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Long Term Subclinical Effects of Parainfluenza (SENDAI) Infection on Immune Cells of Aging Mice[1] (40198)

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Sendai virus, Myxovirus parainfluenza type 1, has been found in 44% of the animal colonies tested in our country (1). In spite of its high prevalence, studies of infections caused by it have been limited to the epidemiology of the disease (1–4) and to the lung pathology associated with it (5, 6). To date, no one has studied the long term effects of subclinical infection at the cellular level. There are two major reasons why such a study is of significant interest. The first is that publications from laboratories with infected animal colonies generally neglect to mention the viral infection. Thus, any effect Sendai virus may have on the parameters studied is not only overlooked but also is recorded as the expected norm for the experimental conditions employed, when the observed results might actually be measures of chronic viral infection. For example, in this report it is shown that 55 of the 63 indices tested [i.e. 7 weight, 31 cellular, 18 activity, and 7 autoimmune indices, (7)], were abnormal as late as 8 mo after the disappearance of clinical symptoms of Sendai infection and the cessation of acute deaths even though the mice appeared normal. The second reason for studying the long-term effects of a parainfluenza virus infection in an aging population is that these and related viruses are implicated in human diseases such as multiple sclerosis, systemic lupus erythematosus, Coombs' positive autoimmune hemolytic anemia, etc (for a review see Ref. 8). Indeed, it has been postulated that common respiratory viruses can initiate or accelerate autoimmune diseases in individuals with an extended deficit in T cell activity (8). The results reported here support this hypothesis in that they indicate that the long term sequelae of the viral infection including autoimmune disease were most severe in immunologically immature young, immunodepressed adult, and immunologically deficient old mice.

Materials and methods. Sendai infection was brought into the animal farm of the Gerontology Research Center of the National Institute on Aging, NIH, Baltimore, MD, in January, 1976, by a shipment of infected 5- to 6-week old mice from Bethesda, MD. None of the aging mice had detectable serum antibody titers to the virus prior to the infection, but subsequently had titers of 1:20–1:80 by complement fixation tests performed by Microbiological Associates, Bethesda, MD. Titers to other viruses did not change significantly during this time. At the height of the infection (2–3 weeks postinfection) the old mice appeared ill, but adult and middle-aged mice appeared normal. However, autopsies performed on randomly selected mice revealed pulmonary lesions typical of Sendai infection in mice and influenza infection in humans (5). The following strains, hybrids, and random bred mice were used in this study: $BC3F_1$, $B6D2F_1$, CBA/T6T6, CV1, C3H, C57B1, C57B1/6, and DBA/2. The age of mice when infection occurred ranged from *in utero* to 25 months. Young (<3 months), adult (3–7 months), middle-aged (10–12 months) and old (18–31 months) mice of each strain and hybrid were tested at 1, 2, 3, 5, 6, and 8 months postinfection. Only data obtained from normal mice which were not surgically manipulated are presented in the normative study reported here. Surgical manipulation involved complete thymectomy by controlled suction of mice at 4 weeks of age. Prior to infection, deaths from thymectomy occurred during the surgical procedure, but following Sendai infection, they occurred primarily between 1 and 14 days postoperatively, and to a lesser extent thereafter. Data on weight, cellular, activity, and autoimmune indices from thymectomized and thymectomized mice which were X-irradiated and re-

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constituted with bone marrow will be published separately. A total of 63 indices were determined on each mouse (i.e., 7 weight 31 cellular, 18 activity, and 7 autoimmune indices).

Experimentally determined weight indices included body (g), lymph node (mg), spleen (mg), and thymus weight (mg); calculated weight indices derived from experimental determinations included percent organ weight of body weight for lymph nodes, spleen, and thymus.

Cellular indices included the following experimental determinations: Cells per tissue, percent dead cells at 4° during routine processing for tissue culture, percent T cells, percent B cells, and percent dead cells after 30 min incubation at 37° in culture media with 5% bovine serum albumin for dispersed bone marrow, lymph node, spleen and thymic cells. The following indices were calculated from experimental determinations: cells per mg wet weight for lymph nodes, spleen and thymus; the number of B cells and the number of T cells for bone marrow, lymph nodes, spleen and thymus (9).

Activity assays included the ^{3}H-thymidine incorporation in counts per minute (cpm) of unstimulated background cultures (BKG), a phytohemagglutinin (PHA) dose-cpm response curve (0.03, 0.05, 0.10, 0.25, 0.5, 1.0, 2.0, and 3.0 μg PHA) of stimulated cultures from which the dose eliciting the peak response was determined, the (cpm) response of lipopolysaccharide (LPS) stimulated cultures of lymph node and spleen cells (9), and the number of spleen colony units (CFU-S) per 5×10^4 bone marrow cells (10, 11). The following indices were calculated from experimental determinations: $\log_{10} \text{cpm}_{PHA} - \log_{10} \text{cpm}_{BKG}$, $\log_{10} \text{cpm}_{LPS} - \log_{10} \text{cpm}_{BKG}$, $\log_{10} (\text{cpm}_{PHA} - \text{cpm}_{BKG})$, $\log_{10} (\text{cpm}_{LPS} - \text{cpm}_{BKG})$ for dispersed lymph node and spleen cells, and the total CFU in the femoral bone marrow (11).

Autoimmune indices included: IgM and IgG Coombs' titers, and the percent dead cells after a 30 min incubation at 37° in culture media containing 5% guinea pig complement for bone marrow, lymph node, spleen and thymus cells, and the hematocrit. Direct Coombs' titers were assessed with serially diluted goat antimouse IgG or goat anti-mouse IgM (Meloy).

Results and discussion. Following Sendai infection, mortality was high among untreated old mice and young adult mice that had been rendered T cell deficient by either thymectomy or thymectomy followed by irradiation and bone marrow reconstitution. Thus, for example, mice of the latter group had greater than 80% mortality following Sendai infection, whereas the mortality was 5–10% prior to infection. The death rate from thymectomy alone increased from approximately 5% prior to infection to greater than 30% following infection. Recipient mice for the bone marrow stem cell colony forming unit assay that received 850 R died of disseminated viral infection within 9 days; this was not the case prior to infection.

Particles closely resembling Sendai virus were observed electron microscopically in peripheral blood, spleen, and bone marrow cell suspensions from young mice that had been rendered T cell deficient and from old mice, but *not* from normal adult mice, as late as 8 months after the initial outbreak. Positive controls for these studies consisted of cell suspensions from uninfected mice housed at another institute which were incubated with Sendai virus (NIAID Research Resources Branch, Bethesda, MD). The results of the electron microscopy studies will be published elsewhere.

For a period of 8 months following the Sendai outbreak, three of the seven weight indices tested (i.e. body wt, thymus wt, and percent thymus wt of body wt) were significantly reduced relative to those of mice of comparable ages prior to infection ($P \leq 0.0002$; Table I). On the other hand, lymph node wt, percent lymph node wt of body wt, and percent spleen wt of body wt increased with increasing months following infection ($P \leq 0.0001$). These findings are consistent with chronic infection.

Of the 31 cellular indices tested, 26 of them were affected by Sendai infection (Table I). The following cellular indices decreased with increasing months post Sendai infection: cells per mg wet wt of thymus, lymph node, and spleen; thymus and spleen cellularity; and frequency of B cells in lymph nodes, spleen and bone marrow ($P \leq 0.001$). For example, the frequency of B cells in the spleen of 11 month old mice was 42 ± 3% prior to Sendai infection and 18 ± 3% postinfection. Similar

TABLE I. EFFECT OF SENDAI INFECTION ON ORGAN, CELLULAR, ACTIVITY, AND AUTOIMMUNE INDICES OF MICE OF 8 STRAINS AND HYBRIDS.[a,b]

Index	Relative change	*P* value (≤)	Index	Relative change	*P* value (≤)
Weight					
Body	Decrease	0.0002	B cells, spleen (%)	Decrease	0.0001
Thymus	Decrease	0.0001	B cells, bone marrow (%)	Decrease	0.03
Lymph node	Increase	0.0001	B cells, thymus ($\times 10^6$)	Not significant	
Spleen	Not significant		B cells, lymph node ($\times 10^6$)	Decrease	0.0001
Thymus wt (g)/body wt (g)	Decrease	0.0001	B cells, spleen ($\times 10^6$)	Decrease	0.001
Lymph node wt (g)/body wt (g)	Increase	0.0001	B cells, bone marrow ($\times 10^6$)	Not significant	
Spleen wt (g)/body wt (g)	Increase	0.0003	cells per mg wet wt, thymus	Decrease	0.0002
Cellular[c]			*Activity*[d,e]		
Thymus cellularity ($\times 10^6$)	Decrease	0.0001	$\log_{10}cpm_{PHA}-\log_{10}cpm_{BKG}$, lymph node	Decrease	0.0005
Lymph node cellularity ($\times 10^6$)	Not significant		$\log_{10}cpm_{PHA}-\log_{10}cpm_{BKG}$, spleen	Decrease	0.0001
Spleen cellularity ($\times 10^6$)	Decrease	0.0006	$\log_{10}cpm_{LPS}-\log_{10}cpm_{BKG}$, lymph node	Decrease	0.04
Bone marrow cellularity ($\times 10^6$)	Increase	0.0001	$\log_{10}cpm_{LPS}-\log_{10}cpm_{BKG}$, spleen	Not significant	
Dead cells, thymus (%) at 4°	Increase	0.0001	$\log_{10}$PHA, lymph node	Decrease	0.01
Dead cells, lymph nodes (%) at 4°	Increase	0.0001	$\log_{10}$PHA, spleen	Decrease	0.01
Dead cells, spleen (%) at 4°	Increase	0.0001	$\log_{10}$BKG, lymph node	Decrease	0.02
Dead cells, bone marrow (%) at 4°	Increase	0.0001	$\log_{10}$BKG, spleen	Decrease	0.02
Dead cells, thymus (%) at 37°	Not significant		$\log_{10}$LPS, lymph node	Decrease	0.04
Dead cells, lymph nodes (%) at 37°	Increase	0.0001	$\log_{10}$LPS, spleen	Decrease	0.05
Dead cells, spleen (%) at 37°	Increase	0.0001	PHA dose, lymph node	Decrease	0.05
Dead cells, bone marrow (%) at 37°	Increase	0.0001	PHA dose, spleen	Decrease	0.05
B cells, thymus (%)	Not significant		*Autoimmune*[f]		
B cells, lymph node (%)	Decrease	0.0001	Coombs' titer, IgG	Increase	0.01
			Coombs' titer, IgM	Increase	0.0001
			Dead cells, thymus (%) with complement	Not significant	
			Hematocrit	Decrease	0.06

[a] For statistical analysis, the general linear model procedure was used to determine the effect of time (mo) following Sendai infection on each index. The procedure uses the principal of least squares to fit a fixed-effects model to data and performs linear regression, analysis of variance, analysis of covariance, and partial correlation analysis.

[b] Young (1.5–2 months), adult (3–7 months), middle-aged (10–12 months) and old (18–21, 24–26, or 28–31 months) mice of each of 8 strains were tested prior to the onset of Sendai infection. Six to twelve mice were tested per age group when available. Following Sendai infection, 3–6 mice were tested in each age group, when available, at 1, 2, 3, 5, 6 and 8 months postinfection. Mice which were infected when they were less than 3 months of age are not included in this table and in Figs. 1 and 2. All assays were performed on each individual mouse.

[c] The data for the percent and number of T cells in the thymus, lymph nodes, spleen, and bone marrow are not presented because the high frequency of cell death of untreated T cells at 37° rendered the cytotoxicity assay invalid.

[d] cpm_{BKG} and BKG, counts per minute of unstimulated background culture; cpm_{LPS} and BKG, counts per minute of LPS-stimulated culture; cpm_{PHA}, counts per minute of PHA-stimulated cultures.

[e] The data for the CFU-S per 5×10^4 bone marrow cells and the total CFU-S per femur are not presented because the irradiated recipient test mice died before Day 9, the day of splenic CFU determination.

[f] The data for the percent dead lymph node, spleen, and bone marrow cells after incubation at 37° in medium with 5% guinea pig complement are not presented in this table because they are presented in Fig. 1.

results were obtained with other age groups. On the other hand, bone marrow cellularity, and the frequency of dead cells in all lymphoid tissues increased ($P \leq 0.001$; Fig. 1). The decrease in organ cellularity and frequency of B cells could be the result of the high frequency of cell death following infection and might account for some discrepancies in results between laboratories when Sendai is present in one of them. The frequency of T cells could not be determined because the high frequency of cell death rendered a cy-

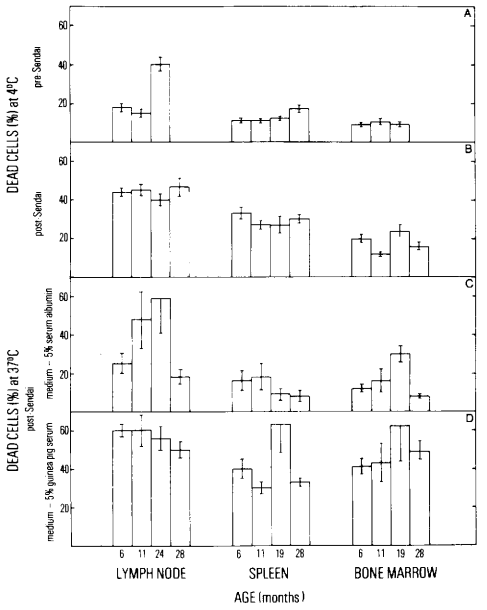

FIG. 1. Frequency of cell death pre- (A) and 6 mo post Sendai (B-D) infection at 4 and 37°. At 4°, the cells were incubated in medium for 30 min. At 37°, cells were incubated in medium with either 5% bovine serum albumin (C) or 5% guinea pig serum as a source of complement (D). Cell viability was determined by the trypan blue dye exclusion technique. Bars indicate SE of the mean. Data presented are composites of all mice examined.

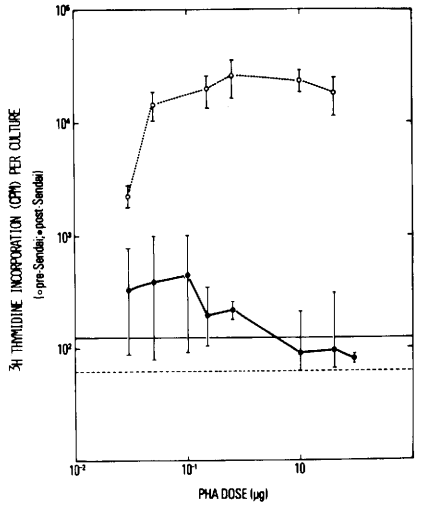

FIG. 2. Effect of Sendai infection on the PHA dose response relationship of adult $BC3F_1$ mouse lymph node cells. The horizontal dotted and solid lines represent background prior to and after infection, respectively. Comparable results were obtained with other strains and hybrids of mice and with other activity indices; e.g. the Spearman correlation coefficient for the PHA response of lymph node cells versus (a) the LPS response of lymph node cells is 0.64 ($P \leq 0.0005$) and (b) the PHA response of spleen cells is 0.45 ($P \leq 0.02$).

totoxicity assay invalid.

Seventeen of the activity indices tested were affected by the subclinical viral infection; e.g., the PHA dose-cpm response curve, culture background (BKG) and LPS response for lymph node and spleen cells (Table I). The PHA response decreased significantly following Sendai infection (Fig. 2). Colony forming units could not be assessed even as late as 6 months postinfection because all adult mice died of disseminated viral infection following X-irradiation.

Autoimmune indices increased following the infection. The IgM and IgG Coombs' titers increased with increasing months post Sendai and with increasing age (Table II) so that by 6 months postinfection, all old mice were Coombs' positive. Table II also shows that mice whose mean age was 7 months at 6 months postinfection were still Coombs' positive. This group consisted of mice which were infected within a month after birth when their T cell immunologic activities were not fully developed (7).

TABLE II. EFFECT OF SENDAI INFECTION ON IgG AND IgM COOMBS' TITERS.[a]

Age when tested (months)	Time after infection (months)			
	3		6	
	IgM	IgG	IgG	IgM
7	15 ± 5	6 ± 3	81 ± 15	6 ± 1
10–12	27 ± 7	20 ± 0	25 ± 9	2 ± 1
18–20	NT	NT	114 ± 22	17 ± 7
23	40 ± 18	15 ± 3	68 ± 11	12 ± 4

[a] Value expressed as the mean ± SE of the mean; sample size, 3–24 mice per group. NT, not tested. Data presented are composites of all mice examined.

The hematocrit of young mice ≤12 months of age remained normal even when the mice became Coombs' positive: the mean hematocrits ±95% confidence intervals of Coombs' negative and positive mice were 41.0 ± 1.9% and 40.0 ± 2.7%, respectively. However, the hematocrit of old mice ≥24 months (all of which were Coombs' positive at 6 months post-Sendai infection) decreased relative to that of young mice at 6 months postinfection

and of old mice which were Coombs' negative at 1 months postinfection; i.e., hematocrits of Coombs' negative old mice were 38.4 ± 2.1% and of Coombs' positive old mice were 33.1 ± 4.0%. Thus, hematocrit correlates with Coombs' positivity only in aged individuals.

It is possible that complement dependent cytotoxicity in the presence of autoantibodies to lymphoid cells was responsible for the high frequency of cell death following infection. The data indicating that the frequency of cell death at 4° is associated with IgM Coombs' titer ($P \leq 0.01$) and that cell death at 37° increases in the presence of complement (Fig. 1D) support this interpretation. However, a cytopathic effect of the virus can not be excluded.

The finding that 55 of the 63 indices tested were still affected 8 months postinfection indicates that studies suggesting that viral infections cause a reduction in cell mediated immunologic activities should be regarded with caution. For example, results purporting to show a decrease in PHA responsiveness (12, 13) could have resulted from increased cell death in culture or from changes in other cellular indices rather than a decrease in PHA-induced responsiveness of T cells. Thus, the results presented here emphasize the urgent need for assessment of cellular viability at the end of culture. This has been overlooked in the past. Further, they indicate that immunologically immature mice suffer severe sequelae following viral infection just as old mice do, suggesting that the immature immune systems of these young mice permit persistence of the virus. Such an interpretation may be of importance to studies of cell-mediated immunity of animals which are chronically infected with viruses; [e.g. Aleutian disease of the mink (14) and NZB mice (15)]. In view of these observations, studies on the effect of viral infection on the immune system should take into consideration the age of individuals, both at the time of infection and at the time of experimentation post infection, so that the long term effects of the virus infection can be differentiated from those of aging.

Epidemiologic studies of mouse breeder colonies indicate that Sendai infection can persist as long as 2 yr and that weanling mice play an important role in perpetuating the virus (2, 16). In this study, it appears that the immunodepressed adult and immunodeficient old mice also acted as "carriers" of the virus, thus perpetuating the subclinical infection. This is consistent with recent evidence suggesting that a cell mediated immune response participates in the eradication of Sendai virus (17) since the T cell component of the immune system is deficient in these mice (7, 18).

Summary. The long-term effects of subclinical parainfluenza virus infection on the immune system of eight strains and hybrids of aging mice were studied. The results demonstrated that 55 of the 63 weight, cellular, activity, and autoimmune indices tested were abnormal as late as 8 months after disappearance of clinical symptoms. Probably the most significant changes were the increase in the fragility of T and B cells and decrease in their proliferative capacities, which were associated with an increase in susceptibility to autoimmune disease. The long term sequelae of the viral infection including autoimmune disease were most severe in immunologically immature young, immunodepressed adult, and immunologically deficient old mice; e.g., the mortality of adult mice rendered T cell deficient by either thymectomy alone or thymectomy followed by irradiation-bone marrow reconstitution was about 5% for both groups prior to infection and increased to 30 and 80%, respectively, after infection. All old mice converted to Coombs' positivity. These findings may be of importance to studies of the pathogenesis of autoimmune disease in aging animals and humans.

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