J. Hyg., Camb. (1978), **80**, 349
Printed in Great Britain

Corynebacterium kutscheri and its alleged avirulent variant in mice

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(*Received* 4 *October* 1977)

SUMMARY

Corynebacterium kutscheri and its alleged avirulent variant were re-examined in C57Bl/6 and Swiss Lynch mice. It was confirmed that while C57Bl/6 mice were resistant and Swiss Lynch susceptible to *C. kutscheri*, the alleged atypical variant was avirulent in both mouse strains. However, following immunosuppression of C57Bl/6 mice with hydrocortisone acetate, it was not possible to reactivate latent *C. kutscheri* or the alleged atypical variant; this was contrary to previous reports. Moreover, sequential hysterectomy derivation over four generations of C57Bl/6 mice did not eliminate their resistance to *C. kutscheri* compared with conventionally born animals. Vaccination with live attenuated *C. kutscheri* protected susceptible mice against virulent challenge; vaccination with the alleged atypical variant afforded no such protection. The suggested role of the alleged avirulent variant in resistance to *C. kutscheri* is challenged and an alternative explanation of such resistance is proposed.

INTRODUCTION

Pseudotuberculosis caused by *Corynebacterium kutscheri* in rodent species is often associated with other infectious processes (Lynch, Pierce-Chase & Dubos, 1965; Topley & Wilson, 1922) or deficiency states (Antopol, 1950; LeMaistre & Tompsett, 1952; Shechmeister & Adler, 1953). In view of this association, the disease in mice was described as a model for those infectious diseases which are activated by physiological stress (Dubos, 1965*a*, *b*).

This suggestion was based largely on the results obtained by Dubos and his co-workers (Fauve, Pierce-Chase & Dubos, 1964; Pierce-Chase, Fauve & Dubos, 1964) in New York and Paris; their work may be summarized as follows. Of various inbred mouse strains, the C57Bl/6 strain was resistant to *C. kutscheri* while the Swiss Lynch strain was extremely susceptible. Further work suggested that C57Bl/6 strain, and a proportion of resistant mouse strains from other sources, were latently infected with *C. kutscheri*, since clinical pseudotuberculosis developed after immunosuppression with hydrocortisone acetate. However, this latent infection was never demonstrated in strains of mice, including Swiss Lynch, which were susceptible to *C. kutscheri*. It was inferred that resistance to *C. kutscheri* in mice was acquired through persistent infection with the organism. During the

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bacteriological examination of latently infected mice, they found, in association with typical *C. kutscheri*, other small gram-positive organisms which produced small translucent colonies and which were completely avirulent for mice, though biochemically similar in some respects to *C. kutscheri*. However, although the authors described distinct differences in colonial morphology and in catalase and urease reactions, and were unable to relate the two organisms on serological grounds, it was proposed that the small translucent colonies were those of an avirulent variant of *C. kutscheri*. A streptomycin-resistant strain of virulent *C. kutscheri* (KSr) was used to latently infect susceptible mice; from which an atypical avirulent form was subsequently isolated and found to be streptomycin resistant (ASr). Susceptible mice infected with high doses of ASr did not show any evidence of clinical disease, but after immunosuppression with hydrocortisone acetate the overt disease developed, from which the streptomycin-resistant virulent form KSr was isolated. They proposed that resistance to *C. kutscheri* in C57Bl/6 mice was due to 'infection immunity', a manifestation of latent *C. kutscheri*, present in these animals as an 'avirulent variant' and that treatment of the animals with cortisone results in the conversion of the avirulent form into virulent *C. kutscheri* and of the latent infection into active corynebacterial disease (Fauve *et al.* 1964).

We re-examined this model and found that while C57Bl/6 mice were markedly resistant compared with Swiss Lynch mice they did not carry latent *C. kutscheri*. Moreover, infection of susceptible mice with the alleged avirulent variant did not render them resistant to virulent challenge.

MATERIALS AND METHODS

Mouse strains

The Swiss Lynch and C57Bl/6 mice used in all experiments were bred from foundation stock kindly supplied by Dr Clara J. Lynch, Rockefeller University, in 1969. The maintenance of these mice is fully described elsewhere (Lynch *et al.* 1965).

Hysterectomy-derivation of C57Bl/6 mice

As most bacterial species do not cross the placenta, the fetus *in utero* can be considered in most cases to be bacteriologically clean (Schaedler, Dubos & Costello, 1965). Although we could never show that C57Bl/6 mice in our Department harboured *C. kutscheri*, a mouse line was nevertheless hysterectomy-derived. Pregnant C57Bl/6 females, judged to be 6 h prepartum, were killed by cervical disarticulation and submerged in 1 % Tego disinfectant (Perkins, Darlow & Short, 1967). The uterus was then aseptically removed and placed in a bowl of disinfectant which was held to 40 °C then transferred to a clean area where each fetus was removed from the submerged uterus. They were then swabbed vigorously with sterile distilled water at 40 °C. This procedure activated the fetus and when the lungs were fully inflated the placenta was detached; the mice were then fostered onto clean susceptible parents. They were subsequently bred in rooms assigned for this

purpose. Following this procedure, C57Bl/6 mice were considered potentially free from *C. kutscheri*. Some animals were derived by four such hysterectomies on successive generations.

Bacterial strains

The *C. kutscheri* strains were obtained from the following sources:

Strain CM1: from Dr C. Pierce-Chase, Rockefeller University, Strain K, isolated from the lung of a mouse, considered to be virulent *C. kutscheri* (Fauve *et al.* 1964; Pierce-Chase *et al.* 1964).

Strain CM9: from Dr N. Wickham, Nicholas Institute, Melbourne, Australia. Isolated from a lung abcess of a mouse.

Strain CM23: from Dr D. K. Blackmore, Laboratory Animals Centre, Carshalton, Surrey. Isolated from a case of pseudotuberculosis in a mouse.

Strain GNS: from Dr C. S. Pierce-Chase, Rockefeller University, Strain A; it had been isolated from the lung of a normal mouse and was considered to be an avirulent variant of *C. kutscheri* (Fauve *et al.* 1964; Pierce-Chase *et al.* 1964).

Development of antibiotic resistant strains

Mutant strains of *C. kutscheri* and GNS resistant to streptomycin sulphate (Glaxo Laboratories) or to Fucidic acid (Fucidin, Leo) were prepared by the gradient plate technique (Shechmeister & Adler, 1953). *C. kutscheri* strains CM23Sr and CM23Sr^{p} were resistant to 50 mg per ml of streptomycin. Strains CM1Fdr, CM23Fdr and GNSFdr were resistant to 100 mg per ml of Fucidin.

Preparation of live vaccine and challenge strains

C. kutscheri strain CM23Fdr was of considerably reduced virulence for mice and was therefore used as a vaccine strain. A single colony was seeded into digest broth with 2% fetal calf serum and agitated gently for 18 h at 37 °C. This was stored in 2 ml volumes in liquid nitrogen in screwtop ampoules (Nunk, U.K.). A similar technique was used to prepare the GNSFdr vaccine.

Initially CM23 was used as a challenge strain, but it was found expedient to use the streptomycin-resistant variant CM23Sr to differentiate challenge organisms from the vaccine strain. Each batch of vaccine and challenge organism was carefully characterized both before freezing and after thawing to ensure that this procedure did not affect viability or virulence. This allowed consistency of both vaccination and challenge to be maintained.

RESULTS

The response of Swiss Lynch and C57Bl/6 mice and their F1 to infection with various strains of C. kutscheri *and with the avirulent variant GNS*

An LD 50 titration was carried out which used six or eight mice of similar age and sex at each dilution of the challenge organism. Initially $\log_{10}$ dilution intervals were used; in later determinations $\log_3$ intervals were used. The results presented in Table 1 show that there was almost a 100-fold difference in LD 50 of all

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Table 1. *LD 50 of different strains of* C. kutscheri *in Swiss Lynch mice, C57Bl/6 and their F1 hybrids*

C. kutscheri strain	Swiss Lynch	C57Bl/6	(Swiss Lynch × C57Bl/6) F1
CM23	3·85*	5·78	4·85
CM23Sr	5·80	> 7·50	> 7·50
CM23Sr^{p}	4·69	6·56	5·63
CM23Fdr	6·96	> 7·50	> 7·50

* Log_{10} LD 50 calculated by the method of Reed & Muench (1938).

Table 2. *Mortality in C57Bl/6 mice following subcutaneous administration of 10 mg of hydrocortisone acetate per mouse*

Sex	Age	Mortality*	Reactivations of latent *C. kutscheri*
Male	4–6 months	26/40†	0
Male	6–12 weeks	6/25	0
Female	3–6 months	29/35	0

* Causes of death were various but none were due to *C. kutscheri*.
† Number of deaths/number of mice in group.

C. kutscheri strains between Swiss Lynch and C57Bl/6 mice. The F1 was of intermediate susceptibility to *C. kutscheri* as determined by LD 50.

Strain CM23Sr was of such reduced virulence that 4×10^7 viable units only occasionally killed resistant C57Bl/6 and F1 mice. This strain was repeatedly passaged through mice which enhanced its virulence about tenfold; this substrain was labelled CM23Sr^{p}. On no occasion did intravenous inoculation of 10^7–10^8 viable units of the streptococcus GNS produce either clinical illness or death in any mouse strain.

Attempts to activate latent C. kutscheri *infection in C57Bl/6 mice with hydrocortisone acetate*

One hundred C57Bl/6 mice which had been bred and maintained in the Department of Pathology, Cambridge, were divided into three groups depending upon age and sex. Each mouse was injected subcutaneously with 10 mg of hydrocortisone acetate (HCA), (Hydrocortistab [Boots, U.K.] or Hydrocortisyl [Roussel, England]). Any animals which died were examined for gross lesions of pseudotuberculosis and bacteriological evidence of *C. kutscheri*. At the end of 8 weeks survivors were similarly examined.

The results presented in Table 2 show that none of the mice which died after treatment with HCA showed either gross pathological or bacteriological evidence of *C. kutscheri* infection. Similarly, those animals which survived for 8 weeks were negative both at autopsy and on bacteriological examination. Most of the animals which died after treatment with HCA showed no visible lesions; of those lesions found, the most common were obesity, spontaneous thoracic haemorrhage and pneumonia.

Table 3. *LD* 50 *of virulent* C. kutscheri *and mortality and reactivations of latent* C. kutscheri *after immuno-suppression in hysterectomy derived and conventionally born C57Bl/6 mice*

Derivation	LD 50 of *C. kutscheri* over 4 generations ± S.E.	Immunosuppression with HCA: Mortality*	Immunosuppression with HCA: Reactivations of latent *C. kutscheri*
Hysterectomy derived	5·67 ± 0·48	5/16†	0/16
Conventionally born	5·49 ± 0·13	14/47	0/47

* Causes of death various but none were due to *C. kutscheri*.
† Number of deaths/number of mice tested.

Bacteriological examination of the dead animals was difficult owing to the accelerated decomposition of the cadavers. This suggested very rapid invasion from the gut, which was borne out by the identification of coliform organisms, *Pseudomonas* species and *Proteus* species from liver, kidney and lung cultures.

Comparative examination of sequentially hysterectomy-derived and conventionally born C57Bl/6 mice

To investigate the role in resistance to *C. kutscheri* of their latent infection with either virulent *C. kutscheri* or the alleged avirulent organism, the C57Bl/6 mouse stock was hysterectomy-derived onto sensitive parents. Four generations of conventionally born and of hysterectomy-derived mice, all originally from a single breeding female, were examined in parallel. Their resistance to *C. kutscheri* was determined by LD 50 titration of strain CM23Sr. Groups of mice from each generation were also immunosuppressed with HCA to confirm their continued freedom from latent *C. kutscheri*.

A summary of the results are presented in Table 3. There was no significant difference in LD 50 between conventionally born and hysterectomy-derived mice over the four generations tested ($P > 0{\cdot}25$). Moreover, latent *C. kutscheri* was not reactivated in any of the immunosuppressed animals.

The survival of Swiss Lynch mice when challenged with virulent C. kutscheri *after immunization with live attentuated* C. kutscheri *or with the alleged variant GNS*

Groups of 48 Swiss Lynch mice were vaccinated either with $10^{5{\cdot}8}$ CM23Fdr or with 10^6 GNSFdr, or served as untreated controls. After 1 month an LD 50 titration of virulent *C. kutscheri* was carried out in each of these groups. The kidneys from animals which received the two highest challenge doses were removed at death or at the termination of the experiment, and the surviving challenge organisms were counted on media which contained streptomycin. None of the mice vaccinated with attenuated *C. kutscheri* died even when challenged with approximately 50 LD 50s of the virulent strain. However, mice vaccinated with the alleged variant and the untreated control mice showed a similar pattern of mortality following challenge (Table 4).

Table 4. *Mortality and LD* 50 *in Swiss Lynch mice after vaccination with* C. kutscheri *or the alleged avirulent variant and challenge with varying doses of virulent* C. kutscheri

Vaccine	Log_{10} challenge dose 6·5	5·5	4·5	3·5	Log_{10} LD 50 *C. kutscheri*
Attenuated *C. kutscheri* CM23Fdr	0/12*	0/12	0/12	0/12	>6·5
Avirulent variant GNSFdr	12/12	9/12	1/12	0/12	5·12
Untreated	12/12	7/12	1/12	0/12	5·31

* Number of deaths/number of mice in group.

Table 5. *Recovery of* C. kutscheri *challenge organisms from the kidneys of vaccinated mice and their controls*

Log_{10} challenge dose	Vaccine: Attenuated *C. kutscheri* CM23Fdr	Alleged variant GNSFdr	Nil (control)
6·5	5·7 ± 0·70*	8·44 ± 0·16	8·60 ± 0·11
5·5	<1·0	8·92 ± 0·12	7·76 ± 0·51

* Results are expressed as mean log_{10} *C. kutscheri* CM23Sr^{p} recovered from kindneys ± S.E.

The recovery of the challenge strain from the kidneys of the three groups of animals is presented in Table 5. There was no statistically significant difference in the numbers of organisms isolated from the kidneys of untreated mice and those vaccinated with the avirulent variant when challenged with either dose ($P > 0{\cdot}25$ in each case); however, the difference between these two groups of mice and those vaccinated with *C. kutscheri* was highly significant ($P < 0{\cdot}001$ in each case). Remarkably few challenge organisms were detected in the kidneys of mice immunized with strain CM23Fdr.

DISCUSSION

We have shown that in general the susceptibilities of the two mouse strains were as described nearly 10 years earlier (Fauve *et al.* 1964; Pierce-Chase *et al.* 1964); Swiss Lynch mice were susceptible to virulent *C. kutscheri* infection whereas C57Bl/6 mice were resistant. There was a difference of nearly 100-fold in LD 50 for *C. kutscheri* between the two parental strains; their F1 hybrid was intermediate. The alleged variant was non-pathogenic to both mouse strains even at very high doses.

Fauve and co-workers activated latent pseudotuberculosis in 100% of C57Bl/6 mice by immunosuppression with hydrocortisone acetate (Fauve *et al.* 1964; Fauve & Pierce-Chase, 1967). They attributed to this latent infection the high resistance of these animals to experimental inoculation of *C. kutscheri*. However, recrudescence of clinical pseudotuberculosis was not observed in any of our

https://doi.org/10.1017/S0022172400024797 Published online by Cambridge University Press

C57Bl/6 mice which had been immunosuppressed with hydrocortisone acetate. Moreover, after serial hysterectomy-derivation, these C57Bl/6 mice remained as resistant as those mice which had been conventionally born. Swiss Lynch mice remained fully susceptible to virulent *C. kutscheri* after infection with the alleged variant, but were resistant after vaccination with live attenuated *C. kutscheri*. From these results we concluded that the alleged variant was not associated with resistance or susceptibility to *C. kutscheri* in these mice.

In a subsequent paper (Hirst & Olds, 1978) we present evidence that the avirulent organism is not a variant of *C. kutscheri* but a streptococcus of Group N. If these two organisms are unrelated how can one explain the previous reports (Fauve *et al.* 1964; Pierce-Chase *et al.* 1964) that cortisone evoked the clinical disease in mice given the alleged variant? We suggest that *C. kutscheri*, which is an insidious infiltrator into mouse colonies even under the best regulated circumstances, may have established itself unbeknown into mouse colonies other than those already heavily infected (Fauve *et al.* 1964; Lynch *et al.* 1965; Pierce-Chase *et al.* 1964). Such a possibility could have accounted for the results of the preliminary experiments reported by Fauve *et al.* (1964) before the use of genetically-marked streptomycin-resistant organisms. Injection of the alleged 'variant' into such mice latently infected with *C. kutscheri* and then subsequent immunosuppression with hydrocortisone acetate could have re-activated a pre-existing latent infection with *C. kutscheri*.

Further, how can one explain the report (Fauve *et al.* 1964) that the Sr genetic marker was found in the alleged variant (ASr) recovered from mice experimentally infected with *C. kutscheri* (KSr)? One could consider gene transfer either by transduction or transformation. However, a more likely explanation is that the Sr gene of ASr arose independently of that of KSr. In their experiments Fauve, Pierce-Chase & Dubos (1964) homogenized organs in fluid containing 50 mg/ml streptomycin: the homogenate was incubated for up to 48 h and then plated on media containing the same concentration of streptomycin. In most cases incubation before plating was essential for the demonstration of streptomycin-resistant strains. We suggest that this provided sufficient selection pressure for streptomycin-resistant mutants. If this were so, ASr would not have been derived from KSr.

In many instances, the Rockefeller workers could not isolate *C. kutscheri* from resistant mouse strains which were presumed to be latently infected. They attributed this to a high level of acquired resistance which prevented the fatal course of the disease. In the absence of positive cultures from mice of resistant strains which died after immunosuppression, they diagnosed pseudotuberculosis on 'the rare appearance of gross lesions and the presence of pleomorphic bacilli' in Giemsa-stained impression smears (Fauve *et al.* 1964). However, we readily isolated *C. kutscheri* from mice which died of pseudotuberculosis; therefore, their negative cultures might imply the absence of *C. kutscheri* from their mice. Further, in mice dead after immunosuppression we found massive post-mortem invasion, predominantly by gut flora. This would make it extremely difficult to diagnose pseudotuberculosis on the morphological appearance of *C. kutscheri* only.

We now propose that the presence of the alleged variant in susceptible Swiss

Lynch mice does not render them resistant to virulent *C. kutscheri*. Moreover, latent infection with either organism is not responsible for the resistance of C57Bl/6 mice. Recent data have shown that there is a major difference in the killing of *C. kutscheri* by mononuclear phagocytes in these two mouse strains (Hirst & Campbell, 1977) which is under genetic control (Hirst & Wallace, 1975, 1976).

We are especially indebted to Dr Cynthia Pierce-Chase for the bacterial strains CM1 and GNS and to Dr Clara J. Lynch for the two mouse strains.

This work was undertaken while one of us (R. G. H.) was the holder of a Wellcome Trust Senior Veterinary Research Training Scholarship and was in receipt of a generous grant from the Marmaduke Shield Fund, Cambridge University.

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