Med Microbiol Immunol (1988) 177:255–263

Medical Microbiology and Immunology

Effect of protein and zinc deficiencies on vaccine efficacy in guinea pigs following pulmonary infection with *Listeria*

Lezlee Graham Coghlan[2,*], Mirta A. Carlomagno[1,**], and David N. McMurray[1,2]

[1] Departments of Medical Microbiology and Immunology, and
[2] Veterinary Public Health, Texas A & M University, College Station, TX 77843, USA

Abstract. Specific pathogen-free guinea pigs were maintained for 3 weeks on purified diets containing 30% protein (ovalbumin) and 50 ppm added zinc (Control-C), 10% protein and 50 ppm added zinc (low protein-LP), or 30% protein and no added zinc (low zinc-LZ). Half of the animals in each diet group were vaccinated intraperitoneally with 2.5×10^3 viable *Listeria monocytogenes* organisms after 8 days of diet treatment. Ten days later, all animals received an aerosol challenge of 250 *L. monocytogenes* organisms and were killed 4 days later. Both zinc and protein deficiency resulted in animals that were growth retarded as compared to controls. Specific nutrient effects were observed as significant reductions in total serum proteins (LP group) and plasma zinc concentrations (LZ group). In vaccinated guinea pigs, both protein and zinc deprivation resulted in significant impairment of delayed-type hypersensitivity (DTH) responses following the intradermal injection of listeria antigen. Diet did not exert a measurable impact on the response of nonvaccinated guinea pigs to pulmonary listeriosis. Prior vaccination allowed both malnourished groups to control the challenge infection successfully as measured by significant reductions in viable bacilli recovered from the lung, spleen and hilar lymph nodes. The diet and vaccine effect varied depending on the tissue examined. Thus, although both protein and zinc deficiencies resulted in loss of peripheral antigen-specific T lymphocyte function (DTH), vaccine efficacy was not impaired.

* *Present address:* University of Texas System Cancer Center, Veterinary Resources Division, Science Park, Bastrop, TX 78602, USA
** *Present address:* Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Rivadavia 1917, Buenos Aires, Argentina

Offprint requests to: D. N. McMurray

Introduction

Protein malnutrition has been associated with defects in both cellular and humoral immunity in humans and animals [1, 9, 14, 28]. Increased susceptibility to infectious disease has been demonstrated in protein-deficient humans and animals [9]. The most striking nutritionally induced aberrations are in cell-mediated immunity [4, 9, 14]. Impairment of delayed-type hypersensitivity (DTH) and in vitro lymphocyte blastogenesis have been almost universally demonstrable in both protein and protein-calorie malnutrition (PCM) [4, 15, 17]. However, some studies have revealed cellular immune enhancement secondary to PCM or protein deprivation. Protein-deficient mice have demonstrated improved peritoneal macrophage phagocytosis of *Listeria monocytogenes* [23]. Acutely starved mice were more resistant to a lethal dose of *L. monocytogenes,* presumably through non-specific activation of macrophages [30]. Superoxide dismutase levels were increased in macrophages harvested from protein-deficient guinea pigs [6]. Protein malnutrition did not influcence Fc-receptor-mediated phagocytosis by alveolar macrophages in tuberculous guinea pigs [19].

Contradictory effects of micronutrients on the cell-mediated immune (CMI) system also have been observed. Zinc deficiency resulted in pronounced depression of thymic-dependent immunological function both in vivo and in vitro [14, 28]. Zinc-deficient animals demonstrated impaired T cell functions, reduced lymphocyte mitogenesis and impaired wound healing [1, 6]. Zinc-deficient guinea pigs controlled infection with an attenuated strain of *Mycobacterium tuberculosis* as well as control animals, although other parameters of cellular immune function were reduced [5, 17].

Few of the previous investigations concerning the effects of nutrition on CMI have included response to primary infectious challenge [24], and even fewer have examined the effect of malnutrition on vaccine efficacy, which is defined as a significant increase in resistance to challenge infection [3, 18, 19]. In this study, the separate effects of protein and zinc deficiencies on cellular immunity and resistance to *L. monocytogenes* in guinea pigs were assessed in vaccinated and non-vaccinated animals.

Materials and methods

Experimental Animals

Juvenile (150–200 g) female outbred albino specific pathogen-free guinea pigs [Hartley-COBS; Crl:(HA)BR] were purchased from Charles River Laboratories (Wilmington, Mass). Animals were housed individually in polycarbonate cages with stainless steel grid floors and feeders, under constant temperature (22.5 °C), relative humidity (50%), and alternating 12-h light/dark cycles. Essentially zinc-free demineralized water was provided *ad libitum*. Body weights were recorded weekly during the experiment.

Diets

Experimental diets (Dyets, Bethlehem, Pa) were isocaloric and met the dietary requirements of the juvenile guinea pig [22], except for the nutrients noted below. The low zinc/high protein diet (LZ) contained 300 g/kg (30%) ovalbumin as the protein source and less than 5 ppm Zn. The low protein/adequate zinc diet (LP) contained 100 g/kg (10%) ovalbumin and 50 ppm zinc. The control diet (C) contained 300 g/kg (30%) ovalbumin and 50 ppm zinc. The diets were biotin-fortified, and zinc was added as $ZnCO_3$ to the otherwise zinc-free salt mix. The exact composition of the diets was reported previously [15]. Animals were adapted to the purified diets by giving them a mixture of powdered commercial guinea pig diet (Wayne Feed Co; Tomball, Tex) and control (C) diet, with the proportion of control diet increasing from 50% to 100% over an 8 day period. After adaptation, each animal was assigned randomly to one of the three experimental diets which was provided fresh every other day.

Bacterial cultures

The EGD strain of *L. monocytogenes* (Trudeau Institute, Saranac Lake, NY), was kindly provided by Dr. B. G. Foster, Texas A & M University. Stock suspensions were prepared as previously described [11] and stored at −20 °C. For experimental use, a sample was thawed and cultured in brain-heart infusion (BHI) broth in a shaker/incubator at 100 rpm. After 18–20 h at 37 °C, dilutions were prepared to vaccinate or infect experimental animals. Viable unit counts were determined by inoculating appropriate dilutions on trypticase soy agar (TSA) plates and incubating for 2–3 days at 37 °C.

Vaccination and aerosol infection

Half of the animals in each dietary group were vaccinated intraperitoneally with 0.5 ml containing approximately 2.5×10^3 viable bacteria eight days following initiation of the experimental diets. An infection chamber described previously [29] was used to challenge all guinea pigs by the respiratory route with *L. monocytogenes* 10 days post-vaccination. Up to 18 animals, selected randomly from all experimental treatments, were infected simultaneously. Previous data indicate that the infective dose received by each animal is irrespective of chamber position [17, 18, 29]. The challenge culture was adjusted empirically so that each animal inhaled and retained approximately 250 viable *L. monocytogenes* as droplet nuclei.

Skin test antigen

Soluble Listeria antigen was prepared by the method of Fulton et al. [7]. BHI broth, supplemented with glucose and casamino acids, was inoculated with the stock culture of *L. monocytogenes*, and incubated in a rotary shaker for 48 h at 37 °C and 100 rpm. The culture was centrifuged at 10,500 *g* for 30 min, the supernatant was saturated

with 75% $(NH_4)_2SO_4$ and centrifuged at 19,700 *g* for 40 min. The precipitate was dissolved in 50 ml Tris-HCl (0.01 M, pH 7.3) and the protein content was determined [12]. The Tris-dissolved precipitate was lyophilized, re-dissolved in tissue culture medium (RPMI 1640, K. C. Biological, Lenexa, Kan) to a final concentration of 250 mg protein/ml, sterilized with a 0.45-μm filter (Nalge, Rochester, NY), fractionated into 20-ml aliquots, and stored at −20 °C.

Skin tests

Each guinea pig was injected intradermally with 25 μg of *L. monocytogenes* antigen in 0.1 ml. Cutaneous reactivity was measured 24 h later as mean diameter of induration, as well as uptake of tritiated thymidine into the skin injection site [10]. All animals received 40 μci of tritiated thymidine (^{3}HTdR) subcutaneously 24 h prior to skin testing. A biopsy punch was used to obtain a uniform skin sample from the test site and a contralateral control site. These tissues were dissolved in 1–2 mls of tetramethylammonium hydroxide (Sigma, St. Louis, Mo) for 24 h at 40 °C. The fluid was mixed with scintillation cocktail, incubated in darkness at 4 °C for 24 h, and counted in a liquid scintillation counter (LS 8000, Beckman Instruments, Irvine, CA). DTH indexes were calculated as counts/min (cpm) of antigen-injected skin over cpm of placebo-injected skin.

Organ bacterial assays

Four days following respiratory challenge, all animals were killed by cervical dislocation and bled immediately by cardiac puncture. Tissue samples (spleen, left caudal lung lobe, and hilar lymph nodes) were collected aseptically, weighed, and placed immediately on ice. Tissues were processed separately in sterile Teflon-glass homogenizers in 4.5 ml physiological saline. The number of viable *L. monocytogenes* in each tissue was determined by colony counts of appropriate dilutions on TSA plates, and expressed as $\log_{10}$/gram (wet weight) tissue.

Total serum protein levels were determined by the method of Lowry [12]. Plasma zinc concentrations were measured by atomic absorption spectrophotometry (Perkin-Elmer, Model 303).

Statistical analysis

All experiments were factorially designed and followed the principles of randomization and proper replication. Analysis of variance was used to test for treatment effects, and Duncan's new multiple range test [27] was employed to determine reliable differences between means. The confidence level was set at 95%.

Table 1. Influence of diet on growth, plasma zinc and total protein concentrations in vaccinated and non-vaccinated guinea pigs challenged with *Listeria monocytogenes*

Dietary treatment	Weight change[a] (g)	Total serum proteins (g/100 ml)	Plasma Zn (μg/ml)
Low zinc (LZ)	+13 ± 4[b]	4.65 ± 0.40[b]	0.39 ± 0.05[b]
Low protein (LP)	−16 ± 3[c]	3.50 ± 0.30[c]	0.59 ± 0.08[c]
Control (C)	+43 ± 6[d]	4.95 ± 0.35[b]	0.76 ± 0.04[d]

[a] After 3 weeks on experimental diets; all values respresent mean ± SEM of 10–14 animals
[b, c, d] Means in each vertical column with the same superscript letter are not significantly different ($P > 0.05$)

Results

Effect of diets on nutritional status

Diet had a significant effect on weight gain (Table 1). No significant differences in weight change were observed between vaccinated and non-vaccinated animals within the same dietary group, so these data are pooled for each diet treatment. Protein-deficient (LP) animals lost weight during the 3-week period. In the LZ group, animals showed slight weight gains, which were significantly less than the controls, which grew steadily.

Table 1 shows that the LP animals had significantly lower total serum protein values than controls or zinc-deprived guinea pigs, which were not different from each other.

Plasma zinc levels were significantly lower in both experimental diet groups when compared to controls (Table 1). Zinc-deficient guinea pigs exhibited the lowest concentrations, with the protein-deprived group falling between the LZ and control groups.

Effect of diet and vaccination on pulmonary listeriosis

Figure 1 illustrates that the numbers of viable *L. monocytogenes* recovered from the lung (panel A), spleen (panel B) and hilar lymph nodes (panel C) four days after aerosol exposure were all affected by vaccination 10 days prior to respiratory challenge. In the lung, previous immunization resulted in significant protection for protein malnourished (LP) and control (C) animals, but not for the guinea pigs deprived of dietary zinc (LZ). In the spleen, bacterial loads were significantly reduced in vaccinated LZ and LP animals, while the control (C) guinea pigs exhibited no beneficial vaccine effect. In these tissues there was no effect of diet per se on levels of viable listeria in either vaccinated or non-vaccinated animals. The hilar lymph nodes draining the lungs of zinc-deficient and control guinea pigs were protected by previous vaccination, while the nodes of protein-deprived, vaccinated animals harbored as many viable bacilli as those of their non-vaccinated counterparts.

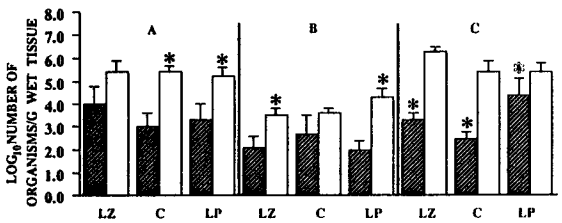

Fig. 1. Effect of diet and vaccination on number of viable *L. monocytogenes* organisms in the lung (*panel A*), spleen (*panel B*) and hilar lymph nodes (*panel C*) 4 days after respiratory challenge in animals maintained on low zinc (*LZ*), low protein (*LP*), or control (*C*) diets for 3 weeks. *Textured bars* indicate vaccinated animals; *open star* indicates significant dietary effect; *solid star* indicates a significant difference due to vaccination ($P < 0.05$). Mean ± SEM of 5–7 observations

Based upon the degree of protection, calculated as the $\log_{10}$ reduction in viable bacterial load achieved by prior vaccination, the zinc-deficient (LZ) guinea pigs expressed protection in the spleen and lymph nodes (1.4 and 3.0 reduction, respectively), while vaccinated LP animals reduced the $\log_{10}$ number of viable listeria by 1.9 and 2.2 in the lung and spleen, respectively.

Delayed hypersensitivity to listeria antigen

Based upon the mean diameter of induration at 24 h (Fig. 2, panel A), both protein- and zinc-deficient, vaccinated guinea pigs developed significantly smaller cutaneous reactions than vaccinated control animals. Diet did not affect the responses of non-vaccinated animals. When the uptake of ^{3}HTdR into the skin test site was quantified as the DTH index (Fig. 2, panel B), only the zinc-deficient guinea pigs, irrespective of vaccination status, were found to have significantly impaired dermal responses. Neither diet nor vaccination influenced the DTH index of the control or protein malnourished groups.

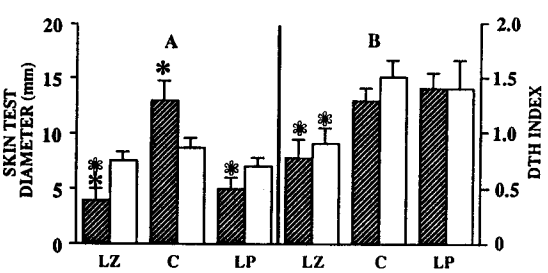

Fig. 2. Skin test diameter (*panel A*) and delayed-type hypersensitivity (DTH) index (*panel B*) following intradermal injection of listeria antigen for animals maintained on a low zinc (*LZ*), low protein (*LP*), or control (*C*) diet for 22 days; *textured bars* indicate vaccinated animals; *open star* indicates significant dietary effect; *solid star* indicates a significant difference due to vaccination ($P < 0.05$). Mean ± SEM of 5–7 observations

Discussion

Juvenile guinea pigs maintained on purified LP or LZ diets for 3 weeks developed clinical syndromes characteristic of their respective dietary deficiency (Table 1). In contrast, animals maintained on the high-protein/zinc-adequate (C) diet demonstrated growth throughout the experiment. Animals maintained on the LP diet suffered the most severe weight losses, were depleted of body fat stores in the coronary grooves and mesentery, and had livers grossly characteristic of fatty infiltration, all indicators of chronic protein deficiency [26]. Some LP animals developed ascites which resulted in the pot-bellied appearance typical of human kwashiorkor [26].

Guinea pigs maintained on LZ diet did not grow normally despite their diet being adequate in all other known nutrients [22]. Growth failure is a common manifestation of zinc deficiency in many species and has been related to decreased food intake secondary to impaired taste acuity [21]. Other characteristics of zinc deficiency observed in LZ animals included patchy alopecia over the caudal back, rump, and extremities. Alopecia has been documented in experimental zinc deficiency in other species [21].

Serum protein levels of LP animals were reflective of the dietary protein content (Table 1) and confirmed previous results with this model [17]. Serum zinc levels were lowest in LZ animals, but LP guinea pigs also demonstrated serum zinc concentrations that were significantly below control levels (Table 1). Secondary zinc deficiency in guinea pigs maintained on the LP diet has been reported previously [17].

The development of resistance to *L. monocytogenes* is dependent upon the bactericidal activity of macrophages activated by lymphokines released from antigen-specific T cells [13, 20]. Alternate mechanisms of macrophage activation have been shown to be protective against *L. monocytogenes* [25]. Wing and his colleagues [30] attributed enhanced resistance to *L. monocytogenes* acutely starved mice to non-specifically activated macrophages.

Resistance to primary aerosol challenge with virulent *Listeria monocytogenes* was essentially unaffected by diet in non-vaccinated guinea pigs (Fig. 1). Vaccinated, malnourished animals controlled accumulation of viable bacilli following respiratory infection. Vaccine efficacy in the nutrient-deficient guinea pig varied depending upon the organ studied. In the LP group, a significant vaccine effect was observed in the lung and spleen, but not in the hilar lymph nodes. Conversely, LZ guinea pigs exhibited vaccine-induced protection in the spleen and lymph nodes, but not in the lung (Fig. 1). These results are similar to those reported in a previous study of resistance to listeriosis in zinc-deficient rats [3].

Studies in mice and rats have linked the development of acquired cellular resistance to *L. monocytogenes* to the host's ability to mount a DTH response [13]. In the guinea pig model, acquired cellular resistance develops prior to DTH response to *L. monocytogenes* [7]. We have observed effective cellular resistance even in anergic guinea pigs harboring either attenuated *M. tuberculosis* H37Ra [17] or *M. bovis* BCG [15]. Cutaneous reactivity to listerin was significantly reduced in both LP and LZ vaccinates when compared to controls (Fig. 2). Vaccinated animals usually had smaller DTH reactions than non-vaccinated animals consuming the same diet, perhaps due to reduced bacterial (and antigenic) loads. Only control animals demonstrated larger skin reactions when vaccinated. Previous investigators [7] reported that peak DTH responses in *L. mono-*

cytogenes-infected guinea pigs occurred 7 days post-infection. Sensitized animals in this experiment were tested 12 to 16 days after vaccination when DTH may be declining. Non-vaccinates had only 4 days to develop cutaneous sensitivity to listerin, but for these animals DTH reactions should have been approaching peak activity. These results support previous findings of reduced cutaneous reactivity in both protein- and zinc-deficient animals [15, 17, 18]. Children with protein-energy malnutrition and secondary zinc-deficiency demonstrated stronger skin test reactions when zinc sulfate ointment was applied to the skin test site [8].

The measurement of induration revealed a significant impairment of DTH in LP guinea pigs, while the uptake of ^{3}HTdR by the skin test site did not (Fig. 2). Both measurements should reflect the accumulation of infiltrating cells at the reaction site. The disparity in the LP animals may have resulted from a normal cellular infiltrate which failed to produce visible induration due to loss of non-specific inflammatory phenomena [2].

We have demonstrated that impairment of a commonly employed assay of cellular immune function (i.e., DTH) may not accurately reflect host antimicrobial defense status. A deficiency of either protein or zinc resulted in guinea pigs that were compromised both biochemically and physically, as well as not responding normally to DTH skin tests. On the other hand, both LP and LZ groups resisted a primary challenge with aerosolized *L. monocytogenes* as well as control animals. Most importantly, both vaccinated, nutrient-deprived groups derived a protective benefit from vaccination which was equal to or greater than well-nourished guinea pigs, depending upon the organ studied.

Acknowledgements. This research was supported, in part, by Public Health Service Grant AI-15495 from the National Institutes of Health. Portions of this study were submitted by L. G. Coghlan in fulfillment of the requirements for the M. S. degree at Texas A & M University. The authors wish to thank Jane Lantz for secretarial support.

References

1. Beisel WR (1982) Single nutrients and immunity. Am J Clin Nutr 25:417–468
2. Bhaskaram P, Reddy V (1982) Cutaneous inflammatory response in Kwashiorkor. Indian J Med Res 76:849–853
3. Carlomagno MA, Coghlan LG, McMurray DN (1986) Chronic zinc deficiency and listeriosis in rats: acquired cellular resistance and response to vaccination. Med Microbiol Immunol 175:271–280
4. Edelman R, Suskind R, Olson RE, Sirishinha S (1973) Mechanisms of defective delayed cutaneous hypersensitivity in children with protein-calorie malnutrition. Lancet I:506–509
5. Fernandes G, Nair M, Onoe K, Tanaka T, Floyd R, Good RA (1979) Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. Proc Natl Acad Sci USA 76: 457–461
6. Fraker PJ , Zwickl CM, Leucke RW (1982) Delayed-type hypersensitivity in zinc-deficient adult mice: impairment and restoration of responsivity to dinitrofluorobenzene. J Nutr 112: 309–313
7. Fulton AM, Dustoor MM, Kasinski JE, Blazkovec AA (1975) Blastogenesis as an in vitro correlate of delayed hypersensitivity in guinea pigs infected with *Listeria monocytogenes.* Infect Immun 12:647–655

8. Golden MHN, Harland PSEG, Golden BE, Jackson AA (1978) Zinc and immunocompetence in protein-energy malnutrition. Lancet I:1226–1228
9. Keusch GT, Wilson CS, Waksal SD (1983) Nutrition, host defenses, and the lymphoid system. In: Gallin JI, Fauci AS (eds) Advances in host defense mechanisms, vol 2. Raven Press, New York, pp 275–359
10. Kostiala AAI (1977) Radiometric ear index test as a measure of delayed-type hypersensitivity in the rat. Immunology 33:561–571
11. Lane FC, Unanue ER (1972) Requirement of thymus (T) lymphocytes for resistance to listeriosis. J Exp Med 135:1104–1111
12. Lowry O, Rosebrough N, Farr A, Randall R (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
13. Mackaness GB (1969) The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J Exp Med 129:973–992
14. McMurray DN (1984) Cell-mediated immunity in nutritional deficiency. Prog Food Nutr Sci 8:193–228
15. McMurray DN, Yetley EA (1983) Response to *Mycobacterium bovis* BCG vaccination in protein- and zinc-deficient guinea pigs. Infect Immun 39:755–761
16. McMurray DN, Yetley EA, Burch T (1981) Effects of malnutrition and BCG vaccination on macrophage activation in guinea pigs. Nutr Res 1:373–384
17. McMurray DN, Carlomagno MA, Cumberland PA (1983) Respiratory infection with attenuated *Mycobacterium tuberculosis* H37Ra in malnourished guinea pigs. Infect Immun 39:793–799
18. McMurray DN, Carlomagno MA, Mintzer CL, Tetzlaff CL (1985) *Mycobacterium bovis* BCG vaccine fails to protect protein-deficient guinea pigs against respiratory challenge with virulent *Mycobacterium tuberculosis*. Infect Immun 50:555–559
19. McMurray DN, Kimball MS, Tetzlaff CL, Mintzer CL (1986) Effects of protein deprivation and BCG vaccination on alveolar macrophage function in pulmonary tuberculosis. Am Rev Respir Dis 133:1081–1085
20. Miki K, Mackaness GB (1964) The passive transfer of acquired resistance to *Listeria monocytogenes*. J Exp Med 120:93–104
21. Miller WJ, Pitts WT, Clifton CM (1964) Experimentally produced zinc deficiency in the goat. J Dairy Science 47:556–559
22. Navia JM, Hunt CE (1976) Nutrition, nutritional diseases and nutrition research applications. In: Wagner JE, Manning PJ (eds) The biology of the guinea pig. Academic Press, New York, pp 235–267
23. Newberne PM, Williams G (1970) Nutritional influences on the course of infection. In: Dunlow RH, Moon HW (eds) Resistance to infectious diseases. Saskatoon Modern Press, University of Saskatchewan, Saskatoon, pp 68–87
25. Petro TM, Watson RR (1982) Dietary stress and development of resistance to *Listeria monocytogenes* in mice. J Nutr 112:1498–1505
25. Ruskin J, McIntosh J, Remington JS (1969) Studies on the mechanisms of resistance to phylogenetically diverse intracellular organisms. J Immunol 103:252–259
26. Smith HA, Jones TC, Hunt RD (1972) Veterinary pathology. Lea and Febiger, Philadelphia, pp 981–1023
27. Steel RGD, Torrie JH (1980) Principles and procedures of statistics. McGraw-Hill New York, pp 213–224
28. Watson RR, McMurray DN (1979) The effects of malnutrition on secretory and cellular immune processes. Crit Rev Food Sci Nutr 12:113–159
29. Wiegeshaus EH, McMurray DN, Grover AA, Harding GE, Smith DW (1970) Host-parasite relationships in experimental airborne tuberculosis. III. Relevance of microbial enumeration to acquired resistance in guinea pigs. Am Rev Respir Dis 102:422–429
30. Wing EJ, Barczynski LK, Boehmer SM (1983) Effect of acute nutritional deprivation on immune function in mice. I. Macrophages. Immunology 48:543–550

Received February 1, 1988