Experimental listeriosis in Rabbits: Biochemical Changes in Serum and Cerebrospinal Fluid

T. PAMUKCU, G.F. YARIM, N. KABAKCI, M. YARIM and Ö. DURU

SUMMARY

This study was focused on serum and cerebrospinal fluid (CSF) biochemical changes in experimental Listeria monocytogenes infection in rabbits. A total of 20 New Zealand White rabbits were used. 14 New Zealand White rabbits were inoculated with Listeria monocytogenes 1/2a (L.m. 1/2a) by intracerebral way and 6 rabbits served as controls. Ten hours after the inoculation of the bacteria the rabbits developed symptoms of meningitis, with stiffness of the neck and fever. The existence and the severity of the inflammatory reaction were controlled by microscopic observation. The presence of bacteria into the cytoplasm of neutrophils and macrophages localised in inflammatory areas was confirmed by immunohistochemical analysis using rabbit polyclonal antibodies. We have analysed in serum and cerebrospinal fluid (CSF) the concentrations of glucose, total protein, and the enzymatic activities of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and lactate dehydrogenase (LDH). After intracerebral inoculation of bacteria the glucose concentrations in the serum and CSF decreased to 428.69 ± 5.50 mg/l and 309.56 ± 3.93 mg/l (mean ± SE) respectively compared to control values of 975.33 ± 84.34 mg/l and 689.22 ± 65.13 mg/l (p ≤ 0.001). Total protein contents, the activities of ASAT, ALAT and LDH in the serum of infected animals increased to 98.92 ± 1.97 g/l, 327.87 ± 22.18 U/l, 292.39 ± 4.66 U/l, 153.28 ± 3.04 U/l respectively compared to 64.42 ± 1.14 g/l, 67.91 ± 7.79 U/l, 74.25 ± 1.79 U/l, 49.35 ± 1.20 U/l in control rabbits. In CSF, increases in total protein concentrations and in enzyme activities were also evidenced in inoculated rabbits. Moreover, serum and CSF LDH activities were correlated with brain injury (p < 0.05).

KEY-WORDS: biochemistry - experimental listeriosis - histology - immunohistochemistry - rabbit.

RÉSUMÉ

Listériose expérimentale chez le lapin : altérations biochimiques du sérum et du liquide céphalo-rachidien. Par T. PAMUKCU, G.F. YARIM, N. KABAKCI, M. YARIM et Ö. DURU.

Ce travail a pour objectif d’évaluer les modifications biochimiques du sérum et du liquide céphalo-rachidien lors d’infection expérimentale par Listeria monocytogenes (L.m.) chez le lapin. Parmi les 20 lapins néozélandais utilisés, 14 ont été inoculés par voie intracérébrale par une suspension de L.m. 1/2a et 6 ont servi de témoins. Dix heures après inoculation, les lapins ont développé des symptômes de méningite (rigidité de la nuque, fièvre). L’existence et la sévérité de la réaction inflammatoire induite ont été évaluées par microscopie. La présence de la bactérie dans le cytoplasme des neutrophiles et des macrophages présents dans les zones inflammatoires a été confirmée par immunohistochimie en utilisant des anticorps polyclonaux de lapins. Les concentrations de glucose et de protéines ainsi que les activités enzymatiques de l’ASAT, de l’ALAT et de la LDH ont été mesurées dans le sérum et dans le liquide céphalo-rachidien. Après inoculation intracrânienne, les concentrations en glucose dans le sérum et dans le liquide céphalo-rachidien sont nettement diminuées (respectivement 428.69 ± 5.50 mg/l et 309.56 ± 3.93 mg/l) par rapport à celles du groupe contrôle (respectivement 975.33 ± 84.34 mg/l et 689.22 ± 65.13 mg/l) (p ≤ 0.001). Les concentrations en protéines et les activités de l’ASAT, de l’ALAT et de la LDH ont été augmentées dans le sérum des animaux infectés (respectivement 98.92 ± 1.97 g/l; 327.87 ± 22.18 U/l; 292.39 ± 4.66 U/l et 153.28 ± 3.04 U/l respectivement comparé à 64.42 ± 1.14 g/l, 67.91 ± 7.79 U/l, 74.25 ± 1.79 U/l, 49.35 ± 1.20 U/l dans le contrôle). Dans le liquide céphalo-rachidien, des augmentations de la concentration protidique et des activités enzymatiques ont également été mises en évidence chez les animaux inoculés. De plus, les activités de la LDH dans le sérum et dans le liquide céphalo-rachidien ont augmenté proportionnellement aux lésions de méningite induite (p < 0.05).

MOTS-CLÉS : biochimie - immunohistochimie - histologie - lystériose expérimentale - lapin.

Introduction

Listeriosis is an infectious disease and a complementary model of an intracytoplasmic organelle infection in the central nervous system (CNS) of domestic and wild animals caused by Listeria monocytogenes and is transferable to humans [7, 16, 35]. Listeria monocytogenes is an ubiquitously occurring non-sporulating gram positive bacterium, is an opportunistic...
pathogen capable of causing meningitis, encephalitis, bacte-
remia and febrile gastroenteritis in animals and humans [8,
32, 34]. In high risk populations, infection can lead to life-
threatening sepsis and meningitis [22]. There are serotypes of Listeria monocytogenes, however, three of them
(1/2a, 1/2b and 4b) cause most of the infections [20]. Strains
of serotype 1/2a are highly heterogenous and thus are easily
differentiated by any of the molecular methods [27].

A variety of clinical manifestations are possible, but bacte-
remia and meningitis are most common [3]. Meningitis pro-
duced in rabbits is a hyperacute disease, whereby most liste-
riae lie extracellularly [14]. Encephalitic lesions are most
severe in the midbrain, less severe in the cerebellum and sel-
don occur in the cerebrum [9, 29].

The diagnostic value of cerebrospinal fluid (CSF) enzyme
activities in bacterial meningitis has been evaluated most
extensively with the enzymes Aspartate aminotransferase
(ASAT-EC 2.6.1.1), Alanine aminotransferase (ALAT-EC
2.6.1.2) and Lactate dehydrogenase (LDH-EC 1.1.1.27) [2,
12, 15, 28, 30].

The aims of the present study were to determine the bio-
chemical changes in the serum and CSF in experimentally
infected rabbits by Listeria monocytogenes and whether a
correlation occurs between the intensity of this experimental
infection and the biochemical changes.

Materials and methods

ANIMALS

Twenty New Zealand White rabbits of both sexes weighing
2.6-3.0 kg were used. Fourteen rabbits were infected by intra-
cerebral inoculation of Listeria monocytogenes 1/2a (L.m.
1/2a) and 6 rabbits served as controls.

INFECTING ORGANISM

The Listeria monocytogenes 1/2a test strain (Dr. Weise,
Bundesgesundheitsamt, Berlin) was inoculated into tryptic
soya broth (Oxoid, Basingstoke, England) with 5 % yeast
extract (TSB-YE) (Oxoid) and incubated at 30°C for 24 hr.
Following incubation, serial dilutions in peptone water of
TSB-YE were dropped on modified Oxford Agar (MOX,
Difco, Detroit, USA) and this was incubated at 35°C for
48 hr. Count of typical round Listeria colonies surrounded
by a black zone on mox were determined as 10^7 cfu/ml. The
fresh, 24 hr culture of L.m. 1/2a in TSB-YE was serially dilu-
ted in saline.

EXPERIMENTAL DESIGN

Anaesthesia was induced by an intramuscular injection of
acepromazine (3 mg/kg), ketamine (30 mg/kg) and xylazine
(15 mg/kg) in rabbits [35]. Animals were infected by injec-
ting the inoculum of 10^7 cfu/ml [24] of saline through a
25-gauge butterfly needle (Abbott, Abbott Park, IL) by intra-
cerebral route from atlanto-occipital region. Six control rab-
bbits were inoculated with saline by the same way.

Ten hours later after injection animals were anaesthetised
again using same anaesthetic recipe. The blood sample of
2 mL was obtained from auricular vein with a 24-gauge intra-
cat (Mediflon, Eastern Medikit Ltd., India), and CSF sample
of 0.5 ml was obtained by puncture of the cisterna magna
with a 25-gauge butterfly needle. After 30 min blood and
CSF samples were centrifuged at 3000 rpm (1550 x g) for
10 min at 4°C and the supernatants were used for biochemi-
ocal analyses. Nine rabbits died in 3rd day. Five rabbits
were euthanized in 4th day after post-inoculation. In necropsy,
only whole brain was removed and the tissues were imme-
diately fixed in Bouin’s fixative overnight at 4°C. Subse-
quently, tissues were dehydrated in ethanol and xylene and
embedded in paraffin wax. Serial sections of each tissue were
cut on a microtome at 5 µm in thickness. The first sections of
the tissues were stained by Haematoxylin and Eosin.

BIOCHEMICAL METHODS

Glucose and total protein concentrations [13] and ASAT,
ALAT and LDH enzyme activities [18] in the serum and
CSF were analysed spectrophotometrically using Sigma
commercial kits (Sigma-Aldrich Chemie GmbH, Eschenstraße 5, 82024 Taufkirchen, Germany) according to the
manufacturer’s instruction.

IMMUNOHISTOCHEMICAL METHODS

Antibodies

The anti-Listeria monocytogenes antibody was produced in
two New Zealand White rabbits of our laboratory. The L.m.
1/2a test strain was inoculated in tryptic soya broth and incu-
bated at 37°C for 18 hours. Kolle flasks of tryptic soya agar
were inoculated with 0.5 ml broth culture and incubated at
37°C. The growth was removed with 5 % formalinized saline
and the cells washed 4 times with normal saline. Pairs of
adult New Zealand White rabbits were injected by intra-
venous way with 0.1, 0.2, 0.4, 0.8 and 1.0 ml of antigen on
successive days. One week after the final inoculation both
rabbits were killed. Serum was collected and stored at -20°C.

Immunohistochemistry for L.m. 1/2a

Immunohistochemical examination was performed for
only the sections of the brain. A universal LSAB 2 horsera-
dish peroxidase (HRP) kit (Dako, Ct. No: K0675, Glostrup,
Denmark) was used for the demonstration of L.m. 1/2a anti-
gen.

The sections were dried overnight at 37 °C and dewaxed in
two changes of xylene for 10 minutes each, rehydrated in
100 %, 95 % and 70 % alcohol and placed in distilled water
for 10 minutes. All steps unless indicated otherwise were per-
formed at room temperature in a dark humidified chamber.
Two changes of tris-buffer (pH 7.4) were used for washing.
For immunohistochemistry, the sections were boiled with
antigen retrieval solution (Dako, Ct. No: S1699, Glostrup,
Denmark) for 40 minutes. Endogenous peroxidase activity
was eliminated from the sections by incubating with 3 %
hydrogen peroxide in absolute methanol for 5 minutes. The
sections were incubated with the primary anti-L.m. 1/2a anti-

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serum at a dilution of 1:1024 for 1 hour at room temperature. They were treated with the anti-rabbit secondary antisera (Dako, Ct. No: K0675, Glostrup, Denmark) in phosphate buffer solution (PBS) for 10 minutes at room temperature, and then streptavidin-peroxidase enzyme for 10 minutes at room temperature. The sections were incubated with 3,3’-diaminobenzidine (DAB) chromogen in hydrogen peroxide for 5-10 minutes. Subsequently the sections were counters- tained lightly with Mayer’s haematoxylin for 1-2 minutes, mounted with aqueous medium under cover slips. Negative controls consisted of sections incubated in the absence of the primary antibody. The localisation of immunoreactive cells was studied using an Olympus BX-50 microscope.

STATISTICAL ANALYSIS

The data obtained from serum and CSF analysis was subjected to the Mann-Whitney test for two independent samples and correlation tests were also performed using the Minitab-User Guide program [3] and Student test. Results were considered as significant when p values were less than 0.05.

Results

MICROSCOPIC FINDINGS

The meninges of all infected animals were inflamed especially in the brain stem (medulla oblongata, pons cerebri, and cranial part of the medulla spinalis) and showed a mild to moderate semi purulent meningitis. Meninges vessels were hyperaemic and thick perivascular inflammatory cell layers were seen. In parenchyma and subependymal region were prominent haemorrhages, microabscesses and necrotic foci. Perivascular mononuclear cell layers were seen in the same areas.

IMMUNOHISTOCHEMICAL FINDINGS

The L.m., immunopositive results were characterised by prominent intracellular brown staining punctate foci in neu- trophils and macrophages within microabscesses and necrotic foci (figure 1). In sections lacking necrosis, positive punctate intracellular staining was present in only a few cells within some but not all multifocal accumulations of macrophages. Negative controls showed no staining.

Immunostaining for L.m. is best visualised in microabscesses and areas of necrosis. Antigen appeared as small circular or ovoid granules or as small rods of variable size, localised in microabscesses and in large foci of malacia, mainly in the cytoplasm of phagocytes. Some cells showed normal form of the Listeria antigen, but in others, there was a diffuse and uniform reaction by immunohistochemistry. The bacte- rial antigen in the tissues examined was always sparse and variable in distribution. In many microabscesses only few cells containing antigen were found and some had no detectable antigen. Small numbers of phagocytes containing bacte- rial antigen were detected in the perivascular cuffs, glial foci and areas of parenchyma free of inflammation. Bacterial antigen was not detected in neurones or neuronal processes.

BIOCHEMICAL FINDINGS

The means of glucose, total protein, ASAT, ALAT and LDH levels and the standard error of the means in serum and CSF collected from infected and control animals are present- ed in the Table I.

Serum glucose, total protein contents and ASAT, ALAT and LDH activities were 428.69 ± 5.50 mg/l, 98.92 ± 1.97 g/l, 327.87 ± 22.18 U/l, 292.39 ± 4.66 U/l, 153.28 ± 3.04 U/l respectively in infected animals as against 975.33 ± 84.34 mg/l, 64.42 ± 1.14 g/l, 67.91 ± 7.79 U/l, 74.25 ± 1.79 U/l, 49.35 ± 1.20 U/l in controls.

CSF glucose and total protein concentrations and ASAT, ALAT, LDH activities were 309.56 ± 3.93 mg/l, 20.12 ± 0.40 g/l, 44.38 ± 3.01 U/l, 38.55 ± 0.62 U/l, 26.00 ± 0.52 U/l respectively in infected rabbits as against 689.22 ± 65.13 mg/l, 4.03 ± 0.27 g/l, 8.88 ± 1.03 U/l, 9.60 ± 0.23 U/l, 6.16 ± 0.18 U/l in control rabbits.

The serum and CSF glucose concentrations in diseased rabbits were lower than in the control rabbits (p ≤ 0.001). Serum and CSF total protein and ASAT, ALAT and LDH activities were found to be significantly higher in infected animals than in healthy animals (p ≤ 0.001). For all biochemical markers, positive correlations were found between results obtained in serum and in CSF in infected rabbits (Table II).

The biochemical results according to the histological scores, i.e. severity of brain damage, are presented in Table III. Decrease of glucose concentrations and increases of total protein concentrations and ASAT, ALAT activities in serum and in CSF were more pronounced in infected rabbits with severe meningitis than in rabbits with mild damage. However, these variations were not statistically significant because of the low size of the groups and because of the scatter- ing of recorded values. Furthermore, LDH activities were significantly more elevated in serum and in CSF of rabbits with severe injury than in the other infected rabbits (p < 0.05).

Discussion

Listeriosis is an important bacterial zoonosis caused by the intracellular pathogen Listeria monocytogenes. Listeriosis has been reported to cause encephalitis, abortion, mastitis, repeat breeding and endometriosis in animals [10, 23].

In the present study the serum and CSF concentrations of glucose were significantly decreased (p ≤ 0.001) and the total protein concentrations, the ASAT, ALAT and LDH activities in infected rabbits were significantly increased (p ≤ 0.001).

Hypoglycaemia due to septicemia should be relatively easy to diagnose [26]. The mechanism is not well defined, but depleted glycogen stores, impaired gluconeogenesis, and increased peripheral use of glucose may be contributing fac- tors [5].

The presence of ASAT in different tissues makes the serum enzyme a non-specific but sensitive marker of soft tissue damage [17]. Activities of the enzymes ASAT and ALAT

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TABLE I. — Serum and cerebrospinal fluid biochemical findings in 14 rabbits with listeriosis and in 6 controls (mean ± SE).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Serum</th>
<th>CSF</th>
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<tbody>
<tr>
<td></td>
<td>Infected (n = 14)</td>
<td>Control (n = 6)</td>
</tr>
<tr>
<td>Glucose mg/l</td>
<td>428.69 ± 5.50</td>
<td>975.33 ± 84.34</td>
</tr>
<tr>
<td>Total protein g/l</td>
<td>98.92 ± 1.97</td>
<td>64.42 ± 1.34</td>
</tr>
<tr>
<td>ASAT U/l</td>
<td>327.87 ± 22.18</td>
<td>67.91 ± 7.79</td>
</tr>
<tr>
<td>ALAT U/l</td>
<td>292.39 ± 4.66</td>
<td>74.25 ± 1.79</td>
</tr>
<tr>
<td>LDH U/l</td>
<td>153.28 ± 3.04</td>
<td>49.35 ± 1.20</td>
</tr>
</tbody>
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TABLE II. — Correlations between biochemical changes in serum and cerebrospinal fluid according to the intensity of meningitis (mild or severe) in rabbits infected by L. m. 1/2a. NS : non significant.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Correlation between serum and cerebrospinal fluid</th>
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<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Glucose mg/l</td>
<td>0.996</td>
</tr>
<tr>
<td>Total protein g/l</td>
<td>0.999</td>
</tr>
<tr>
<td>ASAT U/l</td>
<td>1.000</td>
</tr>
<tr>
<td>ALAT U/l</td>
<td>1.000</td>
</tr>
<tr>
<td>LDH U/l</td>
<td>0.942</td>
</tr>
</tbody>
</table>

TABLE III. — Modifications (mean ± SE) of biochemical markers in serum and in cerebrospinal fluid (CSF) according to the intensity of meningitis (mild or severe) in rabbits infected by L. m. 1/2a. NS : non significant.
increase with the severity of the tissue damage [5, 12]. These findings also were found in our study.

Changes in the ASAT activity in CSF has been found to be relatively non-specific and are of little diagnostic value [5]. So we decided to determine these enzymes activities in both serum and CSF. If the enzyme activity is elevated in both serum and CSF, massive parenchymal brain damage is suggested and the prognosis is poor, whereas if only the CSF level is raised, recovery is probable [5].

KNIGHT et al. (1981) calculated a reference interval for LDH enzyme activity in CSF (0-23.5 U/l) and demonstrated that mean concentrations were higher in humans with bacterial meningitis (805 U/l, n = 73) than in aseptic humans (10.5 U/l, n = 20). In our study, the serum and CSF LDH activities significantly increased and furthermore, increases of LDH activities were significantly highest in infected rabbits with severe brain injury. Consequently, LDH would be a more sensitive and earlier indicator of bacterial meningitis than glucose concentration. As LDH tissue contents are about 500 times greater than those normally found in serum, leakage of the enzyme from even a small mass of damaged tissue can markedly increase the measure of LDH activity in serum [5].

In healthy subject, LDH activity in CSF is normally very much lower than the serum activity. LDH isoenzymes may be released from cells that have infiltrated into the CSF. For example, in bacterial meningitis in humans, the resulting granulocytosis can produce an elevation of LDH-4 and LDH-5 [4, 6]. LDH-1 and LDH-2 isoenzymes are dominant in rabbit leukocytes [39]. It was found any study about the isoenzymes pattern of LDH in the CSF of rabbits with bacterial meningitis.

ABATE et al. (1998) postulated that during neurological diseases, these enzymes are released in CSF as a consequence of tissue damage and/or increased permeability of the blood-brain barrier. Simultaneous measurement of serum and CSF LDH activity and LDH isoenzyme profile can aid in establishing status of the blood-brain barrier [11, 25].

Five LDH isoenzymes have been described in the central nervous system. It was previously shown that isoenzymes enriched in the A subunit (like LDH-5) were abundant in astrocytes while those containing predominantly B subunits (like LDH-1) were plenty of in neurones [37, 38]. LAUGHTON et al. (2000) reported that, the in situ hybridisation of LDH-1 and LDH-5 mRNAs revealed that while LDH-1 mRNA had a much more discrete expression, LDH-5 mRNA had a uniform distribution suggesting a glial localisation.

In conclusion, our findings suggest that ASAT, ALAT and LDH activities in the serum and CSF are significantly altered by Listeria monocytogenes infection. But, although all studied biochemical markers seemed to be more modified in rabbits with severe meningitis, only positive correlations were found between serum or CSF LDH activities and intensity of bacterial meningitis. Further investigations in this area especially in LDH profile in rabbits with bacterial meningitis are desirable.

References
