The Expression of CD14 antigen in Experimental Encephalitic Listeriosis in Rabbits

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SUMMARY

The present study examined the distribution of Listeria monocytogenes 1/2a (L.m. 1/2a) and the antigen CD14 by using immunohistochemical methods in the brain of healthy and experimentally infected rabbits with L.m. 1/2a. The ten New Zealand White rabbits were inoculated by intracerebral route, and 4 rabbits were used for control. Only the brains of the animals were removed during necropsy, and they were analysed by conventional histological techniques. Haematoxylin and Eosin staining all L.m. 1/2a inoculated rabbits presented meningitis or meningoencephalitis. In healthy rabbits, the immunohistochemical staining of brains for CD14 was very slight and seen only in the lumen of the vessels, whereas, in infected rabbits the CD14 immunoreactivity was more prominent especially in inflammation areas of parenchyma and meninges. Moreover, the CD14 positive immunostainings were seen massively in L.m. positive inflamed areas.

The present study provides the immunohistochemical expression of CD14 and L.monocytogenes antigen in the brain and a positive correlation was obtained between CD14 and this infectious agent.

KEY-WORDS: brain - CD14 - immunohistochemistry - Listeria monocytogenes.

RÉSUMÉ

Expression de CD14 dans la forme encéphalitique de la listériose induite expérimentalement chez le lapin. Par N. KABAKCI et M. YARIM.

La distribution de Listeria monocytogenes 1/2a (Lm 1/2a) et de l’antigène CD14 a été étudiée par immunohistochimie dans le cerveau de lapins sains et infectés expérimentalement par Lm 1/2a. Dix lapins New Zealand White ont été inoculés par voie intracérébrale et 4 lapins sains ont servi de contrôles. Après examen nécropsique, les cerveaux des animaux ont été prélevés et examinés par des techniques histologiques conventionnelles. En coloration hémalun-éosine, tous les lapins inoculés ont montré des lésions de méningite ou de méningo-encéphalite. La présence de l’antigène CD14 n’a été détectée par immunohistochimie que très ponctuellement et seulement dans la lumière des vaisseaux chez les lapins sains, alors que chez les lapins infectés, l’immunomarquage a été bien plus intense surtout dans les foyers inflammatoires du parenchyme et des méninges. De surcroit, l’antigène CD14 était plus particulièrement exprimé dans les zones inflammatoires positives pour Lm 1/2a. Cette étude a donc permis de visualiser par histochimie l’expression conjointe de l’antigène CD14 et des antigènes de Lm 1/2a dans le cerveau et d’obtenir une corrélation positive entre ces 2 paramètres.


Introduction

Listeriosis in animals shows five syndromes: (a) infection of the pregnant uterus with subsequent abortion, stillbirth or neonatal death, (b) septicemia with miliary lesions, (c) mastitis, (d) purulent conjunctivitis and keratitis, and (e) encephalitis [8]. Listerial encephalitis is characterised by microabscesses, focal gliosis and perivascular cuffing. The most severe lesions are confined to the brainstem, especially the pons cerebri and medulla oblongata [5]. The use of immunohistochemical method has demonstrated the distribution of Listeria in many tissues in natural cases and experimental listeriosis in animals [7, 9, 11-13, 17, 22]. Activation of microglial cells depend on various pathological conditions of the central nervous system, including infection and inflammation, neurodegenerative disorders, ischemia and traumatic brain injury [6]. The generation of these local immune responses requires signal transduction via binding of receptors. The CD14, a key pattern recognition receptor of the innate immune system, is a membrane antigen on monocytes involved in cellular activation. The recognition and signalling through CD14-dependent pathways require three
FIGURE 1. — The exceedingly low level CD14 immunolabelling with DAB chromogen was obtained only in the lumen of the vessels of negative control sections (arrows). Streptavidin-peroxidase. Bar: 40 µm.

FIGURE 2. — Section of brain from an infected rabbit with L.m. antigen. The CD14 immunoreactivity with DAB chromogen is prominent in vessels (arrows) and macrophages (arrowheads) in meninges. Streptavidin-peroxidase. Bar: 40 µm.

FIGURE 3. — Section of brain from an infected rabbit with L.m. antigen. The CD14 immunoreactivity with DAB chromogen is prominent around of the glial focus and vessels. Positive immunoreactivity is prominent especially in mononuclear cells (arrows). Streptavidin-peroxidase. Bar: 110 µm.

FIGURE 4. — Section of brain from an infected rabbit with L.m. antigen. The CD14 immunoreactivity with DAB chromogen is prominent around of the vessel. Positive immunoreactivity is prominent especially in mononuclear cells (arrows). Streptavidin-peroxidase. Bar: 40 µm.

FIGURE 5. — Section of brain from an infected rabbit with L.m. antigen. The CD14 immunoreactivity with DAB chromogen is exceedingly high level in ependymal and subependymal areas (arrows). Streptavidin-peroxidase. Bar: 110 µm.
LPS-binding protein, a plasma lipid transfer protein, acts on LPS aggregates or bacterial membranes and presents LPS monomers to binding sites on CD14 [21] by facilitating their binding to CD14. When LPS binds to CD14 expressed by monocytes or neutrophils, the cells become activated and release cytokines such as tumor necrosis factor (TNF) and up-regulate cell surface molecules, including adhesion molecules. It is not exactly known what is the origin of the CD14 [18].

Recent research has revealed the detailed mechanisms by which LPS activates mononuclear phagocytes (monocytes and macrophages) [1, 4, 10, 21] and other types of cells to secrete TNF-Alpha, IL-1Beta, IL-6 and other cytokines, which mediate the development of septic shock [14]. SCHIMKE et al. [19], have shown that a mAb to rabbit CD14 that blocks LPS binding and subsequent cell activation offers remarkable protection even when administered after several exposures to LPS.

The aim of the present study was to determine the immunohistochemical distribution of CD14 in the brain of healthy and experimentally infected rabbits by L. monocytogenes and its contribution to brain damage. The second purpose of this study was to identify whether a correlation of CD14 and this infectious agent occurred in the brain.

Materials and methods

PREPARATION OF LM 1/2A TEST STRAIN

The L. m. 1/2a test strain (Dr. Weise, Bundesgesundheitsamt, Berlin) was inoculated into tryptic soya broth (TSB) (Oxoid, Basingstoke, England) with 5 % yeast extract (YE) (Oxoid) and incubated at 30° C for 24 hours. Following incubation, serial dilutions in peptone water of TSB-YE were dropped on modified Oxford Agar (MOX, Difco, Detriot, USA) and this mixture was incubated at 35° C for 24-48 hours. Count of typical round Listeria colonies surrounded by a black zone on mox were determined as $10^7$ cfu/ml. The fresh, 24 hours culture of L. m. 1/2a in TSB-YE was serially diluted in NaCl 0.15M.

ANIMALS AND EXPERIMENTAL INFECTION

Fourteen New Zealand White one-year old rabbits (six of males and eight of females) were used and they had food and water ad libitum. The rabbits were anaesthetised via intramuscular injection of acepromazine (3 mg/kg), ketamine (30 mg/kg) and xylazine (15 mg/kg). Ten rabbits were infected with L.m. serovar 1/2a by injecting the inoculum of $10^7$ cfu/ml in NaCl 0.15M through a 25-gauge butterfly needle (Abbott, Abbott Park, IL) by intracerebral route from atlanto-occipital region. Four control rabbits were treated only with NaCl 0.15M. Three inoculated rabbits died in the 3rd day. Seven rabbits were sacrificed in the 4th day after post-inoculation. In necropsy, only whole brain was removed and immediately fixed in Bouin’s fixative overnight at 4° C. Subsequently, 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.

ANTIBODIES

The anti-L.m. 1/2a antibody was produced in two New Zealand White rabbits in our laboratory:

The L. m. 1/2a test strain was inoculated in tryptic soya broth and incubated at 37° C for 18 hours. Kolle flasks of tryptic soya agar were inoculated with 0.5 ml of broth culture and incubated at 37° C. The growth was removed with 0.5 % formalized saline and the cells washed 4 times with normal saline. Pairs of adult New Zealand White rabbits were injected intravenously 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.

Anti-rabbit CD14 mAb raised against CD14 (provided by Dr. Richard J. Ulevitch, The Scripps Research Institute, La Jolla, CA) was used to demonstrate CD14 positive cells in the central nervous system.

IMMUNOHISTOCHEMISTRY FOR L.M.1/2A AND FOR CD14

Immunohistochemical examination was performed on the sections of the brain. An universal LSAB 2 horse radish peroxidase (HRP) kit (Dako, Glostrup, Denmark) was used for the demonstration of L.m.1/2a antigen.

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 performed 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, Glostrup, Denmark) for 40 minutes. Endogenous peroxidase activity was eliminated from the sections by incubating with 3 % hydrogen peroxide in absolute methanol for five minutes. After blocking nonspecific binding components with 5 % normal goat serum for five minutes, the sections were incubated with the primary antibody (anti Lm 1/2 a-antiserum for detection of Lm 1/2 a and anti-rabbit CD14 monoclonal antibody for detection of CD14) at a dilution of 1:1024 for 60 minutes at room temperature. They were treated with the goat anti-rabbit secondary antiserum in PBS (Dako) for 10 minutes at room temperature, and then with streptavidin-peroxidase enzyme (Dako) for 10 minutes at room temperature. The sections were incubated with 3, 3’-diaminobenzidine (DAB) chromogen (Dako) in hydrogen peroxide for 5-10 minutes. Subsequently the sections were counterstained lightly with Mayer’s haematoxyl in 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.
Results

MICROSCOPIC FINDINGS

The meninges of all infected animals were inflamed especially in the brain stem (pons cerebri, medulla oblongata and cranial part of the medulla spinalis) and showed a mild to moderate non purulent meningitis. The vessels of the meninges were hyperaemic and thick perivascular inflammatory cell layers were seen. Haemorrhages, microabscesses and necrotic foci were prominent in parenchyma and subependymal region. 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 of chromogen in neutrophils and macrophages within microabscesses and necrotic foci. In sections lacking necrosis, positive punctate intracellular staining was present in only a few cells within some, but not all, multifocal accumulations of macrophages.

Antigen appeared as small circular or ovoid granules or as small rods of variable size. Some cells showed normal form of the Listeria antigen, but others were seen diffuse and uniform reaction by immunohistochemistry. The bacterial antigen in the tissues examined was always sparse and variable in distribution. In many microabscesses, only a few cells containing antigen were found and some had no detectable antigen. Small numbers of phagocytes containing bacterial antigen were detected in the perivascular cuffs, glial foci and areas of parenchyma free of inflammation. Bacterial antigen was not detected in neurons or neuronal processes. Negative controls showed no staining.

In negative control sections, the CD14 immunoreactivity was very low and seen only in vessels (figure 1). No meningeval or ventricular inflammation can be observed in control animals. In infected tissues, a massive brown staining in the cell cytoplasm was observed, particularly in inflamed zones (vessels and meninges) (figure 2). Severe immunopositivity of CD14 was seen in vessels (figure 2), in perivascular mononuclear cells and macrophages in the microabscesses and more weakly in neutrophils (figures 3, 4), ependymal cells and subependymal areas (figure 5). CD14 positive immunostaining was prominent especially in the cytoplasm of macrophages and mononuclear cells in inflamed meninges and parenchyma (figures 3-4). Moreover, inflamed areas (microabscesses in vessels and in meninges) positive for Lm antigen were also massively positive for CD14 immunostaining, and the CD14 antigen and bacterial antigen were be evidenced in the same cell types: macrophages and neutrophiles.

Discussion

In the present study, when the immunohistochemical staining method is using for only L.m. antigen, the distribution of the bacteria was localised especially in the brain stem and seen within microabscesses, necrotic foci, perivascular cuffs and glial foci. MARCO et al. [13], KRUEGER et al. [11] and KARADEMIR et al. [9], using an immunohistochemical method, detected L.m. antigen, always in association with histopathological lesions, in the cytoplasm of neutrophils and in macrophages, in which they took the form of small granules of variable size. Although KRUEGER et al. [11], reported that the amount of antigen detected in all cases was sparse and that could result partially from formalin fixation and tissue processing, it seems likely that the amount of bacterial antigen in the brain stem of naturally infected moribund sheep was low. The amount of the bacteria detected in the tissues by immunohistochemistry during experimental encephalitic listeriosis was also low. The bacterial antigen in the tissues examined was always sparse and variable in distribution. In many microabscesses only a few cells containing antigen were found and some had no detectable antigen. KRUEGER et al. [11], found bacteria in neutrophils and macrophages. Nevertheless, MARCO et al. [13], found bacteria more frequently in neutrophils than in macrophages, and OTTER and BLAKEMORE [16], found no bacteria in macrophages.

In this study, the CD14 positive immunostainings were seen massively in L.m. positive inflamed areas and severe immunopositivity of CD14 was seen in microabscesses, necrotic foci, perivascular spaces, ependymal cells and subependymal areas. In addition, the CD14 expressed strongly on monocytes or tissue macrophages and weakly on the neutrophils. Although the origin of the CD14 is not exactly known, CAUWELS [6], reported that three major cell types were likely candidates for CD14 synthesis in the brain: residential microglial cells, astrocytes and infiltrating blood leukocytes. However, BESCHORNER et al. [2,3], reported that no CD14 expression was visible in B-lymphocytes (CD20+), T lymphocytes (CD3+), astrocytes (GFAP +) and/or endothelial cells (CD34+) in ischemic brain lesions.

CAUWELS et al. [6] reported that soluble CD14 (sCD14) started to appear in the cerebrospinal fluid (CSF) 24 hours after intracerebral injection with the meningitis causing agents. Following L.m. inoculation, sCD14 amounts in the CSF typically continued to increase during 72 hours. Regardless of the initiating stimulus, sCD14 concentrations in the CSF were high and persisted associated with inflammation. In normal human blood [6], sCD14 was present at a concentration of 2-3 µg/mL, but increased concentrations (up to 10 µg/mL) seem to be associated with a wide variety of human inflammatory diseases (whatever their origin: infectious, autoimmune, or even traumatic). Simultaneous analysis of serum revealed much lower and only transient increases in sCD14 concentrations than in CSF [6]. This discrepancy between sCD14 concentrations in CSF and serum suggested the possibility of local production during bacterial meningitis, rather than leakage from the blood through the impaired blood-brain barrier. Furthermore, other researchers (NOCKHER et al. [15]) linked that increase cerebrospinal fluid sCD14 to cerebral production by activated or infiltrated macrophages. Their results indicated that sCD14 is produced.
locally in central nervous system and its concentration in cerebrospinal fluid can be a reliable marker for activation of macrophages within the central nervous system during inflammatory processes. In this study, the CD14 immunoreactivity is prominent in the cytoplasm of meningeal macrophages or perivascular mononuclear cells, and in (sub) epidymal cells, although CD14 was described as a membrane antigen, suggesting an increase of CD14 synthesis by these cell types. Besides, the positively of CD14 in vessels was apparent particularly in enflamed areas. These findings tended to confirm a local production of CD14 by inflammatory cells and macrophages after activation by infectious agent. But, as there was no direct correlation between sCD14 and monocyte count in the cerebrospinal fluid, other origins of CD14, like massive release by intrathecal leukocytes, would occur [6]. As consequence, the CD14 concentrations would increase locally and in CSF. The sCD14 could directly interact with LPS and consequently prevent cell activation (neutrophiles, macrophages and other cells) by disturbing complex formation on membrane between LPS, membrane CD14 and Toll-like receptors and could participate to the regulation of inflammatory reaction by controlling cell activation.

As a conclusion, all previous researches conducted on this subject and the findings of the present study leads us to think that the CD14 positive immunostainings were seen massively in L.m. positive inflamed areas and the origin of the CD14 might be macrophages and neutrophils in the inflamed areas.

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References