nutrient broth containing 0·5 mM CaCl₂ were mixed with 0·5 ml. of a phage lysate at a final concentration of 10⁸ p.f.u./ml., incubated at 37° for 30 min, and centrifuged for 15 min. at 2500 g to sediment adsorbed phage. The supernatant fractions were assayed for unadsorbed phage using Mycobacterium sp. ATCC 607 as an indicator in a soft agar layer. The phage counts were made in quintuple sets of Petri dishes, calculated to contain 300 to 400 plaques per dish where there was no adsorption. Each experiment was repeated 5 times.

Electron microscopy. The phage-adsorbed materials obtained by centrifugation as described above were negatively stained with a 2 % sodium silicotungstate solution at pH 7·5 and examined with a Jeol JEM 7 A electron microscope at 80 kv.

RESULTS

Qualitative analyses of the three polymers contained in the cell wall showed different sugar components in each polymer: glucose, arabinose and a slight amount of galactose in the outer lipopolysaccharide; glucose, galactose, mannose and arabinose in the middle lipopolysaccharide-lipoprotein complex; and arabinose and galactose in the inner lipopolysaccharide-mucopeptide complex. The lipid portions of each polymer consist mainly of mycolic acid. Details of the chemical analyses of these polymers are described in previous papers (Imaeda et al. 1968; Kanetsuna, 1968).

Table 1. Decrease of p.f.u. after adsorption on heat-killed cells and cell-wall components

<table>
<thead>
<tr>
<th>Fractions</th>
<th>p.f.u. decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-killed whole cells</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium sp. ATCC 607</td>
<td>48</td>
</tr>
<tr>
<td>M. smegmatis ATCC 14468</td>
<td>90</td>
</tr>
<tr>
<td>M. phlei ATCC 11758</td>
<td>0</td>
</tr>
<tr>
<td>Cell walls (607)</td>
<td>60</td>
</tr>
<tr>
<td>Cell walls (defatted) (607)</td>
<td>0</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>33</td>
</tr>
<tr>
<td>Lipopolysaccharide-lipoprotein</td>
<td>27</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>21</td>
</tr>
<tr>
<td>Lipopolysaccharide-mucopeptide</td>
<td>12</td>
</tr>
<tr>
<td>Polysaccharide-mucopeptide</td>
<td>0</td>
</tr>
</tbody>
</table>

Free p.f.u. decreased as a result of adsorption to various fractions (Table 1). Heat-killed Mycobacterium smegmatis ATCC 14468, which is insusceptible to phage GS-7 showed a higher adsorption rate than the susceptible species Mycobacterium sp. ATCC 607. However, the other insusceptible species, M. phlei, did not adsorb the phage at all.
The cell-wall fraction of the susceptible species adsorbed phage (Pl. 1). After delipidation, however, it ceased to do so.

All three of the main polymers of the cell walls of susceptible hosts adsorbed the phage. Electron microscopy showed phage tails attached to each fraction with a different ultrastructural pattern (Pl. 2a, b, c, d). Two morphologically and chemically different subfractions of the outer lipopolysaccharide, that is, fibrils and amorphous spheres, adsorbed phages equally well (Pl. 2a, b).

Repeated experiments consistently showed the greatest adsorption to the lipopolysaccharide and the least to the lipopolysaccharide-mucopeptide complex. The lipopolysaccharide portion separated from the lipopolysaccharide-lipoprotein complex after pronase treatment showed an adsorption rate similar to that of the whole complex. On the other hand, the delipidated-lipopolysaccharide-mucopeptide complex, i.e., polysaccharide-mucopeptide complex, was negative like the lipid-free cell-wall fraction.

**DISCUSSION**

In studies of the phage-host relationship in Gram-positive and Gram-negative bacteria, cell walls have been found to play a role in the adsorption of phage tails as the first step in infection (Oram & Reiter, 1968; Weibull, 1953). Among various cell-wall components, lipopolysaccharide, lipoprotein or their complexes are believed to be responsible for phage attachment (Fischetti & Zabriskie, 1968; Goebel & Jesaitis, 1952; Jesaitis & Goebel, 1952, 1955; Michael, 1968; Rapin, Kalckar & Alberico, 1968; Vidaver & Brock, 1966; Weidel, Koch & Lohss, 1954; Weidel, Koch & Bobosch, 1954). In the studies of mycobacteriophage mycobacterium interactions, cations and surfactants influence phage adsorption (Sellers, Baxter & Runnals, 1962; White & Knight, 1958). This observation, especially the inhibitory effect of surfactants, suggests that the receptor sites for mycobacteriophages may be lipid-containing substances of cell walls.

The present study shows that all three of the polymers, the lipopolysaccharide, the lipopolysaccharide-lipoprotein and the lipopolysaccharide-mucopeptide complexes, isolated from the cell walls of susceptible mycobacteria adsorb the phage particles. A question arises as to whether or not these adsorptions result from cross-contamination with a single receptor substance. However, the different chemical composition of each polymer excludes this possibility. Moreover, each of these morphologically and chemically different polymers shows adsorbed phage in the electron microscope.

All of these polymers contain lipopolysaccharides, although their sugar components are different from each other. It appears, therefore, that these lipopolysaccharides represent the phage adsorption sites. In fact, the lipopolysaccharide moiety isolated from the lipopolysaccharide-lipoprotein complex shows an adsorption rate equivalent to that of whole complex. Furthermore, degradation of the lipopolysaccharide portion of the lipopolysaccharide-mucopeptide complex by saponification results in complete removal of the receptor site. Although the lipopolysaccharide moiety of the lipopolysaccharide-mucopeptide complex has not been examined, because of the difficulty in separating it from the complex (Kanetsuna, 1968), the evidence described above leads us to conclude that the lipopolysaccharide moieties of all polymers, regardless of their localization within the cell wall, play an equal role in adsorbing the phages. Furthermore, the cell wall loses the receptor site after delipidation, suggesting that lipid, most
Phage tails attached to the surface of the cell wall of *Mycobacterium* sp. ATCC 607. CW, cell wall.

T. IMAEDA AND F. SAN BLAS

(Facing p. 496)
Arrows indicate sites of tail attachment to the various polymers.

A phage attached to a sphere of lipopolysaccharide (LPS). Tail fibres are not clearly observed.

A phage tail attaches on to a fibrillar lipopolysaccharide (LPS).

A phage tail attaches on to the lipopolysaccharide-lipoprotein complex (LPSm–LP).

A phage tail attaches on to the lipopolysaccharide-mucoprotein complex (LPSag-MP).

T. IMAEDA AND F. SAN BLAS
Adsorption of mycobacteriophage

of which is mycolic acid, may be the indispensable element in lipopolysaccharides for phage adsorption.

The different degrees of adsorption among the cell-wall polymers may be explained in terms of specific anatomical localization of the effective lipopolysaccharides in each polymer. Namely, the lipopolysaccharide moiety overlays the lipoprotein matrix, whereas the lipopolysaccharide moiety is mainly embedded in the mucopeptide matrix (Imaeda et al. 1968). These differences in free surfaces of the possible receptor sites of lipopolysaccharides among three polymers are concordant with the phage adsorption rates.

It is of particular interest in the present study that two species of insusceptible hosts show opposite results, i.e. Mycobacterium phlei does not adsorb phage GS-7, while M. smegmatis adsorbs it at a high rate. As observed in the study of the GS-7 phage-M. phlei system (Ward & Redmond, 1962), a high multiplicity of infection of phage GS-7 induces the lysis of M. smegmatis. On the other hand, M. phlei is resistant to infection with this phage even at high m.o.i. (T. Imaeda, & M. Rieber, to be published). Although both species are naturally lysogenized by different phages (Imaeda & Rieber, 1969), the results presented suggest that the insusceptibilities of these two hosts to phage GS-7 may be due to two different mechanisms: the absence of the receptor site in M. phlei (resistant to phage), and the presence of both receptor and repressor substances in M. smegmatis (immune to phage).

The cell-wall components of mycobacteria are qualitatively the same in various species (Imaeda et al. 1968) but mycolic acid is species specific (Kanetsuna, 1968). The lipid, especially mycolic acid, is evidently the phage receptor when it combines with polysaccharides. Thus, it is probable that the lipopolysaccharides of M. phlei do not function as receptors, because their specific mycolic acid is different from that of M. smegmatis and of Mycobacterium sp. ATCC 607.

This investigation was supported by the U.S. Public Health Service Research Grant AI-07888 from the National Institute of Allergy and Infectious Diseases.

REFERENCES


(Received 9 May 1969)