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Neurotropic Activity of a Strain of *Listeria innocua* in Suckling Mice

Neurotrope Aktivität eines Stammes von *Listeria innocua* bei Saugmäusen

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Summary

Strain *Welshimer* of *Listeria innocua* generally considered nonpathogenic, induces non-purulent, periventricularly localized encephalitis on intracerebral injection in suckling mice. It was demonstrated by culture and morphologically that listeriae multiply in the lesions. It is therefore necessary to reconsider the problem of pathogenicity of listeriae in far greater detail.

Zusammenfassung

Listeria innocua, Stamm *Welshimer*, gilt im allgemeinen als avirulent. Dennoch gelang es nach intrazerebraler Inokulation, bei Saugmäusen eine nichteitrige, meistens periventriculäre lokalisierte Enzephalitis hervorzurufen. Die Vermehrung der inokulierten Listerien war morphologisch sowie bakteriologisch nachweisbar. Die Fragen einer Pathogenität der sogenannten avirulenten Listerien sollte neu diskutiert werden.

For many years listeria was studied mainly as an interesting cause of anthro-zoonoses until it was demonstrated, similarly as in *Yersinia pestis*, that it is capable of surviving for a long time outside the human and animal organism. In the investigations of *Welshimer* (20, 21, 22), *Seeliger* et al. (14), *Weiss* and *Seeliger* (19) and many others it was demonstrated that listeria can persist not only in the top layers of soil but also on various cultivated plants, particularly past the vegetative period. *Listeria monocytogenes* and closely related microorganisms (*L. grayi*, *L. murrayi*) are almost unique in this respect. This is reflected even in con-

temporary taxonomy, namely for peculiarities of biological activity and particularly for its pathogenic capacity. Therefore some nonhemolytic strains of *L. monocytogenes* possessing distinct antigenic factors (XI, XV), not inducing monocytosis in rabbits and nonpathogenic in experimental animals have been reclassified as a new species *Listeria innocua* (15). The very broad problem of ecology is reflected in its relation to the mammalian and consequently the human organism where it may persist in the digestive and respiratory tract as a frequent epiphyte which under rather exactly determined conditions is even an expressed pathogen, particularly in ruminants, but also in man. It can even be reasoned that *L. monocytogenes* is one of the possible representatives of an evolutionary line in which originally perhaps a telluric eukaryont by adaptation, selection and genetic transformations becomes a microbe at least potentially pathogenic.

The problem of pathogenicity of listeria became the center of general attention. The vagueness of factors of pathogenicity of the microbe and criteria of its assessment in experiment induced us to investigate the microbiology and morphology of the activity of strain *Welshimer* (classified at that time as *L. monocytogenes*), which is generally considered nonpathogenic after discovering that the suckling mouse on intracerebral inoculation is an extraordinarily sensitive animal and therefore suitable for studies in this direction.

Material and Methods

Strain *Welshimer*, isolated by *J. H. Welshimer* from plants, was supplied by Dr. *Elischerová* (Bratislava), designated S-12 similar to serotype 4 of *L. monocytogenes* but further unclassified (*Donker-Voet*), considered nonvirulent for mice (3). Subsequent antigenic analysis in the *Listeria* Reference Laboratory-Würzburg revealed that this strain possesses two distinct O-antigens characteristic of *L. innocua* (15).

The strain was maintained on blood agar, in experiments the first cerebral passage was used. The strain does not hemolyze sheep and rabbit erythrocytes. It is not lethal to mice in doses of 10^9 cells on intraperitoneal injection. The Anton test is negative (guinea pig, rabbit). Tryptone Soya Broth (TSB) cultures of strain *Welshimer*, incubated at 37 °C for 5 or 18 hours were used for inoculations. The cells were alive and fully vital according to their growth curve.

Animals

Two to four day old conventional mice were reared in groups of 10–12 animals nursed by two mothers. The mice were inoculated intracerebrally with 0.03 ml TSB culture of strain *Welshimer* (37 °C) containing 3×10^4 to 3×10^6 CFU. Control animals were inoculated in the same manner with saline to rule out adnate bacterial or viral infestation.

Mice were observed for 5–11 days. Positive clinical signs of encephalitis were considered unrest, ataxia, tremor, convulsions and eventually terminal paralyzes which were usually manifest on the 5th day following inoculation either spontaneously or after rotation.

Histology

Surviving mice were sacrificed 5–11 days following inoculation. Both hemispheres were fixed in 10% formol and prepared by the usual paraffin technique. Sections were stained with hematoxylin-eosine and Gram's stain. The remainder of the brain, particularly the cerebellum and medulla oblongata, was examined bacteriologically using blood agar and TSB incubated in parallel at 37 °C and 4 °C. Cultures were examined and evaluated after 48 hours, eventually 6 months (cultures maintained at 4 °C).

For electron microscopical examination a group of 5 mice was inoculated in like manner and sacrificed the 5th day. The site of inoculation was apparent in the hemispheres as a subtle brownish focus. This site was removed, fixed in 2.5% isotonic glutaraldehyde pH 7.4, osmified, dehydrated, embedded in Durcupan and after selection of semithin sections stained with toluidine blue thin sections were made of picked material and stained with uranyl acetate and lead citrate.

Results

Clinical signs of encephalitis were apparent in eleven out of thirty nine inoculated mice in 5 to 7 days.

Listeria was isolated from brains in 82% of the 39 mice, i. e. in cultures at 37 °C in 15 mice and at 4 °C in another 17 mice.

Histological examination revealed signs of nonpurulent, predominantly periventricular situated encephalitis in 14 out of a total of 23 animals examined.



Fig. 1. Destruction of ependyma in lateral ventricle. Inflammatory infiltration. Basophilic plasm of macrophages in the ventricle contains bacteria. Stained with hematoxylin-cosine. 400 ×.

In advanced cases there was destruction and desquamation of the ependyma of lateral ventricles (Fig. 1). Periventricularly and also freely in ventricles in the neighborhood of focuses of damaged ependyma were increased numbers of mononuclear cells containing in basophilic cytoplasm numerous fine Gram-positive listeriae. In adjacent periventricular spaces of small venules was observed cloak-like lymphoid infiltration which was apparent even in distant parts and in basilar meninges (Fig. 2). Multiplication of listeriae was demonstrated even in the cytoplasm of macrophages of the stroma of plexus chorioideus (Fig. 3).



Fig. 2. Lymphoid infiltration of leptomeninges. Stained with hematoxylin-eosine. 400 \times .

Electron microscopy of selected cases revealed a macrophage reaction at the site of injection. The cytoplasm of macrophages contained a number of disintegrated bacterial cell walls encircling dissolved or clarified plasma of listeriae (Fig. 4). The bacteria were rarely found with dense and in places condensed plasma, or occasionally indications of septa and signs of division (Fig. 5). Beside bacterial cell-wall material the macrophage plasma contained usual dense lysosomes, and other partially lamellar structures which did not resemble bacterial cell walls.



Fig. 3. Gram-positive listeriae in macrophages of plexus chorioideus. Gram's stain. 1000 \times .

Discussion

The greatest surprise of our experiment was that the listeria strain usually considered nonpathogenic caused encephalitis in suckling mice after intracerebral injection.

Criteria of pathogenicity of microbes are viewed by various workers differently. *Weiss* and *Seeliger* (19) consider a strain virulent when it kills conventional mice within 3 weeks after intraperitoneal injection of 0.5 ml of an 18 hour broth culture and listeriae can be recovered from organs.

Khan, Seeman and *Woodbine* (4) class among virulent strains those which on intraperitoneal injection into conventional mice either kill them within 10 days (expressed in LD_{50}) or can be recovered from organs of even symptomless mice sacrificed 10 days after injection.

Ralovich, Emödy and *Merö* (13) consider those strains pathogenic that kill 15–20 g mice on intraperitoneal injection of 0.5 ml of a Lewinthal broth culture

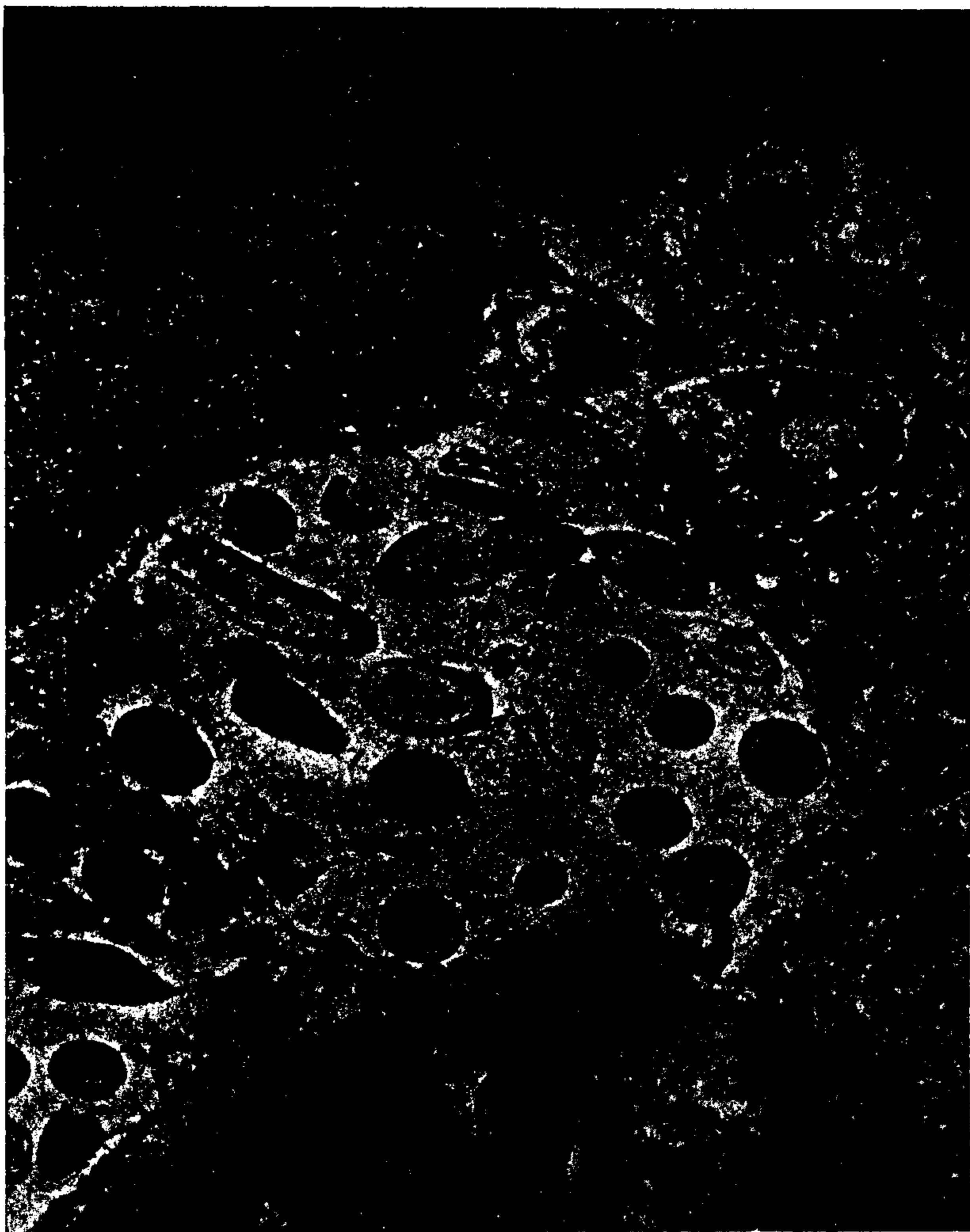


Fig. 4. Prevalence of listerial cell wall material with lysis of plasm. 5,500 \times .

incubated at 37 °C for 24 hours, induces purulent keratoconjunctivitis in guinea pigs and are hemolytic.

In 11 day old chick embryos on intravenous injection there are only several hundred virulent bacterial cells in the LD₅₀, in weakly virulent strains the LD₅₀ contains already 3×10^6 cells. On infection of the yolk sac the LD₅₀ of a virulent strain is less than 60,000 cells. With nonvirulent strains not even a dose of 3×10^6 cells has a lethal effect. Lethal tests with all hemolytic strains of *L. monocytogenes*



Fig. 5. Dividing septum in listerial cell wall. 11,600 \times

the authors (13) tested were positive while the effect of nonhemolytic strains was zero. It is understandable that virulent pathogenic strains are usually recovered from pathologic products such as abscesses and cerebrospinal fluid.

Differences between criteria established on the action of listeriae in lethality tests and those based on positive isolations determined some time after their ino-

cultivation are reflected in that some investigators (16, 4) prefer the term infectivity (i. e. the possibility of positive reisolation after a certain period from organs of even symptomless animals) rather than pathogenicity.

Obviously, the term infectivity expresses far better the conditions we followed in our experiments. The elimination of listeriae by macrophages is an immunologically determined process requiring specific information of macrophages mediated by T lymphocytes evidently by means of the so-called arming factor (10). If we were able to demonstrate morphologically and by isolation the multiplication of listeriae in the brain of suckling mice after intracerebral inoculation and by electron microscopy find after 5 days dividing bacterial cells it can be held to be a reflection of the fact that the relationship of the macroorganism's defense to the ability of listeriae to multiply is in favour of the bacteria and that the strain of *L. innocua* (*Welshimer*) can be considered pathogenic (infective) for the suckling mouse on intracerebral injection.

Problems of formal pathogenesis of morphological features caused by inflammatory changes in the brain must, of course, be investigated in view of the effect of biologically active surface factors and products of the bacterial cells. These problems are first of all correlated with the production of hemolysin and phospholipase by listeria. Thus strains isolated from the respiratory and intestinal tract of symptomless adults lack the mentioned exoproducts, i. e. hemolysin and phospholipase (5, 1). They are incapable of causing distinct signs of inflammation, nevertheless their persistence is a similar expression of the macroorganism's inability of eliminating listeriae just as in our experiments.

We (11, 6) and *Srivastava, Siddique* et al. (17, 2) described a further factor, a complex glycolipid-protein substrate designated by us E_i , according to the separation procedure employed. This factor in its chemical structure and biological effect in animals is in part similar to endotoxin of Gram-negative bacteria (7). Intracutaneous injection in rabbits (12) showed that this product has distinct pyogenic and necrotizing properties which after all are not characteristic of listerial lesions. In a still unpublished report (9) we attempted to explain the eventual significance of the factor E_i after intracerebral injection of listeriae for the appearance and development of the produced encephalitis. In this arrangement the nature of changes after intracerebral injection of E_i in suckling mice is the same as the skin reaction in rabbits after dermal administration. A necrotic and purulent encephalitis was induced as against nonpurulent encephalitis following injection of living whole listeriae. On the other hand the amount of E_i administered in rabbits and in preliminary experiments in mice were incomparably greater than the amount which can be released from injected or propagated and ultimately disintegrated listeriae during the experiment. Moreover, compared to hemolysin and phospholipase, generally considered decisive factors of pathogenicity and virulence, E_i is equally present even in some strains considered nonpathogenic (*Welshimer, L. murrayi*) although in smaller amounts.

To what extent *Stanley's* lipid MPA can be considered a factor supporting pathogenic properties is not clear (18, 8). Likewise, it seems improbable that there may be further, hitherto unknown factors that potentiate and modify the pathogenic activity of the microorganism.

The basic expression of pathogenicity of listeria is, of course, a nonpurulent lymphoid monocytic, or even granulomatous reaction, which was reproduced in our experiments.

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