Sendai Virus Infection of Mice with Protein Malnutrition

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Sendai virus pneumonia was produced in BALB/c mice fed protein-deficient diets in an effort to understand the severity of viral pneumonia in infants in developing countries. Animals on the deficient diet became clinically malnourished, and some aspects of cellular immunity were altered. In protein-deprived animals, the 50% lethal dose of intranasally administered Sendai virus was over 1,000-fold lower, pulmonary virus titers were higher, the infection was prolonged, and lung infection was established at a lower inoculum than in normal animals.

Acute respiratory infections are the leading cause of morbidity and mortality in children under 5 years of age in many developing countries (8, 18; Editorial, *Lancet* ii: 699-701). Malnutrition probably plays some role in this peculiar susceptibility (11, 14, 17). The mechanism of this effect is obscure. There is a widespread assumption that the major reason for the high mortality is an increased incidence of severe bacterial pneumonia (15). There is, however, little or no information about the severity of pure viral respiratory infection in this group.

Because there have been few systematic investigations of respiratory viral infections in malnourished animals (13, 16), we decided to examine a model of protein malnutrition in the BALB/c mouse and chose Sendai virus as the respiratory pathogen.

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Female BALB/c mice 21 days old were purchased from Charles River Breeding Laboratories, Inc., Kingston, N.Y. They were free of antibodies to Sendai virus and other mouse pathogens. On their arrival at the research facility, animals were weighed and placed in groups of five in autoclaved cages with filter tops (Microisolator cages; Lab Products).

Purified, defined rodent diets were acquired from Zeigler Bros. Inc. The normal diet (1) had 20% protein based on casein, contained 4,000 Kcal/kg of body weight, and was complemented with DL-methionine. Diets containing 4, 3, and 2% protein were supplemented with starch to maintain equivalent caloric contents. Diets and water were offered ad libitum, and consumption was measured by weighing the food in each cage when first offered and 22 to 26 h afterwards. Total serum protein was quantified by the biuret reaction (5). Albumin was analyzed by the method of Doumas et al. (3).

To measure delayed-type hypersensitivity, animals were contact sensitized to 2,4,6-trinitro-1-chlorobenzene (TNCB; picryl chloride; BDH, Poole, England; the generous gift of Martin Dorf, Harvard Medical School) by the single cutaneous application of 0.1 ml of 5% solution in acetone (7, 10). The mice were challenged 5 days later by 0.01 ml of 1% TNCB in acetone on the dorsal and ventral surfaces of the ear. Control animals received acetone alone. After 24 h, the

thicknesses of both ears were measured in millimeters with a caliper.

Two weeks after the institution of diets, splenocytes removed from five animals in each group were cultured at 5×10^5 cells per well in 96-well U-bottomed plates (Nunc, Roskilde, Denmark) in a final 200- μ l volume of RPMI 1640 supplemented with 1% normal mouse serum, antibiotics, L-glutamine, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer, and 5×10^5 M 2-mercaptoethanol. Triplicate cultures were established from individual mice against medium alone or a panel of antigens or mitogens at various doses, including concanavalin A (0.1 mg/ml; Miles Yeda), lipopolysaccharide (1.0% Difco Laboratories, Detroit, Mich.), and irradiated allogeneic cells [(B6 $\times$ D2)F₁, at 10^5 per well]. After 6 days, each well was pulsed with 1 μ Ci of [³H]deoxythymidine (Dupont, NEN Research Products, Boston, Mass.). Approximately 18 h later, the plates were processed for liquid scintillation counting.

Sendai virus (American Tissue Culture Collection, Rockville, Md.) was grown in MA104 cells (Whittaker, M.A. Bioproducts). Mice were inoculated intranasally with doses of 1.2×10^6 to 1.15×10^5 PFU per animal in a volume of 25 μ l after light intraperitoneal pentobarbital anesthesia. After infection, the animals were kept in groups of five in filter-top cages (Microisolator cages; Lab Products). On chosen days, animals were sacrificed, and the lungs were removed and prepared separately as 10% (wt/vol) suspensions. MA104 cells in 24-well plates were inoculated with 100 μ l of 10-fold dilutions of individual lung homogenates. Methyl cellulose (1.5%; Mallinckrodt, Inc., St. Louis, Mo.) mixed 1:1 with minimum essential medium without fetal bovine serum plus trypsin (0.5 μ g/ml; Calbiochem-Behring, La Jolla, Calif.) was used as an overlay. After 48 h of incubation, cells were fixed with 10% formaldehyde and stained with crystal violet. Plaques were counted under a dissecting microscope.

Malnutrition model. The body weights of animals receiving different diets are shown in Fig. 1. We chose the 2% protein diet administered for 2 weeks prior to viral infection for further experiments. Interestingly, the 20% protein diet group initially ate more than the malnourished group (180 versus 120 mg/g of body weight per day), but this decreased during the 2-week period. In contrast, the amount eaten by mice on the 2% protein diet increased until at the end of the 2-week period, the positions were reversed (120 versus 155 mg/g). The body lengths of the mice 2 weeks after starting their diet illustrate the delay in their growth. In the 20 and

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FIG. 1. Body weight changes in BALB/c mice on different diets. Female mice, 21 days of age, received four different diets. All diets had the same contents of calories and nutrients with the exception of protein. The mice were weighed separately every day. Each point plotted is the mean of the weights of 10 mice per diet expressed as a percentage of the initial weight.

2% protein groups (59 mice per group) the body lengths were 8.29 ± 0.19 and 7.46 ± 0.20 cm (mean $\pm$ standard deviation), respectively (paired *t* test, two tail; $P < 0.001$). Analysis of the total serum protein and albumin levels after 2 weeks also showed significant differences between the same two groups (18 mice per group). The serum protein levels were (mean $\pm$ standard deviation) 4.40 ± 0.28 and 3.78 ± 0.37 g/100 ml, respectively; serum albumin levels were 2.34 ± 0.17 and 1.97 ± 0.23 g/100 ml, respectively (both comparisons by paired *t* test, two tail; $P < 0.001$). Edema of the extremities was not seen. Except for some mild hyperexcitability, the animals appeared otherwise normal. Thus, the model avoided the pitfall seen by others, that animals on deficient diets eat less and less and become emaciated (6, 9). At the same time, depressed serum protein and albumin levels mimicked the findings in kwashiorkor.

Immunologic studies. In a measurement of delayed-type hypersensitivity 24 h after TNCB challenge on clipped chest or abdomen, all mice on the normal diet showed inflammation, swelling, and stiffness of the sensitized ear in comparison with the control ear. Ear thicknesses of acetone-treated controls (10 mice) and of the TNCB challenge group (18 mice) were (mean $\pm$ standard deviation) 0.022 ± 0.002 and 0.055 ± 0.004 mm, respectively (paired *t* test, two tail; $P < 0.001$). In contrast, in the malnourished mice there were no measurable differences between the ear thicknesses. Ear thicknesses of acetone-treated controls (10 mice) and of the TNCB challenge group (18 mice) were (mean $\pm$ standard deviation) 0.022 ± 0.004 and 0.026 ± 0.004 mm, respectively (paired *t* test, two tail; insignificant). This demonstrated an absence of contact sensitivity in the malnourished group.

In vitro stimulation of splenocytes with various B- and T-cell mitogens demonstrated a mixed pattern of proliferative responses (Fig. 2). Splenocytes from the malnourished mice proliferated more than those of the control group in response to lipopolysaccharide (a B-cell mitogen) and allogeneic cells (a specific T-cell stimulus), but a similar or slightly poorer proliferative response to concanavalin A (a

FIG. 2. Proliferation of splenocytes of BALB/c mice 2 weeks after institution of diets. Five animals in each diet group were tested individually following 2 weeks of conditioning. Splenocytes were stimulated in triplicate with media or media supplemented with concanavalin A (CON-A; 0.1 mg/ml), lipopolysaccharide (LPS; 1.0%), or alloantigen [ALLO; 10^5 irradiated (B6 $\times$ D2) F_1 splenocytes]. Means and standard errors of means from five individuals were calculated at each datum point. Background incorporation of splenocytes with media was subtracted from results for stimulated groups.

nonspecific T-cell mitogen) was observed. All the malnourished animals had grossly atrophic spleens and thymuses, and recovery of mononuclear cells from these organs was approximately 25% that from the age-matched control animals (data not shown).

Sendai virus infection. On day 2 after intranasal inoculation with a high dose of virus (1.2×10^4 PFU per animal), the mice appeared lethargic, with dull fur and evident dyspnea.

FIG. 3. Survival of BALB/c mice after intranasal infection with Sendai virus. Kaplan-Meier plot of mortality after high-dose inoculum (1.2×10^4 PFU per animal). Four animals were sacrificed each day for viral titrations, and one animal from each group was sacrificed for histology 7 days after infection.

TABLE 1. Mortality of control and malnourished BALB/c mice after intranasal inoculation with Sendai virus

Virus inoculum (PFU)	No. of inoculated mice dead after (<i>n</i> = 5):			
	10 days		20 days	
	Normal diet (20% protein)	Deficient diet (2% protein)	Normal diet (20% protein)	Deficient diet (2% protein)
1.2×10^0	0	0	0	0
1.2×10^1	0	0	0	0
1.2×10^2	0	2	0	5
1.2×10^3	0	5	0	5
1.2×10^4	1	5	1	5
1.2×10^5	1	5	1	5

All signs were more marked in the malnourished group. By day 7, animals started to die in both groups. The mortality in the malnourished mice on day 7 was 50%, in contrast to 2.2% in the normal group, and increased over the next few days (Fig. 3). All animals but one in the malnourished group died before day 10.

Mortality was lower in both diet groups with smaller virus inocula. At each level except the lowest two, however, differences between the well-nourished and protein-deficient groups were seen (Table 1). The observed 50% lethal dose for the malnourished mice was more than 1,000-fold lower than in the normal mice.

Infectious virus in lung homogenates among the two groups was measured following inoculation with 1.2×10^4 or 1.2×10^2 PFU per animal (Fig. 4). In both instances, Sendai virus replicated to higher titers and for a longer time in members of the malnourished group. With the higher inoculum it was not possible to reach the point of virus clearance from the lungs of malnourished animals because of the high mortality in this group. At the lower inoculum, virus was cleared 4 days earlier in the control mice (day 6) than in the malnourished group (day 10).

Finally, we examined the susceptibility of the two groups of animals to low virus inocula. It appeared that malnourished animals could be infected with as little as 1.2 PFU per

FIG. 5. Susceptibility of BALB/c mice to intranasal inoculation of Sendai virus 2 weeks after institution of diets. The mice were sacrificed 3 days postinfection, and titers of the lung virus were determined. Each point represents the mean and the standard deviation of titrations in four mice.

animal, a level below that which would establish pulmonary infection in the well-nourished group (Fig. 5).

The histopathologic features of the pneumonia were also distinctly affected and will be the subject of a separate report.

It appears likely that perturbations in the immune status of the animals were to some extent responsible for the profound changes we observed. Regulatory cell changes may well be involved, but their nature is not clear. Although, as observed by others (2, 12), the thymus and spleen became atrophic and contact sensitivity disappeared, proliferation of splenocytes in response to mitogens and allogeneic cells was only slightly affected by the low-protein diet. Rather than depression of responses we saw an increase in proliferation after exposure to lipopolysaccharide (a nonspecific B-cell mitogen) or allogeneic cells (a specific T-cell stimulus).

FIG. 4. Viral recovery from BALB/c mouse lungs after intranasal infection with Sendai virus at 1.2×10^4 (A) and 1.2×10^2 (B) PFU per mouse. Inoculation was performed 2 weeks after institution of diets. Each point represents the mean and standard deviation for four animals, with the exception of the last point for malnourished mice inoculated at 1.2×10^4 PFU, for which only three animals were sacrificed.

Several observations suggest that malnutrition contributed both to a reduction in some of the beneficial effects of the immune system and to an increase in some of the harmful effects. The higher titers of virus in the lung and the delayed clearance of virus suggest an impairment in those portions of the immune system which promote recovery. Normal mice which succumbed in this model died at least a day after culturable virus disappeared from the lungs and with a marked mononuclear infiltrate, suggesting that pulmonary immunopathology may be part of the mechanism of death. Nude mice, in contrast, carry Sendai virus in the lungs for as long as 70 days without symptoms or mortality (4). In view of this fact, the lethal pneumonia seen in the mice on a 2% protein diet appears likely to have had, at least in part, an immunopathogenic origin.

This model offers an opportunity to study severe respiratory infection in malnourished hosts, to examine the separate contributions of viral and bacterial infection, and to dissect the various contributing components of the immune response.

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