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Pneumococcal pulmonary infection, septicaemia and survival in young zinc–depleted mice*

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The aim of the present study was to explore whether mice fed a diet low in Zn (2·0 mg Zn/kg diet) for a relatively short period of time were more prone to severe *Streptococcus pneumoniae* infection than mice fed a normal diet (25 mg elemental Zn/kg). The Zn-deficient mice were compared with mice in two Zn-adequate control groups; one pair-fed and another with free access to the diet. After 2 weeks feeding, the mice were infected intranasally under anaesthesia with a suspension containing about 10^7 pneumococci. Clinical status was observed every day and blood samples were examined for *S. pneumoniae* every second day for a week. All infected mice examined carried the infecting strain intranasally. The survival time and time before positive blood culture were significantly shorter in the Zn-depleted group than in the pair-fed Zn-adequate group (hazard ratios 15·6 and 3·2, $P < 0{\cdot}0001$ and $P = 0{\cdot}045$ respectively). At the end of the observation period, ten of the twelve mice in the Zn-deficient group were dead while one of twelve and two of twelve were dead in the two Zn-adequate control groups. This study shows that even acutely-induced Zn deficiency dramatically increases the risk of serious pneumococcal infection in mice.

***Streptococcus pneumoniae*: Sepsis: Zinc deficiency: Mice**

Acute lower respiratory infection with *Streptococcus pneumoniae* is an important cause of death in young children of developing countries (Leowski, 1986; International Conference on Acute Respiratory Infections, 1998). Important risk factors are low birth-weight and protein–energy malnutrition. Deficiencies of micronutrients, including Zn, frequently accompany protein–energy malnutrition and are believed to play a role in the increased risk of severe infectious disease in protein–energy malnutrition (Cunningham Rundles, 1982; Walsh *et al.* 1994). Children with protein–energy malnutrition show atrophic changes of the thymolymphatic system, depressed cell-mediated immunity, and increased susceptibility to infections (Cunningham Rundles, 1982). Children with Zn deficiency (ZD) have similar immunological changes and thymic atrophy is reversed when malnourished children are supplemented with Zn (Cunningham Rundles, 1982, 1996; Wellinghausen *et al.* 1997). Zn supplementation in children with suspected ZD has resulted in reduced morbidity from important infectious diseases including diarrhoea (Walsh *et al.* 1994; Sazawal *et al.* 1996) and lower respiratory-tract infections (Sazawal *et al.* 1998). The diet in many developing countries is low in bioavailable Zn (Walsh *et al.* 1994), and children experience repeated infections, especially diarrhoea, which further impair Zn status. Other common causes of ZD are malabsorption, extensive burns, chronic renal diseases and sickle cell disease (Prasad, 1985; Walsh *et al.* 1994). There is evidence that that mild or marginal deficiency of Zn is widespread even in developed countries (Prasad, 1988).

Experimental suboptimal Zn intake in animals and human subjects has been shown to impair immune responses to

Abbreviation: ZD, zinc deficiency.

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T-cell independent and T-cell dependent antigen challenge (Fraker *et al.* 1987; Keen & Gershwin, 1990; Wellinghausen *et al.* 1997). There is also a significant loss of splenocytes, macrophages and peripheral leucocytes, and reduced thymus weight (Fraker *et al.* 1987; Walsh *et al.* 1994; Wellinghausen *et al.* 1997). The extrapolation of these effects on resistance to pneumococcal infection in human subjects is, however, not straightforward. It depends on the effect on the host as well as on the nutrient need of the bacteria. Whether Zn supplementation reduces the risk of pneumonia, septicaemia or death from pneumococcal infection is difficult to detect through intervention studies, because of the limitations of clinical and bacteriological diagnosis (Garenne *et al.* 1992). Furthermore, this disease has a relatively low incidence rate compared with other important childhood diseases (Garenne *et al.* 1992). Thus, a large sample size is required to examine whether Zn supplementation reduces the high morbidity and case fatality rate of pneumococcal disease.

The present study was designed to explore whether young Zn-deficient mice are at increased risk of lethal pneumonia and/or septicaemia when challenged with invasive *Streptococcus pneumoniae.*

Materials and methods

Overall design

The susceptibility to severe pneumococcal infection in a group of mice fed a diet on a low-Zn content (group 1) was compared with that in two groups of mice fed a Zn-adequate diet. The control mice in group 2 were pair-fed with a Zn-adequate diet in amounts equal to the daily average of what the mice in group 1 had eaten in the previous 2 d. The mice in the other control group (group 3) were given free access to the Zn-adequate diet. After 2 weeks experimental feeding, the mice were challenged intranasally with an invasive strain of *S. pneumoniae*. The measured outcomes were survival and the presence of *S. pneumoniae* in blood, lung homogenates, and nasal washes over the subsequent 7 d. The study protocol was approved by the local officer of the Experimental Animal Board under the Norwegian Ministry of Agriculture, and the experiment was in conformity with the laws and regulations controlling experiments with live animals in Norway.

Animals

Female BALB/c mice (Bomholtgård Ltd, Bomholt, Denmark) obtained at 5 weeks of age were allowed to acclimatize for 1 week before the experimental feeding started. At 6 weeks of age, they were matched for weight and divided into three groups of twelve mice per group. At this time, the average weight was 16·2 g. All thirty-six mice were challenged with *S. pneumoniae*. Two additional control groups were used: six uninfected mice were given the Zn-deficient diet and bled at the same time points as the infected mice to determine whether the bleeding itself would increase the fatality rate of the Zn-depleted mice. Moreover, three mice given free access to the Zn-adequate diet were not challenged by the infecting strain and used as uninfected controls for the nasal carriage experiment.

Cages

Metabolic cages from Scanbur (Köge, Denmark) were used for the pair feeding. These cages have a stainless-steel meshed floor to prevent recycling of Zn from body wastes, and feeding chambers to determine the food consumption. Because of the small size of the mice, the feeding chambers were modified with the placement of 50 ml plastic centrifuge tubes (Sarstedt, Nümbrecht, Germany) inside the chambers to avoid spillage. To reduce exposure to environmental Zn, the cages were washed with HCl (100 ml/l) before use and with deionised water at regular intervals throughout the study.

Feeding

Experimental and control diets (Altromin c1040; Altromin, Lage, Germany) had the same composition except for their Zn content. Diet composition (except Zn) in control and experimental fodder (g/kg); protein 174, carbohydrate 594, fibre 43, fat 50, crude ash 53, lysine 15, methonine 11, cystine 4, threonine 9, tryptophan 2, arginine 12, histidine 6, isoleucine 9, leucine 17, phenylalanine 13, valine 12, alanine 10, aspartic acid 18, glutamic acid 25, proline 7, serine 8, tyrosine 12. The diet provided 3·564 MJ/kg. Vitamin and mineral composition were as follows (mg/kg): retinol 1500, cholecalciferol 500, α-tocopherol 163, menadione 10, thiamin 20, riboflavin 20, pyridoxine 15, vitamin B_{12} 0·03, nicotinic acid 50, pantothenic acid 50, folic acid 10, biotin 0·2, choline chloride 1000, *p*-aminobenzoic acid 100, *myo*-inositol 100, ascorbic acid 20, Ca 9509, P 15 000, MG 759, Na 2669, K 7286, S 3169, chloride 4071, Fe 172, Mn 100, Cu 5·2, I 0·45, Mo 0·2, fluoride 3·6, Se 0·43, Co 0·1. As Zn intake has marked effects on growth and appetite, precautions were taken to avoid differences in the intake of other nutrients by pair feeding the mice in group 2. Thus, the mice that received the Zn-deficient fodder (group 1, 2·0 mg elemental Zn/kg diet) were given free access to the diet while the group 2 mice were offered the Zn-adequate diet (25 mg zinc/kg diet) restrictively, i.e. the daily average of what the mice in group 1 had eaten in the previous 2 d. Because of the slower growth of the mice in group 1, the amount of food offered to the pair-fed mice in group 2 was corrected for body weight. The twelve mice in group 3 and the three non-infected mice were placed in separate standard cages and had free access to the Zn-adequate diet. Free access to deionised water was given to all mice. The mice in group 1 and 2 and the six non-infected Zn-depleted mice were housed individually.

Bacteria and culture conditions

Strain DBL2 of *S. pneumoniae* serotype 2 produces invasive infections in mice following intranasal infection (A Brooks-Walter and D Briles, unpublished results). Bacteria from a stock culture kept at −70°C were grown in Todd–Hewitt broth (Difco Laboratories, Detroit, MI, USA) to mid-logarithmic phase and stored frozen at −70°C in 1 ml

aliquots. The aliquots were thawed immediately prior to challenge. The inoculum dose was confirmed before freezing and after thawing; there were no differences in the concentration of viable bacteria, expressed as colony forming units/ml, at these two measurements. Bacteria were confirmed to be *S. pneumoniae* by colony morphology and optochin sensitivity. The number of viable bacteria from the infection batch, lung homogenate, blood, and nose washes were determined by quantitative culture of serial dilutions on blood agar with 5 μg gentamicin/ml incubated overnight at 37°C in 8 % CO_2 atmosphere. The bacterial counts were expressed as $\log_{10}$ colony forming units/lung and $\log_{10}$ colony forming units/100 μl nasal wash. The detection limit for bacteraemia was 67 bacteria/ml whole blood.

Challenge

After 14 d experimental feeding, the mice were anaesthetised with isoflurane (Forene; Abbot Laboratories, Chicago, IL, USA) in a double plenum induction chamber for small animals (International Marketing Services, North Andover, MA, USA). Immediately after induction of anaesthesia, when breathing was slow and deep, a suspension containing 9×10^8 bacteria/ml were introduced intranasally. Because the average body weight in the Zn-depleted group was significantly lower than in the two control groups, the inoculum size was corrected for body weight by giving 2 μl suspension/body weight (26–43 μl/mouse). The optimal infecting dose to obtain pulmonary infection and septicaemia was determined by prior experiments on female BALB/c mice of the same age.

Collection of samples

Blood (75 μl) was collected in heparinised capillary tubes (Vitrex, Herlev, Denmark) on days 1, 3, 5 and 7 after challenge by puncture of the lateral hind leg vein. Others have shown that repeated bleeding of such volumes does not affect the survival time after pneumococcal challenge (Aaberge *et al.* 1992). On day 7, the blood was collected by heart puncture after killing with a subcutaneous overdose of Hypnorm–Vival ('Hypnorm' (fenytyl citrate and fluanisone), Janssen Pharmaceutica, Beerse, Belgium; 'Vival', (diazepam), AL, Oslo, Norway). The throat and thorax were opened aseptically and sterile PBS was injected into the epipharynx through an incision in the proximal larynx. The larynx was clamped to avoid contamination between the lungs and the epipharynx. The first 100 μl wash from the nostrils was inoculated on blood agar. The lungs were dissected free and the left lung homogenised and cultured on blood agar.

Zinc analysis

The left femoral bone was dissected free from the surrounding tissues using stainless-steel scissors and stored in −20°C before Zn determination. Prior to element analysis, lyophilised tissue and feed samples were wet digested in a Milestone microwave laboratory system (Milestone; Milestone, Bergamo Italy) by addition of 2 ml HNO_3 acid (650 ml/l, Ultrapure; Merck, Damstadt, Germany) and 0·5 ml H_2O_2 (300 ml/l; Merck) to samples of 0·2 g dried material. The concentration of Zn was determined with a Perkin-Elmer 3300 flame atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). The accuracy and precision of the element analyses were controlled by concomitant analysis of a standard reference material.

Statistical analysis

Survival after infection in the three groups was described by the Kaplan–Meyer method while Cox proportional hazards regression was used to compare the survival time and time before bacteraemia between group 1 and 2, the hazard ratio being interpreted as the relative death rate (Rosner, 1989). The central tendency and the spread of the bacterial counts from the lung homogenates, nose washes and blood specimens were expressed as mean values their standard errors of the $\log_{10}$-transformed values. Body weights and Zn contents of bone were expressed as arithmetic mean values with their standard errors and differences analysed using one-way ANOVA with Bonferroni multiple comparison between groups, after testing for equality of variances with Levene's test. The statistical analyses were performed using the SPSS Statistical Package, release 6.1 (SPSS, Chicago, IL, USA).

Results

Body weights and tissue zinc content

There were significant differences in body weights ($P < 0{\cdot}001$), but no significant differences in bone Zn contents between the Zn-depleted mice and the mice in the two groups receiving normal food (Table 1).

Bacteraemia

Five of the twelve Zn-depleted mice (group 1) grew pneumococci in the blood as early as the first day after infection while bacteraemia was present in only one of twelve mice in each of the Zn-adequate groups (group 2 and 3) (Fig. 1). Where bacteraemia was present, the counts ranged from 480 to $7{\cdot}3 \times 10^6$ bacteria/ml heparinised blood. There was a significant difference in the time to bacteraemia between the Zn-depleted mice and the pair-fed Zn-adequate mice in group 2 (hazard ratio 3·2, $P = 0{\cdot}045$). Of the Zn-depleted mice that died before the end of the observation period, there was on an average 2 d between the first positive blood culture and death. Seven of the twenty-four mice in group 2 and 3 had positive blood cultures but only three of these died (after an average of 2 d from a positive blood culture) before the end of the observation period. The other four were alive for an average of 4·5 d (until the day of killing) after testing positive for pneumococci in their blood.

Effect of zinc deprivation on survival

At the end of the observation period, ten of the twelve mice in group 1 were dead while one out of twelve and two out of twelve were dead in the two Zn-adequate groups (group 2

Table 1. Bone zinc concentration at death and body weight after 14 d of feeding diets with two different levels of zinc (Mean values with their standard errors)

Group*	*n*	Bone zinc content (μg/g)†		Body weight (g) on day of challenge	
		Mean	SEM	Mean	SEM
Group 1: zinc depleted	12	50·8	4·7	15*** †††	0·4
Group 2: zinc-adequate, pair fed	12	48·6	6	18·3	0·3
Group 3: zinc-adequate, free access	12	48·4	2	20·7	0·2

* The mice were fed a diet containing 2·0 mg Zn/kg (group 1, Zn-deficient diet) or a diet containing 25 mg Zn/kg (group 2 and 3, Zn-adequate diet). Group 2 were fed restrictedly to control for differences in energy intake in mice with low and normal intakes of Zn (pair feeding). For details of diet and procedures, see p. 302.
† There were no significant differences between the groups.
Mean value was significantly different from that of group 2: ***$P < 0{\cdot}001$ (ANOVA with Bonferroni-adjusted P values).
Mean value was significantly different from that of group 3: †††$P < 0{\cdot}001$ (ANOVA with Bonferroni-adjusted P values).

and group 3 respectively). There was a substantial and highly significant difference in survival time between the Zn-depleted mice and the pair-fed Zn-adequate mice in group 2 (hazard ratio 15·6, $P < 0{\cdot}0001$) (Fig. 2). The six non-infected mice fed the Zn-deficient diet that were bled 75 μl every other day survived during the 7 d observation period.

Lung infections

The culture results of the lung homogenates from all mice still alive on day 7 and from mice whose lungs could be processed within 1 h of spontaneous death are given in Table 2. Of the mice eligible for lung cultivation, six of the eight mice in the Zn-depleted group *v.* only five of the twenty-four mice in the two Zn-adequate groups had detectable pneumococci in the lung tissue.

Nasal carriage

All mice that survived until day 7 carried the infecting strain nasally (Table 3). There was no significant difference in bacterial counts between the groups. However, only two Zn-depleted mice survived until day 7, when the nasal carriage assay was performed. The nasal washes of the mice that had been given sterile PBS instead of bacterial suspension did not grow *S. pneumoniae* (Table 3).

Discussion

Recent clinical trials have shown that Zn supplementation reduces morbidity and enhances growth in moderately malnourished children (Walsh *et al.* 1994; Brown *et al.* 1998). An intervention trial in 6–35-month-old children in India showed a 45 % (95 % CI 10, 67) lower incidence of

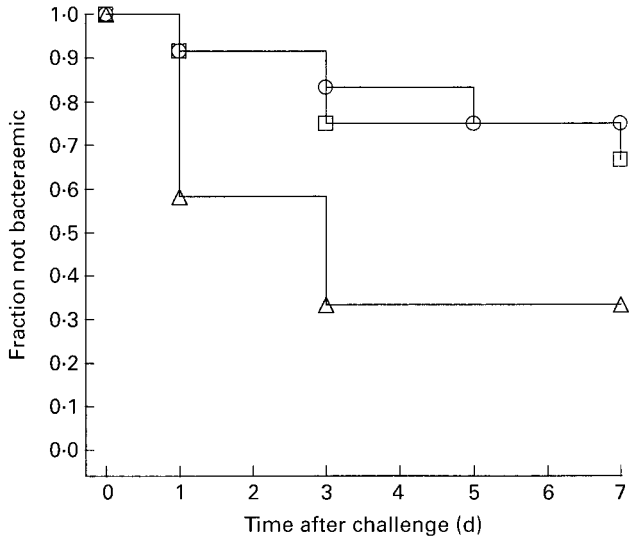

Fig. 1. Kaplan–Meyer plot of time (d) after pneumococcal challenge until acquisition of bacteraemia in mice fed diets with different concentrations of zinc. ○, Zinc-adequate diet, pair-fed; □, zinc-adequate diet, free access; △, low-zinc diet (*n* 12 per group). For details of diets and procedures, see p. 302. Hazard ratio between mice receiving a diet with low-zinc concentration and pair-fed mice receiving a zinc-adequate diet 3·2, $P = 0{\cdot}045$.

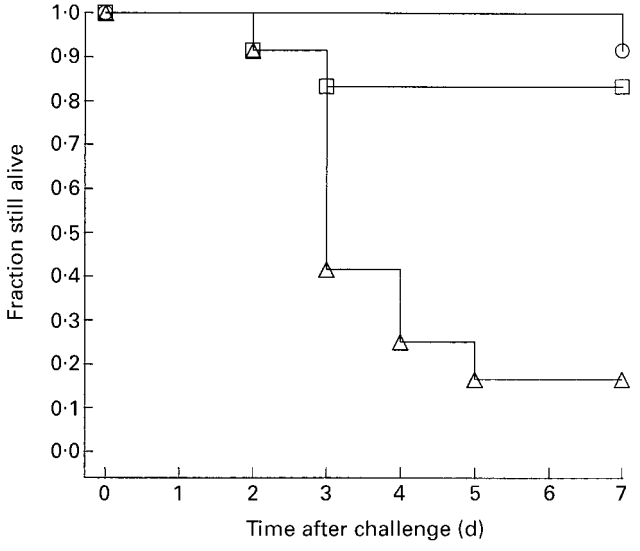

Fig. 2. Kaplan–Meyer plot of survival after pneumococcal challenge in mice fed diets with different concentrations of zinc. ○, Zinc-adequate diet, pair-fed; □, zinc-adequate diet, free access; △, low-zinc diet (*n* 12 per group). For details of diets and procedures, see p. 302. Hazard ratio between mice receiving a diet with low-zinc concentration and pair-fed mice receiving a zinc-adequate diet 15·6, $P < 0{\cdot}0001$.

Table 2. No. of mice and colony forming units (cfu)/lung in three groups of experimentally fed mice testing positive for pneumococci after intranasal challenge with *Streptococcus pneumoniae**

(Mean values with their standard errors)

	n		$\log_{10}$ cfu/lung	
Group	Tested positive	Examined	Mean	SEM
Group 1: zinc depleted	6	8	6·8	0·4
Group 2: zinc-adequate, pair fed	3	12	4·4	0·8
Group 3: zinc-adequate, free access	2	12	5·1	2·2

* For details of diets and procedures, see p. 302.

acute lower respiratory infections in Zn-supplemented children (Sazawal *et al.* 1998). These findings have made Zn a candidate for routine supplementation and fortification of foods for susceptible populations (UNICEF, United Nations International Children's Emergency Fund, 1998). Before such programmes can be initiated, however, more information is needed on the effect of Zn deprivation and on the impact of Zn supplementation on health and disease (UNICEF, United Nations International Children's Emergency Fund, 1998).

Severe ZD increases the chance of a fatal outcome for many infectious diseases, as seen in the rare inherited disorder acrodermatitis enteropatica (Prasad, 1985; Walsh *et al.* 1994). Previous studies have defined severe ZD in mice by a >25 % difference in body weight, presence of parakeratosis of the ears and tail, redness around the anus, and diarrhoea (Walsh *et al.* 1994; King *et al.* 1995). These earlier studies also demonstrated reduced Zn content in the tissues of mice fed a low-Zn diet (King & Fraker, 1991; Walsh *et al.* 1994).

The aim of the present study was to evaluate the effect of acutely-induced ZD. Other mouse studies involving Zn depletion have used deprivation times of at least 4 weeks (King & Fraker, 1991; Walsh *et al.* 1994). In another mouse experiment where Zn deprivation lasted for 7 weeks, the reduced Zn intake was reflected in lower femur Zn content than observed in the present study (TA Strand, A Maage & H Sommerfelt, unpublished results). In this present experiment, however, there were no significant differences in the bone Zn content between the groups. This could be an indication that the mice were not severely Zn deficient, an interpretation which is in agreement with the fact that none of our mice had any of the signs of severe ZD mentioned earlier. On the other hand, the 14 d period of Zn deprivation may be too short to induce changes in bone Zn levels. Moreover, the Zn-deficient animals had a more severe infection during which Zn may have been displaced to bone tissue (Walsh *et al.* 1994), perhaps masking a low-Zn status.

There was a substantial and significant difference in the body weights of the Zn-depleted (group 1) and the Zn-replete (group 2 and 3) mice, that was detected 2 d after the experimental feeding started. ZD affects growth in two ways; by a direct effect on cell division, and by reduced appetite, anorexia being among the earliest signs of ZD. Malnutrition *per se* is known to have negative effects on the immune system (Cunningham Rundles, 1982). Therefore, by pair feeding we ensured that, except for Zn, the mice in group 2 had the same nutrient intake/g body weight as the group 1 mice. To increase the accuracy of the food consumption estimations, the average intake in group 2 mice of the past 2 d was used. This offset may theoretically have contributed to the weight differences. However, the mean body weight (Table 1) in the pair-fed group was between the Zn-deficient and the non-deficient, free access group, indicating that pair feeding was successful.

The strain used for infection was able to induce nasal carriage and lethal infection in Zn-adequate and Zn-depleted mice. Mice that did not die from the infection carried the strain when killed and no pneumococci were found in the nasal washes of the non-infected mice.

In the present study, the 14 d suboptimal Zn intake resulted in a striking increase in the fatality rate. The findings of positive lung and blood cultures in the infected mice strongly suggest that Zn depletion increased the

Table 3. Colony forming units (cfu)/100 μl nasal wash in three groups of experimentally-fed mice testing positive for pneumococci after challenge with *Streptococcus pneumoniae**

(Mean values with their standard errors)

	n		$\log_{10}$ cfu/100 μl	
Group	Carriers	Examined	Mean	SEM
Group 1: zinc depleted	2	2	4·0	0·2
Group 2: zinc adequate, pair fed	11	11	4·0	0·3
Group 3: zinc-adequate, free access	10	10	4·2	0·2
Control: zinc adequate, free access†	0	3	–	–

* For details of diets and procedures, see p. 302.
† Control: Zn-adequate mice given sterile PBS instead of pneumococcal suspension.

susceptibility to severe invasive pneumococcal infection and death. This is the first direct demonstration of increased severity of pneumococcal infection in Zn-depleted individuals.

Theoretically, repeated bleeding of the mice in group 1 could have affected their survival. However, five of the twelve mice in this group had pneumococci in the blood even on the first day after challenge as compared with one of twelve mice in each of the Zn-adequate groups. This rather striking difference was observed before any blood sampling had taken place. Moreover, none of the six non-infected Zn-depleted mice died in spite of undergoing the same blood sampling regimen. Therefore, the difference in survival could not be attributed to repeated bleeding.

There may be several reasons for the high fatality rate in the Zn-depleted mice, as Zn is essential for the normal function of many organ systems. Zn is crucial for cell division and regeneration (Walsh *et al.* 1994); the labile immune cells and cells found in epithelial linings are affected by ZD (Walsh *et al.* 1994). All of the mice with detectable bacteraemia early in the observational period died within 3 d of a positive blood culture. This short time span indicates that mechanisms other than, or in addition to, those mediated by antigen-specific immune responses may be responsible for the increased susceptibility to lethal pneumococcal infection in ZD. A compromised mucosal integrity in the respiratory tract could facilitate bacterial access to the bloodstream and lead to increased leakage of protein-rich fluid into the alveolar space, thereby enhancing pneumococcal multiplication. Earlier experiments of ZD, although using longer periods of deprivation, have shown reduced phagocytic activity and reduced spleen cellularity (Sugarman, 1983; Wirth *et al.* 1989; Keen & Gershwin, 1990; King & Fraker, 1991; Wellinghausen *et al.* 1997). The relative contribution of impaired innate and immunological defence mechanisms in ZD needs to be clarified.

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