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Lymphocytes Responding to Primary and Re-infection of Parainfluenza (Sendai) Virus in Mice

Hoan Jong Lee

Department of Pediatrics, Seoul National University College of Medicine, Seoul 110-744, Korea

=Abstract=**The cells recovered in bronchoalveolar lavage (BAL) fluid after primary and re-infection of Sendai virus in mice were analyzed. In primary infection, total cell counts recovered in the BAL began to increase on day 5, reached a peak on day 7 of infection. One day after infection, there was a remarkable neutrophil response. Lymphocytes began to increase on the third day after infection, reaching a peak on day 5. Preinfection lymphocytes were largely CD4-bearing. Most of the lymphocytes on day 3 and day 5 were Thy 1.2-positive, but double negative for CD4 and CD8 which were thought to be natural killer cells. CD8 cells began to increase on day 5, reached a plateau of 60% since day 7, which coincided with disappearance of virus in the lung. Peripheral differential white cell counts showed a striking lymphopenia lasting 5 days, and CD4 bearing cells predominated throughout the post-infection period. After re-infection, lymphocyte response was more brisk, reaching a peak on day 3, but less prominent compared to that of primary infection. The most remarkable change was observed in the population of B-cells, reaching a plateau of 30% since day 3 after infection.**

In conclusion, there is a major influx of natural killer cells into the lung in the early phase, CD8 cells in the late phase of primary Sendai virus infection, and B-cells in re-infection. These changes are not reflected in the peripheral blood.

Key Words: *Sendai virus, Lymphocyte, Flow cytometer*

INTRODUCTION

Acute respiratory infections are the leading cause of morbidity and mortality in children under five years of age in most of developing world (Berman and McIntosh 1985;Leoiski 1986). There is a widespread assumption that the major reason for the high mortality is an increased incidence of severe bacterial pneumonia (Shann 1986). It is well known, however, that viral lower respiratory infections are also frequent in children (Berman *et al.* 1983), and there is little or no information about the severity of pure viral respiratory infections. The difficulty of studying the subject in the field and assuring that bacterial superinfection has not played a role suggests that animal models may be extremely useful in dissecting the elements of the problem.

While influenza is a well studied system in the mouse, it is not as suitable as Sendai virus as a model for viral pneumonia in infants. Sendai virus is a natural murine pathogen and is widely used in animal experiments as a model of respiratory viral infection (Tyrrell and Coid 1970). It produces an interstitial pneumonia which, histologically, is extremely similar to viral pneumonia and bronchiolitis in children. In addition, the virus is biochemically almost identical to parainfluenza virus type 1, a major pediatric pathogen, and in the same virus genus as the other parainfluenza viruses and the same family as respiratory syncytial virus (RSV).

There is considerable background information about the role of the immune system in protection against and recovery from Sendai virus pneumonia (Kast *et al.* 1986; Iwai *et al.* 1988).

To better elucidate the inflammatory and immune process involved in pure viral respiratory infection, analysis of cells recovered in bronchoalveolar lavage or collagenase-treated whole lungs as well as peripheral blood leukocytes was performed in terms of total cell counts, differential cell counts, and lymphocyte subtypes, using a mouse model infected with Sendai virus.

MATERIALS AND METHODS

Mice: Three to five weeks old, specific pathogen free, female BALB/c mice were used for all experiments.

Virus inoculation: Sendai virus (American Tissue Culture Collection, Rockville, Md) was grown in MA 104 cells (Whittaker, MA Bioproducts). Mice were inoculated intranasally with a dose of 1×10^3 PFU per animal in a volume of 25 ul after light intraperitoneal pentobarbital anesthesia. Five mice were sacrificed one day before inoculation as a baseline, and on every other day up to the thirteenth day. For rechallenge experiment, the survived mice were inoculated with a dose of 1×10^4 PFU per animal at 29th day after the first inoculation, and followed until the seventh day.

Bronchoalveolar lavage (BAL): BAL was performed in mice anesthetized with intraperitoneal pentobarbital. The trachea was exposed and cannulated with 22-gauge intravenous catheter. 0.8 ml of Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS) with 0.06 mM EDTA was introduced and withdrawn through the catheter. This procedure was repeated with 5 changes of the solution per mouse. Out of 4 ml used, more than 3.5 ml was usually recovered.

Peripheral blood cells and cells from collagenase-treated total lung: The deeply anesthetized mice were bled through the axillary vessels. The blood was collected with heparinized Pasteur pipette. Then the lungs were taken after removal of hilar lymph nodes, and prepared as described by Taylor *et al.* (1985). Following filtration through sterile muslin, lymphocytes were separated with a Ficoll-Hypaque gradient. Macrophages and B-lymphocytes were partially removed by incubating twice in a Petri-dish for 1 hour.

Cell preparation: Cells recovered in BAL fluid of each mouse were counted by hemocytometer. Viability, judged by trypan blue exclusion, usually exceeded 95%. Then cells from 5 mice were pooled for differential cell counts and determination of lymphocyte subtypes. Differential cell counts were performed on Millipore filters exactly as described by Saltini *et al.* (1984), with 0.5 to 1.5 ml of BAL fluid, depending on total cell counts. The preparations were stained with hematoxylin-eosin and observed by light microscopy with mineral oil (1,000 x). More than 300 cells were counted each time, and a mean value was obtained from 3 determinations on each slide. The remaining cells were prepared for flow cytometric analysis.

Peripheral blood leukocytes were counted from each mouse by hemocytometer, and differential cell counts was done on Wright-stained smear. Then, the blood was pooled for subtype determination of lymphocytes by flow cytometer.

Fluorescent antibody staining: Standard method was used for direct or indirect staining of 50 ul of cells at 4×10^6 cells/ml. The following antibodies were used for staining; Thy 1.2-FITC (clone 30-H12, Becton Dickinson Immunocytometry System), Thy 1.2-PE (clone 30-H12, Pharmingen, San Diego, CA), anti-L_3T_4-PE (anti-CD4, clone GK 1.5, Becton Dickinson Immunocytometry System), anti-Lyt.2-FITC (anti-CD8, clone 53-6.7, Becton Dickinson Immunocytometry System), anti-CD3-FITC (clone 145-2C11), anti-$\alpha\beta$ TCR-biotin (clone H57-597, a generous gift from Dr. R T Kubo, National Jewish Hospital, Denver, CO) and strepavidin-PE (Becton Dickinson Immunocytometry System), goat anti-mouse Ig-FITC (for staining of B-cells, polyclonal, Becton Dickinson Immunocytometry System), and anti-Mac 1 (clone M1/705, Accurate Chemical & Scientific Corp., Westbury, NY) and goat anti-rat Ig $F(ab')_2$-FITC (Caltac Lab., San Francisco, CA).

For isotype control, the following antibodies were used; Rat IgG 2a-FITC and rat IgG 2b-FITC (Pharmingen, San Diego, CA), clone HMT 3.1, clone HMT 3.2, goat IgG-FITC (Sigma, St. Louis, MD). After

staining, cells were incubated in 2 ml of FACS lysing solution (Becton Dickinson Immunocytometry Systems) and finally fixed with 2% paraformaldehyde in PBS.

Flow cytometric analysis: A FACScan flow cytometer (Becton Dickinson Immunocytometry System) and the Consort-30 program (Becton Dickinson Immunocytometry System) were used for data acquisition and analysis. For lymphocyte subtype measurements, cell populations were gated on the light scatter diagram on the basis of cell size (forward scatter count) and cell granularity (side scatter count) using a polygonal gate. In preliminary experiments, the cells within the gate represented at least 95% of the lymphocytes in the BAL fluids obtained on day 7 after viral inoculation, and less than 5% of the cells in this gate stained positively with anti-macrophage antibody. Gated cells were then analyzed for fluorescence, and the percentage of each subtype were counted. A minimum of 10,000 cells were analyzed from each tube.

Virus titration: Virus was titrated after lavage. The lungs were homogenized with 7 ml Tenbroeck tissue grinders (Wheaton, Millville, NJ). MA 104 cells in 24 well plates were inoculated with 100 ul of 10-fold dilutions of individual lung homogenates. Methylcellulose (1.5%, Mallinekrodt Inc., St. Louis, MO) mixed 1 : 1 with double strength minimum essential medium without fetal bovine serum plus trypsin (0.5 ug/ml, Calbiochem-Behring, La Jolla, CA) was used as an overlay. After 48 hour of incubation, cells were fixed with 10% formaldehyde and stained with crystal violet. Plaques were counted under a dissecting microscope.

RESULTS

Mortality and virus titer of the lung

Mice began to die since 6 days after the primary viral inoculation, and the overall survival rate by Kaplan-Meyer method was 78% (Fig. 1). The virus was isolated till day 5 after infection (Fig. 2). There was no mortality and virus was never isolated after re-challenge on day 29 of the primary infection.

Total cell counts and differential cell counts

One day before infection, the total cell counts

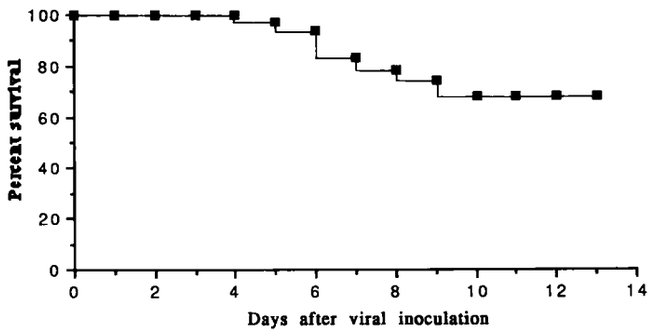

Fig. 1. Survival of BALB/c mice after primary intranasal infection with 1 × 10^3 PFU of Sendai virus (Kaplan-Meier method). There was no mortality when rechallenged with 1 × 10^4 PFU of Sendai virus 28 days after the primary inoculation.

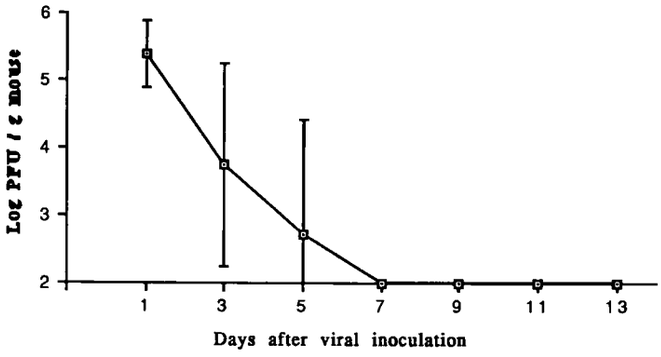

Fig. 2. Virus titer of BALB/c mouse lungs after primary intranasal inoculation of 1 × 10^3 PFU of Sendai virus. Each point represents the mean ± standard deviation for 5 mice. Virus was never recovered after rechallenge.

recovered in BAL fluid was 38.0 ± 20.9 × 10^4 per mouse (mean ± standard deviation). Total cell counts were relatively stable till day 3, then increased abruptly on day 5, reached a peak of about 5-fold above the baseline level (213.7 ± 61.9 × 10^4) on day 7, and decreased gradually thereafter (Fig. 3).

In uninfected mice, more than 97% of the BAL cells were macrophages, and lymphocytes and neutrophils were less than 2%, respectively. One day after infection, There was a remarkable neutrophil response reaching 58.2%. The neutrophil response persisted till day 3. As the percentage and absolute count of neutrophils increased during the first 3 days after infection without significant increase of total cell counts, the percentage and absolute counts of macrophages decreased initi-

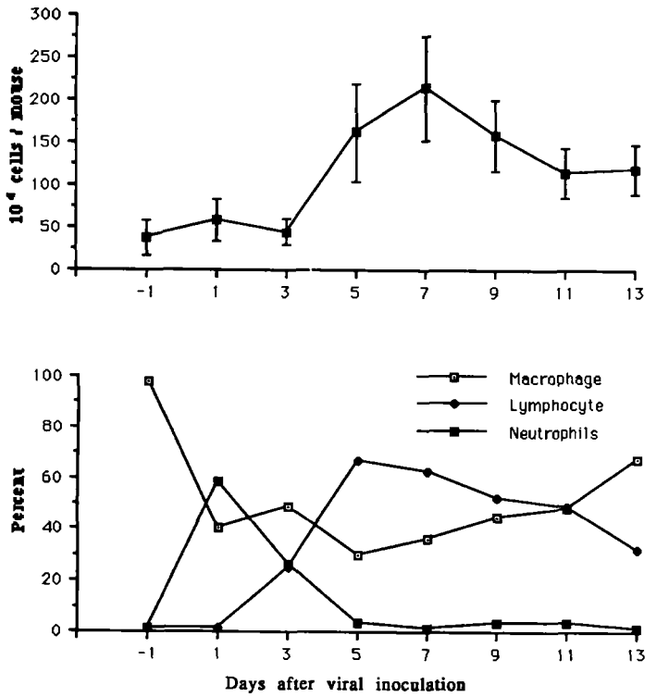

Fig. 3. Changes in total cell counts (upper diagram) and differential cell counts (lower diagram) in the bronchoalveolar lavage fluid of BALB/c mice after primary intranasal inoculation of Sendai virus, 1 × 10^3 PFU/mouse.

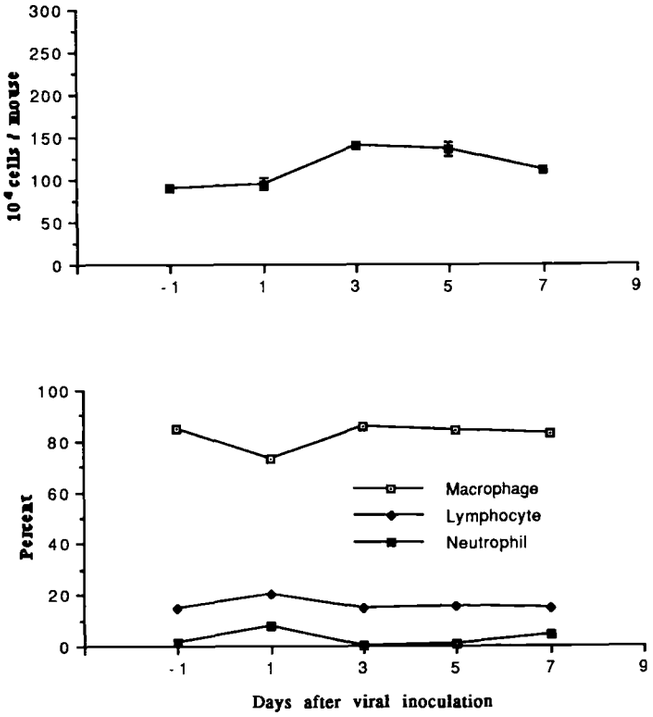

Fig. 4. Changes in total cell counts (upper diagram) and differential cell counts (lower diagram) recovered in the bronchoalveolar lavage fluid after rechallenge with 1 × 10^4 PFU of intranasal Sendai virus 28 days after the primary inoculation.

ally, but began to increase since the 5th day after infection.

The most remarkable change was observed in lymphocytes. The lymphocytes were negligible in the uninfected and on the 1st day after infection. The percentage of the lymphocytes began to increase since the 3rd day, peaked on day 5 at 72.6%, and decreased gradually thereafter. On day 13 it was 31.6%.

One day before re-challenge on day 29 after the primary inoculation, the total cells in BAL fluid was 88.9 ± 3.2 × 10^4 per mouse. Total cell counts began to rise next day, and peaked on day 3 at one and half of the baseline level (139.1 ± 4.7 × 10^4) and decreased thereafter. Though the peak was earlier, the magnitude of increase was far less compared to that after primary infection.

Lymphocytes and neutrophils constituted about 15%, and 1%, respectively, one day before re-challenge. There was slight increase in the percentage of lymphocytes and neutrophils on day 1 and returned to baseline level by day 5 (Fig. 4).

Lymphocytes subpopulations

BAL cells were stained with antibodies against Thy 1.2, L3T4 (CD4) and Lyt.2 (CD8). Thy 1.2^+ cells constituted 40-60% of the gated BAL lymphocytes before infection and on day 1, but reached over 80% since day 5. Before infection and during the early period of infection, CD4-bearing cells were dominant. Beginning on day 5, CD8-bearing cells became more frequent than CD4-bearing cells (Fig. 5). The CD4-bearing cells reached a plateau of 60% since day 7. Interestingly, on day 3 and day 5, more than half of the lymphocytes were Thy 1.2-positive but negative for both L3T4 and Lyt.2 markers. Surface immunoglobulin-bearing cells (B-cells) were negligible before infection and during the early period of infection, and slowly increased reaching 9% on day 13.

To further characterize the lymphocytes, cells obtained by collagenase treatment of whole lungs were stained with antibodies including those

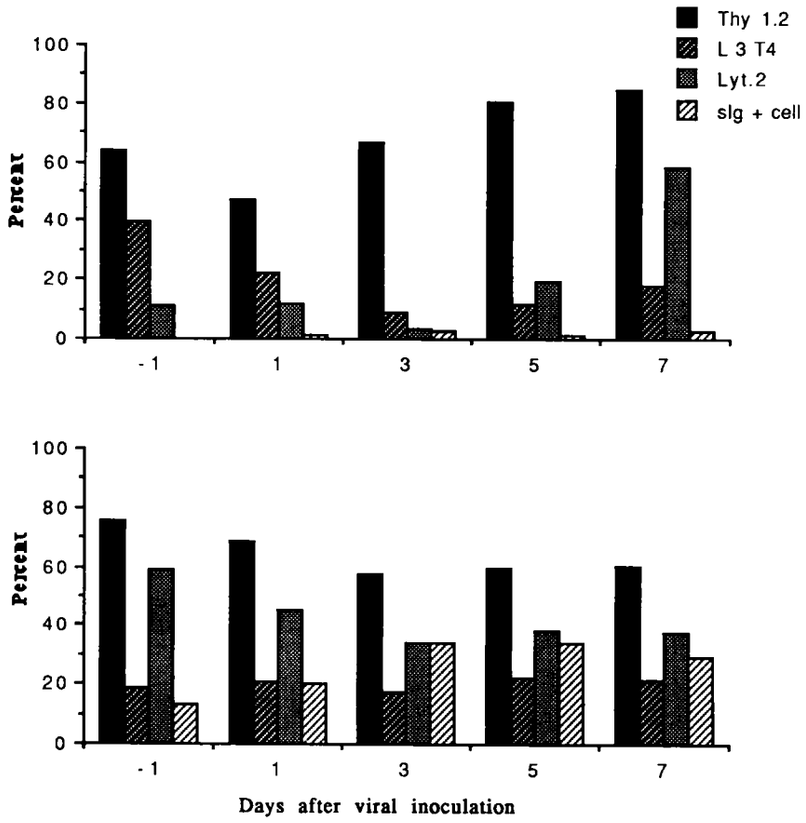

Fig. 5. Changes in Thy 1.2, L3T4 (CD4), Lyt.2 (CD8) positive cells and surface immunoglobulin bearing cells (sIg + cell) in the bronchoalveolar lavage fluid after primary infection (upper diagram) and re-infection (lower diagram) with Sendai virus. Lymphocytes subpopulations were gated on forward and side scatter diagram, and then analyzed for fluorescence using a FACScan flow cytometer.

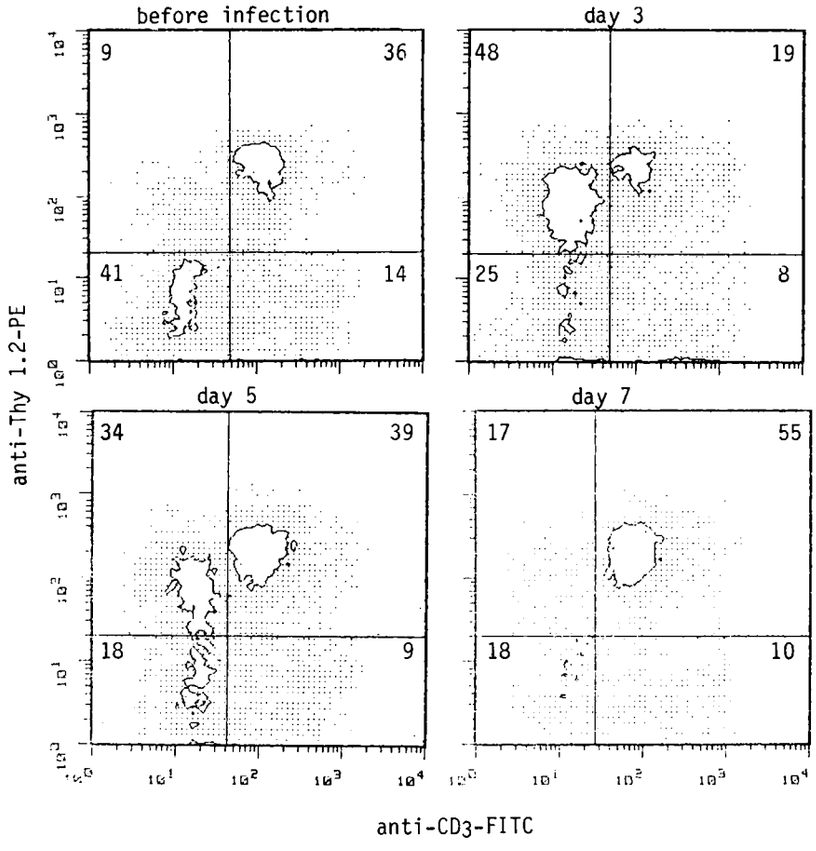

Fig. 6. Contour diagrams of collagenase-treated lung lymphocytes stained with anti-CD3-FITC and anti-Thy 1.2-PE. The values on each quadrants are corrected for isotype control.

against Thy 1.2, CD3 and αβ-TCR, which showed that 47.8% on day 3 and 34.2% on day 5 were Thy 1.2-positive, but CD3-negative in double stained preparations with antibodies against Thy 1.2 and CD3 (Fig. 6). CD3-positive, αβ-TCR-negative (presumptive γδ-TCR cells) constituted 12.7% before infection and there was no significant change during 7 day period after infection (data not shown). These findings suggest that Thy 1.2^+, $CD4^-$, $CD8^-$ cells appearing in the BAL fluid on day 3 and day 5 are most probably natural killer cells.

Before re-challenge, Thy 1.2^+ cells were 76.0%, $L3T4^+$ cells 18.7%, $Lyt.2^+$ cells 58.7%, and B-cells 13%. With re-challenge, most remarkable change was observed in B-cells, which reached a plateau of 30% since day 3. As B-cells increased, $Lyt.2^+$ cells decreased in proportion (Fig. 5).

Peripheral blood

With primary inoculation, there was a remarkable leukopenia in the peripheral blood lastion 5 days after infection (Fig. 7). Differential cell count showed that there was relative neutrophilia and the leukopenia was mainly due to decrease in lymphocyte count.

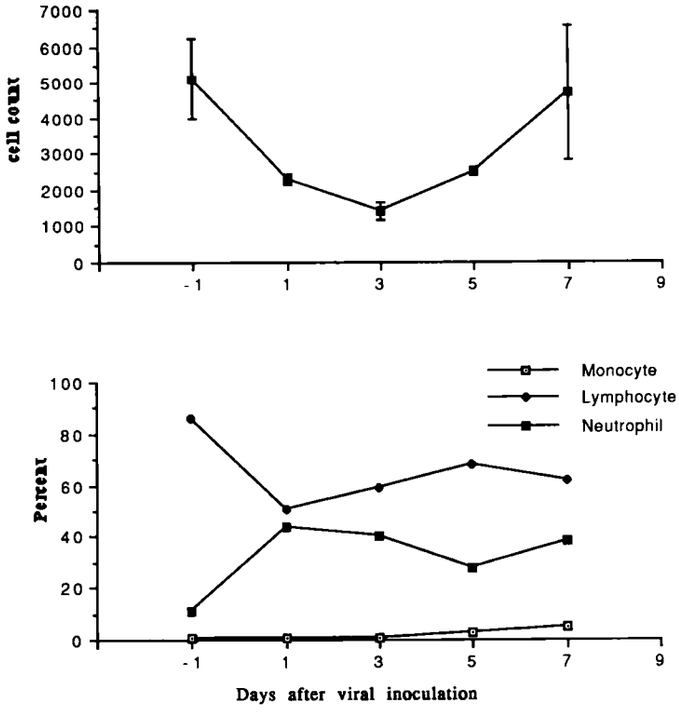

Fig. 7. Changes in total cell counts (upper diagram) and differential cell counts (lower diagram) in the peripheral blood of BALB/c mice after primary intranasal inoculation of Sendai virus, 1 × 10^3 PFU/mouse.

In contrast to lymphocyte subtypes in the BAL or pulmonary lymphocytes, $L3T4^+$ cells dominated through out the observation period, before infection to day 7 after infection (Fig. 8).

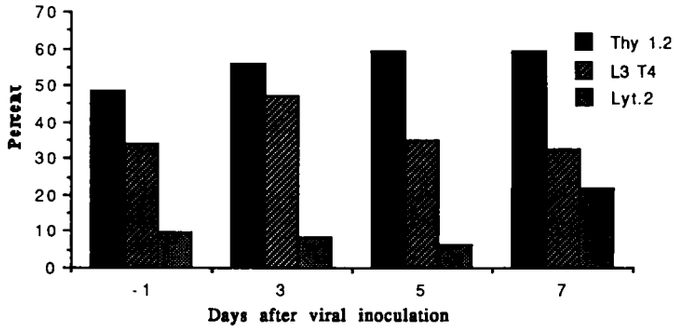

Fig. 8. Changes in Thy 1.2, L3T4 (CD4), Lyt.2 (CD8) positive cells in the peripheral blood after primary intranasal inoculation of Sendai virus, 1 × 10^3 PFU/mouse.

DISCUSSION

Recent development of bronchoalveolar lavage and flow cytometer, and availability of various monoclonal antibodies against murine lymphocyte surface markers enabled me to examine the quantitative changes of lymphocytes in the inflammatory exudate in the virus infected lung.

This report describes the analysis of cells involved in the inflammation of the mouse lung induced by Sendai virus. The initial response was characterized by increase in the percentage of neutrophils 1 day after infection, though there was no increase in total cell counts recovered in the BAL fluid. Total BAL cells began to increase on day 5, reached a peak on day 7, and decreased thereafter. Three to five days after infection, BAL cells were characterized by dominance of Thy 1.2^+, $CD4^-$, $CD8^-$ lymphocytes. These cells were proved to be CD3-negative in collagenase-treated whole lung cell preparation, and, therefore, thought to be probably natural killer cells. Recent studies suggest a putative protective role for γδ-T cells in infectious diseases (Haas *et al.* 1990; Cerdig *et al.* 1990). The percentage of CD3-positive, αβ-TCR-negative (tentative γδ-TCR cells) did not change significantly during the first 7 days after infection.

In uninfected mice, helper/inducer T-cells (CD4) were more numerous than suppressor/cytotoxic T-cells (CD8). But the ratio was reversed since 5 days after infection. Since 7 days after infection CD8-positive cells became the dominant cell type, constituting 60-80% of the lymphocytes, throughout the experiment period. This rise of $CD8^+$ cells is consistent with the reported appeararance of cytotoxic activity around day 7 to 9 in a functional assay of pulmonary lymphocytes in RSV infected mouse lung (Taylor *et al.* 1985), as well as the appearance of CD8 cells in the murine airway on day 9 and 14 after RSV infection (Oppenshaw 1989). The increase of CD8 cells on day 7 after infection paralleled with the clearance of virus from the lung. This temporal association of the increase of cytotoxic T-cells and virus clearance are in accord with results of adoptive transfer experiments. Furthermore, in adoptive transfer experiments, influenza virus-infected recipients of T cell-enriched populations of immune spleen cells cleared virus more rapidly than recipients of T-cell populations from naive mice (Schulman *et al.* 1977). Virus clearance and recovery proceed more rapidly if the adoptively transferred cells were sensitized with the identical viruses (Yap and Ada 1978). Moreover, the transferred effector population requiring enhanced virus clearance express the Ly 2^+ 3^+ phenotype (Yap *et al.* 1978).

Despite the temporal association between the increase of CD8 cells and clearance of viruses, the specificity of the accumulated CD8 cells has yet to be elucidated. It has been shown that the frequency of CTL precursors (CTLP) generating effectors for lymphocytic choriomeningitis virus (LCMV) infected targets ranged from 1 : 2,000 to 1 : 3,500 (Ceredig *et al.* 1987), while it was estimated to be 1 : 80 to 1 : 320 in mice infected intracerebrally with the more lytic vaccinia virus (Hurwitz *et al.* 1983). Another issue is whether the increased cellularity developing in the lung of mice infected with virus is the results of greater proliferation of cells already within the lung, or reflected recruitment from the recirculating pool of lymphocytes. Lynch *et al.* (1989) have reported that recruitment is the predominant reason for the rise of lymphocytes numbers of cervical lymph node in LCMV infection.

Ceredig *et al.* (1987) have suggested that most of the T lymphocytes extravasating into the CSF of mice with LCMV are passive participants recruited as a consequence of the function of relatively few LCMV-specific effector cells.

Although it is generally agreed that CD8 cells are important for the elimination of virus from the infected tissue, the role of CD4 cell in the process is unclear. Though the idea that T-T help provided by CD4-positive T lymphocytes is essential for the CD8-positive T cell response (Bennink *et al.* 1981; Erard *et al.* 1985) gained great acceptance as a consequence of the in vitro studies using defined condition, the results of in vivo experiments are conflicting. Elimination of the $CD4^+$ T cells by treatment of mice with monoclonal antibody did not modify either the pathogenesis of lymphocytic meningitis, or the expression of activation markers on $CD8^+$ T cells (Lynch *et al.* 1989), while co-operation between cytotoxic and helper T lymphocytes was important in protection against lethal Sendai virus infection (Kast *et al.* 1986), and $CD4^+$ and $CD8^+$ subsets are cooperatively responsible for efficient clearance of Sendai virus from the lung (Iwai *et al.* 1988).

The remarkable increase of $CD8^+$ cells as effector cells in the virus infected mouse lungs (the result of this study; Oppenshaw 1989) is in contrast with the minimal increase of lymphocytes on primary challenge with sheep red blood cells (RBC) or dominance of $CD4^+$ cells on rechallenge with sheep RBC (Curtis and Kaltreider 1989).

The changes of cell types in the inflammatory exudate of the Sendai virus-infected lung does not reflect the situation in the blood. Though there was remarkable shifts in the dominant lymphocyte subtypes over the 7 day period in the lung, $CD4^+$ cells dominated in the peripheral blood throughout the experiment period. There thus seems to be selective recruitment, or retention of responding cells depending on the stages of inflammation.

On re-challenge with Sendai virus 4 weeks after the initial infection, cell response was more brisk peaking 3 day after re-infection, and the changes in the percentages of lymphocytes and neutrophils were not so dramatic compared to those after initial infection. As for lymphocyte subtypes, the

most remarkable changes were increase of B-cells reaching a plateau around 30% since 3 days after re-challenge with relative decrease of $CD8^+$ T cells.

The regulation of cellular changes in the inflammatory exudate is probably mediated by lymphokines released from macrophages or lymphocytes. Further experiments will be needed to elucidate the mechanisms involved in inflammation induced by viral infection.

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=국 문 초 록=

Parainfluenza (Sendai) 바이러스 감염 쥐의 폐 림프구에 관한 연구

서울대학교 의과대학 소아과학교실

이 환 종

Sendai virus를 실험적으로 초감염 및 재감염시킨 BALB/c 쥐의 폐세척액(bronchoalveolar lavage fluid)에서 발견되는 세포들을 분석하였다. 초감염시 폐세척액내의 총백혈구수는 감염 5일째에 증가하기 시작하여 7일에 최고치에 도달하였다. 감별계산상 감염 후 1일째에 현저한 중성구 반응이 있었으며, 림프구는 3일째에 증가하기 시작하여 5일째에 정점에 도달하였다. 감염 전에는 CD4 림프구가 주된 림프구이었다. 감염 후 3일과 5일째의 Thy 1.2^+ 림프구는 $CD4^-$, $CD8^-$으로서, 이들은 CD3 역시 음성으로 natural killer cell일 것으로 생각되었다. $CD8^+$ 림프구는 폐에서 바이러스가 없어지는 7일째부터 약 60%에서 plateau를 형성하였다. 말초혈액에서는 감염 후 5일간 현저한 림프구의 감소를 보였으며, 감염 후 전기간을 통하여 $CD4^+$ 림프구가 주종을 이루었다. 재감염시 폐림프구는 3일째에 정점에 도달하여 초감염시보다 빨리 반응하였으나 그 정도는 덜 하였다. 가장 현저한 변화는 B 림프구의 증가였다. 결론적으로, BALB/c 쥐에서 Sendai virus의 초감염 초기에는 주로 natural killer cell, 후기에는 CD8 림프구가 반응하며, 재감염시에는 B 림프구가 반응하였다. 이러한 변화는 말초혈액에는 나타나지 않았다.