Studies on the Pathogenicity of *Listeria monocytogenes*

II. Influence of substances isolated from cells of *Listeria monocytogenes* on experimental listeriosis in white mice

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With 5 figures

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In a previous report (1) a substance of protein nature which markedly enhances listeric infection in white mice was described. This substance isolated from bacterial cells of *Listeria monocytogenes* was partially purified, its chemical and biologic properties were described. So far as has been determined we are dealing with a complex of proteins, polysaccharides, nucleoproteins and lipids. Since it hitherto has not been possible to further isolate the individual components, it was necessary to implement other methods in isolating some of these components and determine to what degree they also enhance listeric infection.

**Material & Methods**

Cultivation and preparation of bacterial cells of *L. monocytogenes* strain ŠE was carried out following the same technic as described earlier (1).

Preparation of protein from *L. m.* (L protein):

According to the method used in isolating streptococcal M-protein (2), a protein substance, appearing to be a chemical individual, was extracted with 0.1 n HCl at 37°C from glycine lysate (GL) of *L. m.* (1). After a thrice repeated 24 hour extraction a soluble protein was reprecipitated by adapting the pH to 4.0–5.0 with 1 n NaOH, and again dissolved in M/15 phosphate buffer of pH = 7.2. Reprecipitation with 1 n HCl was repeated 3–7 times. pH was measured with glass electrode in supernatants, following centrifugation of precipitated protein. We centrifuged at 3,500 r. p. m. After repeated dissolution of the precipitate in phosphate buffer (pH = 7.2) the protein was lyophilized.

Preparation of polysaccharides:

A soluble polysaccharide was obtained from microbial cells by extraction with NaCl at 100°C and precipitation with methanol (3).

Preparation of nucleoproteins:

According to the method of Jones (4) nucleoproteins were precipitated from mechanically disrupted cells of *L. m.*, following extraction with 0.001 M sodium arsenate. A 2% solution of cetyl-pyridinium-ammonium bromide was used in the precipitation. The
nucleoprotein complex with quaternary base, following thrice repeated reprecipitation by diluting the 1n solution of NaCl (in which the complex is soluble) with 2 volumes of distilled water, was dissociated by precipitating 1 volume of the complex in 1n NaCl with 9 volumes of 96% ethanol. The obtained white flaky precipitate, following storage for 24 hours at 4°C, was centrifuged and dissolved in saline. Precipitation was then repeated once more and the resulting solution was tested for the presence of detergents by growth inhibition of L. m. on blood agar. The result was negative.

Preparation of a protein-free extract:

An attempt has been made, employing Boivin’s method modified by Webster et al. (5), to extract with 0.5% TCA and by cold precipitation with ethanol to obtain from washed cells of listeria a substance analogous with the so called endotoxin of Gram-negative bacteria. Further purification of the thus obtained preparation was not successful (precipitation with 0.3M ethanol in a saturated solution of NaCl) because its properties differed from those of the antigenic complex of the majority of Gram-negative bacilli.

Analyses:

Proteins were determined by the biuret reaction in Weichselbaum’s modification (6), and in nucleoproteins with phenol reagent (7). Polysaccharides were determined with alphanaphtol according to DISCHE (8), DNA with diphenylamine according to SEVAG et al. (9) and with cystein sulphuric acid according to DISCHE (10). Absorption spectrum of L protein and nucleoprotein was measured with Zeiss’ universal spectrophotometr (1). Determination of rhamnose was made employing DISCHE and SHETTLES’ reaction (II) with cystein in sulphuric acid, measurements of characteristic maximums in the UV range using 10mg% of rhamnose p.a. (MERCK) as a standard, and then by chromatography. In paper chromatography Whatman I paper was used in systems of n-butanol – acetic acid – water (4:1:5), and phenol – ethanol – water (2:1:1) with 0.1% 8-oxyeholin according to KOLOUŠEK (12). Ninyhydrin and isatin were used in detecting aminoacids, and anilin-phtalate in the case of sugars. Hydrolysis for the chromatographic analysis was made with 6n HCl, and 2n H2SO4, respectively. Electroforesis was made to control the homogeneity of the isolated protein under the same conditions as in our previous report (1).

Activity was determined as described earlier (1) and expressed by an index and its logarithm. Solutions, unless otherwise specified, were adapted to correspond to 0.1% of undialysable substances.

Attempt of preparing antisera:

Three rabbits weighing at an average 2500g were immunized with an initial dose of 2ml/of 0.1% solution of L protein intravenously. Simultaneously the same was injected intramuscularly with lanolin-mineral oil as adjuvant in three separate doses (14). The animals were bled by cardiac puncture after a three-week interval. Every experiment was terminated on the 14th day.

Results

L protein.

The extracted and precipitated protein readily dissolves at pH 7.2, giving a clear, foaming, brownish solution reminiscent of blood serum. Change of pH to 5.0 produces turbidity and rapid floeculation. In this way by repeated precipitation we obtained a substance which furnished in electrophoresis a high gradient of a mobility close to albumins (9.4 x 10^-3). A less mobile gradient found in other preparations, prepared in the same way at various times, most probably represents impurities adsorbed during precipitation (Fig. 1). Impurities were also found during analysis of polysaccharides and DNA as outlined in Table 2.

The protein is biuret positive and results of analysis correspond to the dry solids determined by usual methods. By two-dimensional chromatography in the hydrolysate the following amino-acids were found: lysin, histidine, ornitine, arginine, serine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, valine, tryptophan, phenylalanine and isoleucine. Some of these amino-acids may be component parts of accompanying substances. The UV spectrum
also discloses a small amount of nucleic acid (Fig. 2). The spectral zone of aromatic amino- 
acids of the protein, however, is much more prominent than in the complex preparation 
MA₂ (1). Table 2 and chromatograms of various preparations show that we are dealing with 
an inconsistent admixture of polysaccharides and nucleoproteins, of which the former is ex- 
tracted in the second, and especially in the third step. Therefore we discarded the third yield 
in some batches. In biological experiments we considered the substance, after elimina- 
ting the activity of polysaccharides and nucleoproteins, as practically homog- 
genous. Further purification and detailed studies on the chemical properties 
and composition of this hitherto undescribed protein, reminiscent of M protein, 
will be the subject of another paper.

This protein is not lethal on intravenous administration to 
mice and rabbits. A mild inflammatory reaction is produced on 
intradermal injection. In repeated experiments on enhancement of experimental listeriosis in mice we found the protein's activity to be in the limits of significance determined by us. We obtained positive results with certain not very active preparations only after raising the dose 6 and 10 times, leading to a rise of the index by 2 logarithms (Table 1).

Results of repeated experiments with various preparations prepared in a period of one year demonstrated on the average low activity of L protein. A certain variation in the results lies either in the nature of the agent, i.e. its

Fig. 1. Electrophoresis of L protein (batch E).

Fig. 2. Ultra violet spectrum of L protein (batch E) dissolved in phosphate buffer (pH = 7.2).
production which depends on varying metabolic conditions during cultivation (quality of nutrient medium, especially meat water), or in the seasonal changes in the sensitivity of experimental animals (14).

Table 2

<table>
<thead>
<tr>
<th>Batch</th>
<th>Proteins %</th>
<th>Polysaccharides %</th>
<th>DNA %</th>
<th>Index</th>
<th>Log</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95.0</td>
<td>5.0</td>
<td>—</td>
<td>3</td>
<td>0.477</td>
</tr>
<tr>
<td>B</td>
<td>94.0</td>
<td>6.0</td>
<td>—</td>
<td>772</td>
<td>2.893</td>
</tr>
<tr>
<td>C</td>
<td>92.0</td>
<td>1.5</td>
<td>—</td>
<td>318</td>
<td>2.502</td>
</tr>
<tr>
<td>D</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>18</td>
<td>1.248</td>
</tr>
<tr>
<td>E</td>
<td>90.0</td>
<td>8.0</td>
<td>2.0</td>
<td>59</td>
<td>1.770</td>
</tr>
</tbody>
</table>

Doses of 250 μg dry weight were used.

L protein may also be extracted from a partially purified protein preparation MA₂ described earlier (1). This substance designated as LMA₂, has the same chemical properties as L protein extracted from glycine lysate in so far as isoelectric point, amino-acid content and the capacity to be precipitated with ammonium sulfate are concerned. (L protein is salted out in almost 90% with ammonium sulfate of 50%/saturation, as will be described in detail elsewhere.) Most probably we are dealing with an identical substance of greater purity in regard to polysaccharide content. (The amount of polysaccharides was decreased by precipitation with methanol and ammonium sulfate (1)). The effect of preparation LMA₂, similarly as other preparations of L protein, on enhancement of infection is small, even lesser than of MA₂ from which it was derived. The remainder of the protein complex MA₂ after extraction

![Fig. 3. Outline of preparations derived from glycine lysate. Full circles represent relative activity expressed by log_index. Encircled disks represent relative activity below the limit of significance.](image-url)
with 0.1 n HCl is less active than the original MA₂. This is probably due to partial denaturation of a not readily soluble mixture of proteins damaged by prolonged action of hydrochloric acid. This also may point to the individuality of MA₂. Table 3 and Fig. 3 illustrate results of these experiments.

L protein induces mild production of antibodies in rabbits. After intramuscular injection of L protein, followed by immunization with adjuvants, precipitins appeared in 1:10 antigen dilution. Agglutinins O and H were not demonstrated in serums of immunized rabbits.

**Table 3**
Comparison of activity and chemical composition of various preparations with L protein

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Proteins %</th>
<th>Polysaccharides %</th>
<th>DNA %</th>
<th>Index</th>
<th>Log</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>58.0</td>
<td>17.8</td>
<td>6.5</td>
<td>16,980</td>
<td>4.231</td>
</tr>
<tr>
<td>MA₂</td>
<td>82.5</td>
<td>2.5</td>
<td>4.6</td>
<td>15,870</td>
<td>4.198</td>
</tr>
<tr>
<td>L protein</td>
<td>90.0</td>
<td>8.0</td>
<td>2.0</td>
<td>59</td>
<td>1.770</td>
</tr>
<tr>
<td>LMA₂</td>
<td>95.0</td>
<td>0.0</td>
<td>5.0</td>
<td>23</td>
<td>1.346</td>
</tr>
<tr>
<td>MA₂-L*)</td>
<td>86.9</td>
<td>3.9</td>
<td>6.1</td>
<td>228</td>
<td>2.357</td>
</tr>
</tbody>
</table>

*) MA₂-L is a residue after extraction of L protein from MA₂.

**Polysaccharides.**

On isolation a colorless, clear and viscous solution containing 580 mg of polysaccharides was obtained and then diluted to a concentration of 0.1 %. Following hydrolysis of a sample with 6 n HCl of a biuret negative preparation amino-acids were demonstrated on chromatography confirming the presence of proteins. With Dische’s cystein test methylpentoses were found to be present in the amount of almost 10 % of the total polysaccharides (Fig. 4). Paper chromatography disclosed the presence of rhamnose, an obligatory component in Gram-negative microbes, in the hydrolysate. Activity is outlined in Table 4.
Boivin's extract.

This preparation, because of the identical method used in its preparation, was designated as Boivin's extract. It does not enhance listeric infection on administration of usual doses (Table 4).

Nucleoproteins.

An opalescent solution of nucleoproteins containing 36 mg % of deoxyribonucleic acid, 175 mg % proteins (phenol reagent) and 3 mg % of phosphorus, still gave positive reactions for DNA with cystein-sulphuric acid, and precipitated with sulphosalicylic acid and TCA. UV spectrum of this preparation is given in Fig. 5. Enhancement of experimental listerial infection was not demonstrated although a much higher dose was employed.

Table 4

Slightly active and inactive components in bacterial bodies of L. monocytogenes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (µg)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; controls</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; test</th>
<th>Index</th>
<th>Log</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharides</td>
<td>250</td>
<td>8,043,000</td>
<td>168,700</td>
<td>47</td>
<td>1.669</td>
</tr>
<tr>
<td>Boivin's extract</td>
<td>250</td>
<td>1,349,000</td>
<td>46,420</td>
<td>29</td>
<td>1.420</td>
</tr>
<tr>
<td>Nucleoproteins</td>
<td>1000</td>
<td>179,500</td>
<td>158,500</td>
<td>1.1</td>
<td>0.054</td>
</tr>
</tbody>
</table>

Discussion

This report supplements a previous paper in which a protein or protein-like agent, isolated from bacterial bodies of a virulent strain of Listeria monocytogenes and capable of enhancing listeric infection in white mice on simultaneous administration with infecting dose, was described. The activity of a partial purificate of this agent first of all depends on its protein component other than nucleoproteins. It has not been hitherto possible to obtain a homogenous substance. The most active purificate still contained 2.5 % polysaccharides and 4.6 % nucleic acids, beside lipids. The activity of lipids has not been so far fully studied. Moreover, it is evident that repeated purification, according to the method employed by us, partially denatured the active agent. Therefore, an attempt has been made to prove, per exclusionem, that certain other more readily isolated and homogenious components of listerial cells do not influence experimental infection. Nucleoproteins proved to be inactive. An attempt to isolate from Gram-positive L. m., a substance similar to and analogous in its activity with the polypeptide-glycolipid endotoxic substance of Gram-negative enterobacteriaceae, according to Boivin, was unsuccessful. An almost pure polysaccharide was isolated by us from listeriae according to Fuller. This compound did not prove to be significant in its action. On the other hand a similar compound isolated by a different method is claimed by Stanley to be highly toxic. The investigation of a practically homogenous protein substance designated as L-protein, isolated from listerial bodies following the rather harsh technic originally employed by Hirst and Lancefield in obtaining the important factor of virulence of pyogenic streptococci - M protein, was prompted by certain analogies between these two microbes as studied by us. L-protein proved to be active in a small degree. Therefore it will be proper to further investigate its activity using preparations isolated by more delicate methods. The exclusion and elimination of all other components of the bacterial
cell in respect to their activity clearly demonstrates that the agent influencing listeric infection necessarily has to be sought for in the complex protein substance of listeriae designated as MA2.

Thanks are due to PhDr M. ŠTASTNÝ for carrying out electrophoresis, to Prof. Dr. J. KOŠRIK for reviewing the chemical data in the report, and to Dr. M. CHÝLE for the translation and assistance in the preparation of the manuscript.

Summary

An attempt has been made to isolate some components from bacterial cells of Listeria monocytogenes. Tests were made on white mice. Chemical properties and activity of the components are described.

Employing the method of acid extraction of M protein according to HIRST and LANCEFIELD, a similar protein substance designated as L protein was obtained. Its chemical homogenicity was sufficient in determining its activity. Individual batches of L-protein varied in their activity but did not even equal the activity of the original substances from which they were derived.

The activity of the polysaccharide fraction was not prominent.

Nucleoproteins, isolated according to a modified method of Jones, proved to be inactive.

Isolation of a substance analogous to Boivin's extract was unsuccessful.

Zusammenfassung


Résumé

Etudes sur la pathogénité de Listeria monocytogenes II° rapport

F. Patocka et collab.


Resumen

Estudios sobre la patogenicidad de la Listeria monocytogenes

II. Comunicación

F. Patocka y otros

Se hizo un ensayo para el aislamiento de partes integrantes de las células bacterianas de Listeria monocytogenes y ensayos en ratones blancos. Son descritas las propiedades químicas y la actividad de las partes integrantes. Por aplicación de la extracción ácida de proteína-M según HIRST y LANCEFIELD fue obtenida una proteína parecida y denominada proteína-L. Su homogeneidad química bastó para determinar la actividad. Algunas
Изучение патогенности Listeria monocytogenes.

Сообщение 2-е
Ф. Патоцка и др.

Выводы
Авторы попытались изолировать составные части бактерийных клеток Listeria monocytogenes и для этой цели поставили опыты на белых мышах. Химические свойства и активность составных частей описываются. Применением кислотной экстракции М-протеина по Hirst и Lancefield ими получен аналогичный протеин, называемый L-протеином. Химическая гомогенность его достаточна для определения активности. Части L-протеина колебались в активности, но далеко не достигали активности оригинального вещества, из которого они получены. Активность полисахаридной фракции была незначительной. Изолированные по методу нуклеопротеины казались неактивными. Изоляция вещества аналогично вытяжке Boivin осталась безуспешной.

References


Prof. Dr. F. Patočka, Dr. M. Mára and Dr. J. Schindler, Laboratory for Special Medical Microbiology and Immunology, Medical Faculty, Charles University, Prague.