Release of *Mycoplasma pneumoniae* Substances After Phagocytosis by Guinea Pig Alveolar Macrophages

M. KIST,* E. JACOBS, AND W. BREDT

Institut für Allgemeine Hygiene und Bakteriologie, Zentrum für Hygiene, Universität Freiburg, D-7800 Freiburg, West Germany

Received 27 July 1981/Accepted 4 December 1981

Antibody-opsonized *Mycoplasma pneumoniae* cells with various radioactive markers were sedimented onto monolayers of guinea pig alveolar macrophages (AM). After 2 h of incubation, about 50% of the activity of [³H]palmitate-labeled mycoplasmas was associated with AM. Nonspecific attachment of the opsonized mycoplasmas to AM-free plastic surface areas was negligible. The occurrence of phagocytosis was proven by electron microscopy and monitoring of AM surface-bound antigen by ¹²⁵I-labeled F(ab)₂ fragments. The activity of [³H]palmitic-acid-labeled mycoplasmas was only slowly released into the supernatant. About 55% of the activity remained AM-associated up to 70 h after phagocytosis. After phagocytosis of [³H]thymidine-labeled cells, about 70% of the radioactivity found in the supernatant was released within 8 h. More than 80% of this activity was non-precipitable by trichloracetic acid. [³H]-amino acid-labeled proteins was released to 50% within 8 h. Supernatants and AM were tested for *M. pneumoniae* antigen with enzyme-linked immunosorbent assay. Considerable amounts of antigenically active material could be found in the supernatant within 8 h. This antigen was totally inactivated by heat (80°C). Trypsin treatment (1 mg/ml, 10 min) reduced the antigenicity by 80%. The results suggest a selective release of microbial material after phagocytosis.

Alveolar macrophages (AM) are the first line of cellular defense against infection from *Mycoplasma pneumoniae*. The interaction of these phagocytes (6, 8, 12) or of macrophages of other origins (1, 5, 7, 9, 10, 14) with mycoplasmas has been studied by several authors, but effective phagocytosis has only been observed after opsonization (5, 6, 8, 9, 10, 12, 14). However, little is known about the fate of the ingested microbial substances. The release of [³H]thymidine was used as marker of cellular destruction (9), but no sufficient data are available on other mycoplasmal components, such as membrane glycolipids or proteins. Even less is known about the biological activity of such native or modified molecules released by the macrophage. These molecules may still be able to activate complement (2, 3), react with antibody, or attach to host cell membranes. This study describes the characteristics of a phagocytosis system using guinea pig AM and *M. pneumoniae*.

**MATERIALS AND METHODS**

*Mycoplasmas*. *M. pneumoniae* strain FH was grown in Roux bottles in 100-ml Hayflicks medium containing 1,000 U/ml of penicillin, 0.05% thallium acetate, and 1% glucose. Radioactive substances were added according to the experimental schedule. A frozen stock inoculum (−70°C) containing about 10⁶ colony-forming units per ml was used for each culture. After 48 h at 37°C, the glass adherent mycoplasmas were washed four times with 5 ml of cold, sterile phosphate-buffered saline (PBS), pH 7.2, and harvested into 3 ml of tissue culture medium (TCM) as described below. The suspension was diluted to an optical density of 0.15 at 660 nm before being used.

**Radioactive labeling of mycoplasma cells**. For experiments with labeled *M. pneumoniae* cells, 50 µCi of [³H]palmitic acid or 150 µCi of [⁶-³H]thymidine was added to 100 ml of the growth medium before incubation. The mycoplasmas were harvested as previously described and tested for specific activity. For protein labeling, the glass adherent mycoplasmas of one Roux bottle were harvested into 1 ml of amino acid-free TCM containing 2% horse serum and substituted with 0.1 µCi of a [³H]-amino acid mixture or 1 µCi with [¹⁴C]methionine, respectively. After 4 h of incubation at 37°C, the mycoplasma cells were centrifuged and washed three times with PBS. Radioactive amino acids not bound to the proteins of mycoplasma cells were separated by passage of the suspension through a Sephadex G-25 column (9.1-ml bed volume).

**Preparation and labeling of anti- *M. pneumoniae* F(ab)₂ fragments with ¹²⁵I**. Rabbit anti-*M. pneumoniae* immunoglobulin G (IgG; 50 mg) purified by gel filtration was digested with 3 mg of pepsin in 0.1 M sodium acetate buffer, pH 4.25. The digestion products were gel filtrated (Sephacryl S 300), and the antibody activity of the F(ab)₂ fraction was determined in an indirect immunofluorescent assay on glass-grown *M. pneumoniae* cells. The absence of Fc fragments was proven.
by the negative complement fixation test with *M. pneumoniae* as antigen. F(ab)₂ fragments (0.5 mg) were iodinated with 0.5 μCi of carrier-free ¹²⁵I (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) by using the chloramin T method (11). Unbound ¹²⁵I was removed by gel filtration on Bio-Gel P10 (1 by 30 cm). The specific antibody activity of iodinated F(ab)₂ fragments was again tested by immunofluorescence on *M. pneumoniae* and the absence of binding onto AM monolayers.

**AM.** The lungs of anaesthetized guinea pigs were washed four times with 20 ml of cold TCM containing antibiotics. The cells were centrifuged in siliconized vials for 10 min at 300 × g, resuspended in 10 ml of TCM, and adjusted to 10⁷ cells per ml. Samples of this suspension (2 ml) were put into plastic petri dishes (Nuncolon, Nunc, Denmark; 35 by 8 mm) and allowed to ice and then treated with a CO₂ atmosphere. After 2 h the cultures were vigorously shaken four times with PBS to remove nonadherent cells (about 40%). These monolayers were incubated for 15 to 20 h at 37°C in a CO₂ atmosphere before use and considered to be AM.

The viability of the cultures was monitored by measuring the lactate dehydrogenase concentration of the supernatant and by using the trypsin blue exclusion test.

**Chemicals and media.** Medium 199 (Flow Laboratories, Inc., Rockville, Md.) with glutamine and sodium bicarbonate, and 2 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was used as TCM. The ³H-labeled substances were obtained from Amershams-Buchler (Braunschweig, Germany). As coating buffer for enzyme-linked immunosorbent assay, a sodium carbonate-bicarbonate buffer (pH 9.6) was used. As a substrate buffer, a diethanolamine buffer (pH 9.8) was used (97 ml of diethanolamine, 100 mg of MgCl₂·6H₂O, and 800 ml of distilled water). Cytochalasin B, trypsin, and trypsin inhibitor were purchased from Serva (Heidelberg, West Germany).

**Phagocytosis.** The *M. pneumoniae* suspension was opsonized by rabbit anti-*M. pneumoniae* antiserum in a final concentration of 1:200 (15 min at 37°C) with intermittent mixing. Amounts of 900 μl each of these suspensions were centrifuged onto AM monolayers (300 × g for 10 min at room temperature). After 2 h of incubation at 37°C, the cultures were washed four times with 2 ml of sterile PBS and refilled with 2 ml of TCM. This was termed zero hour.

**Electron microscopy.** After 2 h of phagocytosis, AM were washed three times with PBS (2 ml), fixed in situ with 3% glutaraldehyde in PBS (pH 7.2) for 30 min on ice, and then treated with a 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.2) for 2 h. During dehydration with increasing concentrations of ethanol, the cultures were pretreated with 0.5% uranyl acetate and 1% phosphotungstic acid at the 70% ethanol step. After centrifugation (800 × g for 10 min), the pellet was embedded in Epon. Sections were cut on the LKB microtome, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiscop 102.

**RESULTS**

**Characteristics of the phagocytosis system.** About 50 to 75% of the harvested alveolar cells became adherent after 2 h of cultivation, and 90% of them excluded trypsin blue. During the phagocytosis experiments, the viability decreased, usually within 48 h, to about 70% of the adherent cells. Additionally, the number of glass-attached AM was reduced to about 80% after 20 h and about 70% after 48 h. The effectiveness of the phagocytosis system was tested with [³H]palmitic acid-labeled mycoplasmas. After centrifugation, incubation (2 h), and washing, about 50% of the activity was found to be associated with the AM monolayer (Fig. 1A). An additional complement treatment (10% guinea pig serum) in an experiment with [³H]thymidine-labeled mycoplasmas removed only a minor part of this AM-associated activity, suggesting that most of the mycoplasmas were internalized or tightly bound to the macrophage surface.

**Electron microscopy.** Numerous mycoplasmas were found in phagocytic vacuoles (Fig. 2). In
of radioactivity applied M. phages. (B) ly bound of phagocytosis (not surrounding cumulatively. plotted substances.

AM face-bound beginning of only were included in antigen. macrophage surface. The mycoplasma material has remained to the surface. The mycoplasma antigenicity of the total activity was reduced by about 20% to 40%.

Release of [3H]palmitic acid-labeled substances. In contrast to the thymidine experiments, the activity of the [3H]palmitic acid-labeled mycoplasmas was only slowly released into the supernatant after phagocytosis (Fig. 1B). About 55% of the radioactive material remained associated with the adherent AM for as long as 70 h. After centrifugation of the supernatants at 12,000 × g (5 min), 14 to 28% of the activity was found in the pellet, probably representing detached AM.

Release of [3H]amino acid-labeled substances. The release pattern of amino acid-labeled mycoplasmas was comparable to that of thymidine. Within the first 8 h, about 50% of the activity was found in the supernatant (Fig. 4). After 20 h, only 20 to 28% of the total activity was still bound to the macrophages.

Antigen detection. Supernatants and residual AM monolayers were tested for M. pneumoniae antigen. The occurrence of antigenically active material in the supernatant is shown in Fig. 5. Apparently most (about 80%) of the detectable material was released within 8 h.

Characteristics of released and AM-associated antigen. The antigenic activity found in the supernatant (4 h) was destroyed by being heated at 90°C for 10 min. Additional experiments showed that the inactivation began at 54°C and was completed at 80°C. When the activity of the AM-associated antigen (4 h) was tested under comparable conditions, antigenicity was reduced to 10% at 80°C, and no further reduction was seen at 100°C. This corresponds with the results obtained on sonicated suspensions of whole M. pneumoniae cells. The antigenicity of the supernatant was reduced to 20% by treatment with 1 mg of trypsin per ml (10 min at 37°C). Furthermore, ammonium sulfate in a concentration of

![FIG. 1. Phagocytosis of [3H]palmitic acid-labeled M. pneumoniae by guinea pig AM. The results of two experiments (I and II) are shown. (A) Characteristics of the phagocytosis system. Symbols: a, total amount of radioactivity applied per petri dish; b, AM-associated activity after 2 h of phagocytosis; c, non-specifically bound activity in control dishes without macrophages. (B) Release of [3H]palmitic acid-labeled substances. Sampling of supernatant started after 2 h of phagocytosis (not included in time) and removal of mycoplasma suspension. The supernatant activity was plotted cumulatively.]

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FIG. 2. Electron micrograph of AM after 2 h of phagocytosis of opsonized *M. pneumoniae* (Mp). Abbreviations: CM, cytoplasmic membrane; N, nucleus. Bar = 1 μm.

80% (wt/vol) precipitated 86% of the antigenic activity.

**DISCUSSION**

Quantitative studies of phagocytosis systems are often hampered because some of the microorganisms are not ingested, thereby influencing the results of viable counts and other parameters. This problem is even more complicated with mycoplasmas, especially *M. pneumoniae*. Their ingestion cannot be monitored sufficiently by light microscopy, and furthermore, they stick effectively to those areas of the plastic surface which are not covered by the macrophages. Four systems are conceivable for maximal phagocytosis of *M. pneumoniae*: (i) phagocytosis with both reaction partners in suspension (8); (ii) macrophages sedimented onto sheets of glass-grown mycoplasmas (1); (iii) monolayers of macrophages infected with mycoplasmas which are subsequently opsonized (5, 6, 8, 9, 10, 12); and (iv) monolayers of macrophages onto which opsonized mycoplasmas are sedimented. Preliminary experiments suggested the use of the last method, mainly because the portion of mycoplasmas attaching "unspecifically" could be kept to a minimum. At the same time the macrophages could be precultivated for longer times, reducing the number of nonphagocytic cells to a minimum. On the other hand, opsonized mycoplasmas often form small aggregates and are therefore more difficult to quantitate.

**TABLE 1. Effect of cytochalasin B on internalization of *M. pneumoniae* by AM**

<table>
<thead>
<tr>
<th>Time (h) after end of phagocytosis period</th>
<th>No inhibitor</th>
<th>Cytochalasin B (10 μg/ml)</th>
<th>Controla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM-associated mycoplasmas (³H cpm)</td>
<td>Mycoplasma antigen on AM surface (¹²⁵I cpm)</td>
<td>AM-associated mycoplasmas (³H cpm)</td>
</tr>
<tr>
<td>0</td>
<td>14,335 ± 325b</td>
<td>1,578 ± 96</td>
<td>8,784 ± 278</td>
</tr>
<tr>
<td>4</td>
<td>11,179 ± 537</td>
<td>1,210 ± 95</td>
<td>6,762 ± 141</td>
</tr>
<tr>
<td>22</td>
<td>10,048 ± 652</td>
<td>230 ± 24</td>
<td>7,313 ± 154</td>
</tr>
</tbody>
</table>

a AM without mycoplasmas treated with F (ab)₂ fragments.
b Mean value cpm ± SD.
FIG. 3. Release of $[^{3}H]$thymidine-labeled substances from M. pneumoniae after phagocytosis by guinea pig AM. The phagocytosis took 2 h, and the time of release began after removal of the mycoplasma suspension. The results of two experiments (I and II) are shown.

 acids during the first hours of phagocytosis suggest a relatively fast release, mainly of cytoplasmic material. A different pattern was observed with the palmite label. Apparently, the mycoplasma lipids are retained within the macrophage for a much longer time. They may be stored there in the form of intact mycoplasma substance, as suggested by the antigen content of the macrophages, or the labeled fatty acid could possibly be used by the phagocyte for its own lipid synthesis.

The substances released into the supernatant in the hours after phagocytosis are at least partially mycoplasma antigens with properties which indicate their protein nature. They are apparently not degraded by the action of lysosomal enzymes and are therefore immediately available for further biological interactions with the humoral and cellular factors of the host. The fate and further role of the substances retained in the macrophage for the time of observation is unknown. The few data available suggest that a considerable part of these materials are of non-protein nature, consisting possibly of membrane lipids, but they may also contain some membrane proteins, which at least partly may be retained within the phagocytic cell.

The fast or delayed release of products of the phagocytic process could have some biological importance for the interaction between parasite and host in the early stages and in the sequelae of infectious diseases. Besides being spread

FIG. 4. Release of $^{3}$H-aminio acid mixture and $[^{14}C]$methionine-labeled substances after 2 h of phagocytosis. Time of release began after removal of mycoplasma suspension. (A) Characteristics of macrophages. Symbols: a, AM-associated activity after 2 h of phagocytosis; b, AM-associated activity after 20 h. (B) Characteristics of the supernatant showing release of $^{3}$H-aminio acids (I) and $[^{14}C]$methionine-(II) labeled substances. Cumulative plotting of the supernatant activity was done.
FIG. 5. Release of M. pneumoniae antigen from AM after 2 h of phagocytosis. Cumulative plotting was done, and the amount of antigen was measured by enzyme-linked immunosorbent assay. Symbols: ●, supernatant; ○, supernatant heated at 90°C for 10 min; Δ, control (release from dishes treated with M. pneumoniae suspension, but without AM). Mean values were calculated from triplicate tests. The standard deviation was 7 to 10%.

throughout the tissues of the host, these substances may act as immunogenic stimuli, reacting with effector systems such as complement (2, 3), and they could be toxic for the host cell membrane, alter the surface of the host cell, or interfere with immunological reactions. They therefore certainly deserve further investigation.

LITERATURE CITED