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Extensive lymphopenia due to apoptosis of uninfected lymphocytes in acute measles patients

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Summary. Infection with measles virus induces a transient immunosuppression, which occasionally results in fatal opportunistic infections. To obtain fundamental information about the mechanism, we examined peripheral blood mononuclear cells (PBMC) from acute measles patients aged from infants to 35 years old, obtained at various times from incubation periods to 103 days after onset of rash, for the number of lymphocyte subsets by flowcytometry. The data were analyzed for relationships between aging of the patients and the severity of immunosuppression.

In classical measles cases, infected lymphocytes detected as a minor pupulation during the incubation period disappeared soon after onset of rash, whereas in the cases of serious illness, the infected cells persisted longer after the rash. At the onset of rash, remarkable lymphopenia had already occurred in all measles cases with reduction in cell numbers of $CD4^+$T cells, $CD8^+$T cells, B cells, neutrophils, and monocytes. In contrast, natural killer (NK) cells were increased in number and activated, which might be a response compensatory for the lymphopenia. Apoptosis-associated molecules such as CD95(Fas) and TNF-related apoptosis-inducing ligand-receptor (TRAIL-R) were highly expressed on the cell surface of most surviving non-infected lymphocytes, and DNA fragmentation was also observed upon incubation in vitro. These results suggested that the profound lymphopenia was primarily due to extended death of non-infected blood cells caused by apoptosis. The severity and duration of the lumphopenia were age-dependent; less severe in young children whereas much severer in infants under one year of age as well as adolescents and adults. From these results, it was suggested that remarkable lymphopenia due to apoptosis of uninfected cells is one of the

principal causes for immunosuppression induced by measles virus infection, and is correlated with the age-dependent severity of the disease.

Introduction

Measles virus infection induces a transient immune suppression to the patients, which often provokes opportunistic infections leading to fatal outcome [23, 34] and exacerbates pre-existing chronic infections such as tuberculosis [5]. However, the precise mechanisms underlying the measles virus-induced immunosuppression remain to be clarified. It has been recognized that in measles patients, tuberculin skin reaction, a $CD4^+$T cell-mediated delayed-type allergic reaction against extracellular products of *Mycobacterium tuberculosis*, turns to be negative which lasts for a certain long period even after recovery from clinical measles [16, 31, 32]. Other T cell immunities have also been shown to be impaired in measles virus infections [24, 30]. On the other hand, humoral immunity tends to be retained [7].

Measles virus-infected lymphocytes have been considered to be eliminated from the patients in several ways; necrosis or apoptosis of virus-infected cells, non-specific cell killing by host defense mechanisms and specific immunity to virions or infected cells [9, 12, 29], although the measles virus genome is suggested to persist for a long period in the brain tissues [15]. However, since the number of measles virus-infected cells remains as a minor population, cytolysis of viral-infected lymphocytes could not explain the profound immune suppression.

Down-regulation of cell surface expression of CD46, a complement co-factor, in measles virus-infected lymphocytes has also been suggested to be involved in measles virus-induced immunosuppression [18]. However, CD46, a cellular receptor for certain laboratory strains of measles virus, is distributed in a restricted spectrum of measles virus-permissive cells, and most virus strains recently isolated do not show the down-regulation of CD46 [3, 27]. Therefore, down-regulation of CD46 could not explain the immunosuppression in general. There are many reports suggesting mechanisms by which immune suppression is induced through cytokines in response to the virus infection [14]: yet much of the pathogenesis is not fully understood and left to be elucidated. Cell cycle of lymphocytes has been shown in vitro to be arrested in G1 phase by contact with either measles virus or virus infected cells, and thereby proliferation of the uninfected lymphocyte was suppressed, possibly leading to immunosuppression [8, 20, 28].

It has been controversial as to the mode of peripheral blood cells in acute measles patients. A transient lymphopenia has been shown to be characteristic of measles [2, 4, 6, 11, 33], although several descriptions indicate that the number of lymphocytes in peripheral blood is rather increased [19]. Measles patients often suffer from secondary infections, which may modify the results. Therefore, the mode of drift of each lymphocyte subset in uncomplicated measles patients remains to be studied in detail.

In the present paper, to obtain fundamental information about the mode of immunosuppression in measles patients, we analyzed on molecular level peripheral blood mononuclear cells (PBMC) from measles patients of various ages at

different clinical stages, and show remarkable reduction of uninfected cells of a broad spectrum of lymphocyte subsets.

Materials and methods

Peripheral bloods

A total of 417 peripheral blood samples was obtained, under described informed consent, from 160 measles patients from infant to 35 years of age, at various times ranging from incubation period to 103 days after onset of rash. All the measles cases, which occurred in Tokyo area in 1998/99, did not have any underlying illness, and were confirmed to be primary infections with measles virus. Total number of measles cases analyzed for each age group is described in the respective figure legend. Bloods were also obtained from age-matched healthy individuals as controls. For bleeding, vacuum bleeding tubes containing sodium heparinate (Venoject, Tokyo, Japan) or EDTA-2Na were used. The blood samples were stored at room temperature and processed within 48 h.

Analysis of lymphocytes by flowcytometry

Samples of peripheral blood were lysed for 2 min at room temperature by Lysing Reagent (Beckman Coulter, Miami, FL, USA), and washed twice with phosphate-buffered saline, pH 7.4 (PBS). The resultant white blood cells (WBC) were then stained with fluorescent dye-labeled antibodies to respective cell markers, and fixed with 0.92% of paraformaldehyde at room temperature. The samples were subjected to flowcytometry using Flowcytometer XL (Beckman Coulter). For the staining of cell surface markers and cell adhesion molecules on or within cells, four-color analyses were carried out, using labeled monoclonal antibodies to respective antigens.

For determination of the absolute numbers of PBMC and each lymphocyte subset, 100 μl of total peripheral blood was four-color stained directly with CYTO-STATtetraCHROMEs CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC-5 or CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC-5 (Beckman Coulter) for 15 min at room temperature, and lysed as above except for washing the cells. After fixing as above, the samples were analyzed, after adding an equal volume of internal standard fluorescence particles (Flow-Count, Beckman Coulter) for cell counting. By using this standard, the absolute numbers of lymphocytes, monocytes, granulocytes, T cells, B cells, $CD4^+$T cells and $CD8^+$T cells in 1 μl of peripheral blood were calculated accurately.

NK cells were stained with anti-CD16 and anti-CD56 (Beckman Coulter). CD95(Fas) was stained with phycoerythrin (PE)-labeled anti-CD95 antibody (Immunotech, Miami, FL, USA). Human TNF-related apoptosis-inducing ligand (TRAIL) and its receptor (TRAIL-R) [13] were stained with labeled monoclonal antibodies to the molecules (Pharmingen, San Diego, CA, USA). For determination of absolute number of stem cells in peripheral blood, Stem-Kit (Beckman Coulter) was used. Measles virus-infected cells were stained with anti-measles virus H protein monoclonal antibody (clone B5) [25], followed by treating with FITC-conjugated anti-mouse IgG. For the statistic data analyses, standard deviations were calculated for four or more determinants.

Apoptotic cytolysis of PBMC in measles patients

PBMC were separated from peripheral blood of measles patients by the Ficoll-paque method. Cell suspension (10^5 cells in 100 μl RPMI 1640 medium with 10% FCS) was incubated for 24 h at 37 °C, and was analyzed for DNA fragmentation by the flowcytometry as reported previously [21].

Determination of antibody titers

IgG- [26], and IgM-specific particle agglutination (PA) antibody titers against measles virus were measured using plasma after separation of PBMC. For the determination of anti-measles virus IgM antibody titers, a 96-well microplate coated with anti-human IgM goat antibody received two-fold serially diluted plasma to each well. After washing, gelatin particles coated with measles virus antigens were added, and agglutination pattern was observed (Sato et al., paper in preparation).

Results

Measles virus-infected cells as a minor population

First of all, the number of measles virus-infected cells was determined with PBMC from measles patients at various times. Although only a limited number of blood samples could be available from measles patients in the latent period before onset of skin rash, measles virus-infected cells were detected at as many as 0.01% of the total PBMC during the incubation period (data not shown). The viral antigens were carried by various subsets of lymphocytes including $CD4^+$T, $CD8^+$T, and B cells, and monocytes. Even though the virus could be isolated from PBMC or/and plasma until day 5 after onset of rash, viral-infected cells were no longer detected in the acute stage.

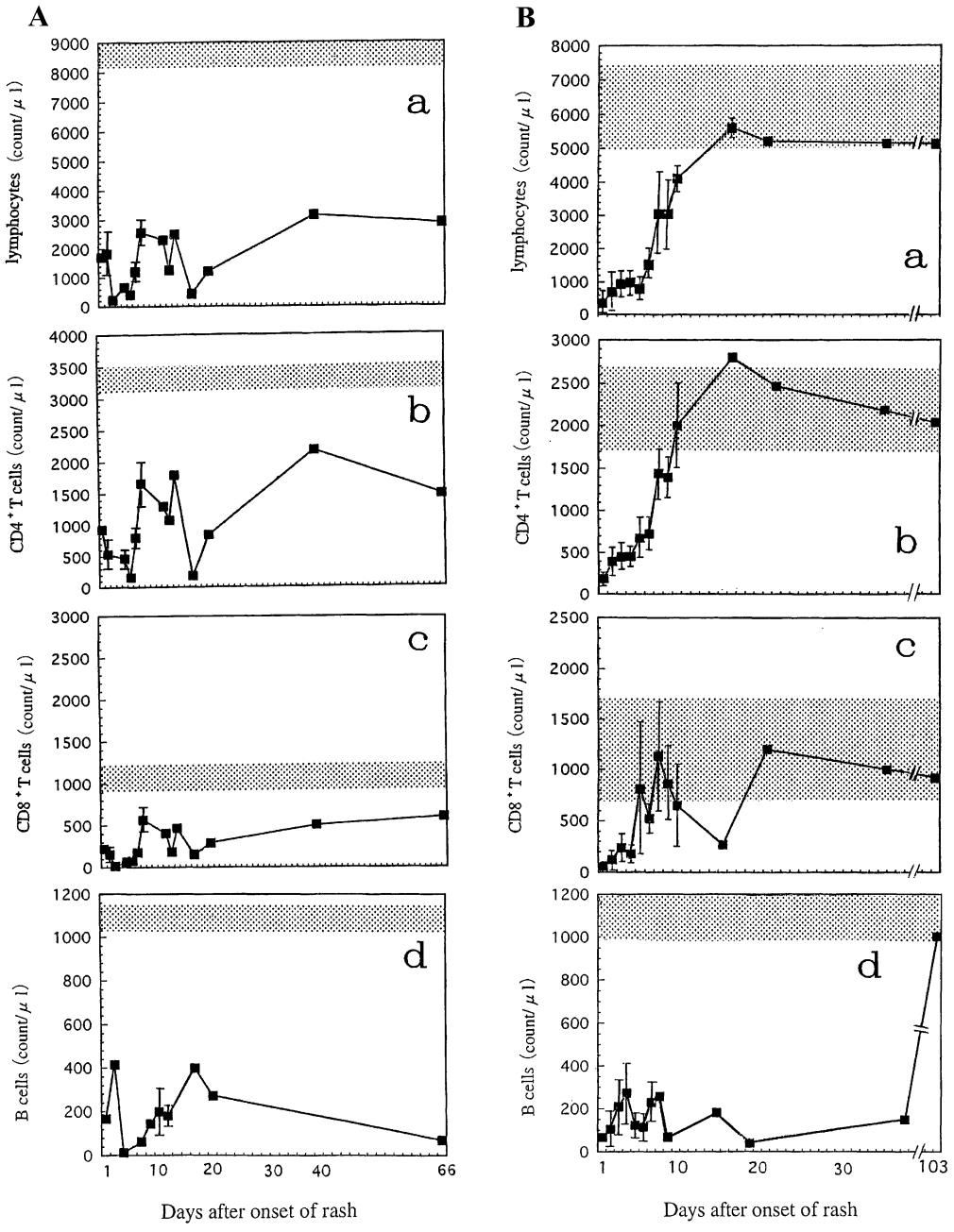
In contrast, in severe measles cases such as infants younger than one year old, fatal cases in any age groups and a 15-year-old boy with measles encephalitis, virus-infected cells remained detectable, even as a minor population, for several days after onset of rash. These results indicated that for ordinary cases, only a small population of PBMC was infected with measles virus, which disappeared rapidly after onset of rash.

From measles patients during the presence of Koplik's spots, 68 strains of measles virus were isolated, of which 33 strains were identified for their genotypes by the RT-PCR method: 28 strains as type D3, and 5 strains as type D5. There was no specific feature between the genotypes and the severity of symptoms, clinical data or results obtained from lymphocyte analyses shown below (data not shown).

Age-dependent lymphopenia due to decrease in the number of uninfected cells

The PBMC of measles patients were analyzed for the absolute numbers of each cell type and subset as described in Materials and methods. The results were classified into five groups according to age of the patients. Since the age group of 7–9 years old showed essentially the same pattern as those for 10–15 years, data from this age group (26 cases) are not shown. For each age group, time courses of the number of total lymphocytes, $CD4^+$T, $CD8^+$T, and B cells are presented in Fig. 1.

In all age groups, the absolute number of total lymphocytes was severely reduced to 1/7 to 1/10 of normal values as early as the onset of rash. Since the number of total WBC was not so severely reduced as lymphocytes, relative



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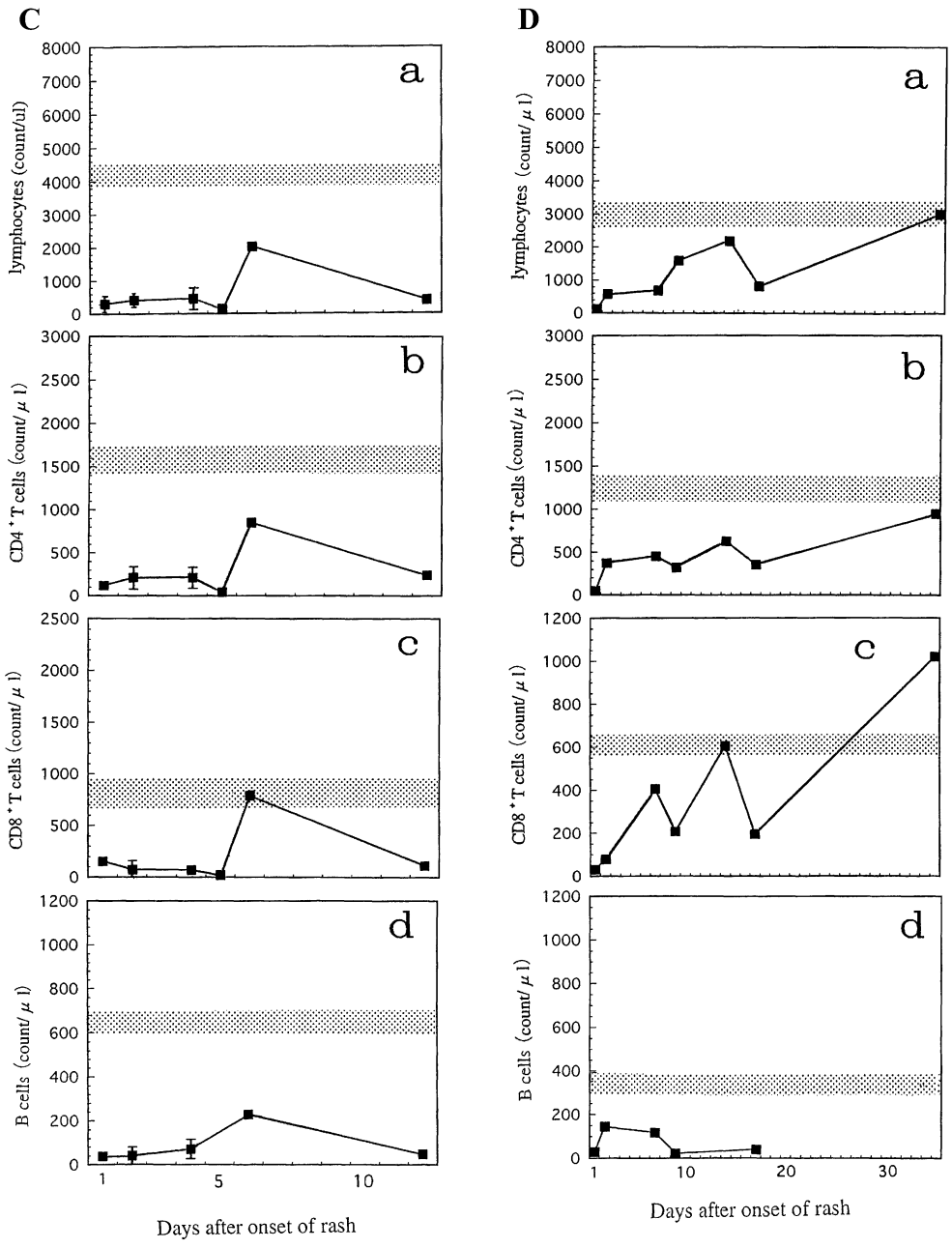

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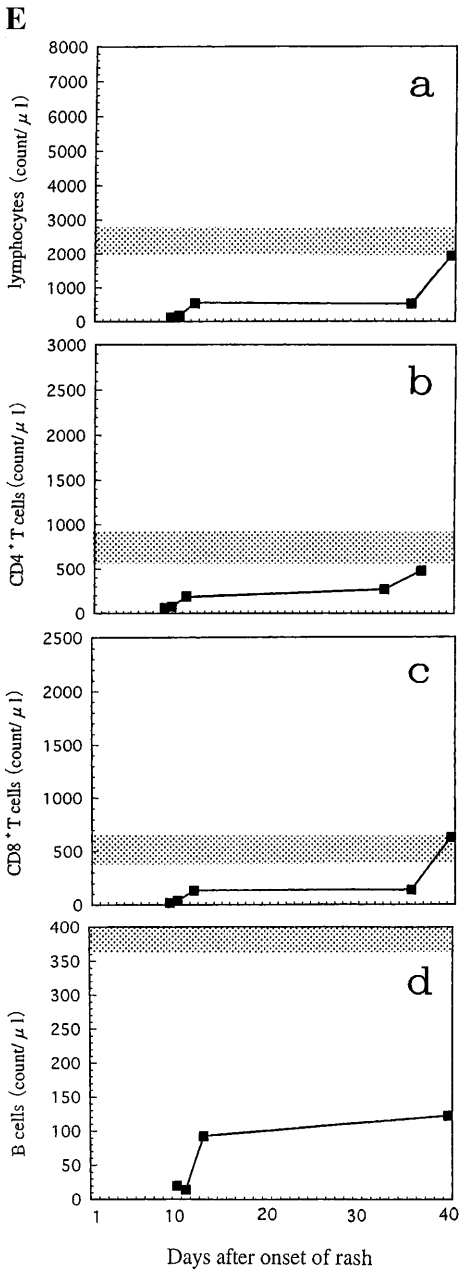

Fig. 1. Time-dependent changes in the absolute number of lymphocytes and their subsets in the peripheral blood of measles patients. Blood samples were collected at the indicated days after onset of rash. The number of total lymphocytes (*a*), $CD4^+$T cells (*b*), $CD8^+$T cells (*c*), and B cells (*d*). Grey zones represent the normal levels of each item in age-matched healthy subjects. Each closed square (■) indicates the average of 3 to 73 samples, and vertical bars indicate standard deviation when the sample number was more than 4. **A** Total 20 patients aged lower than one year old. **B** Total 73 patients aged 1–3 years old. **C** Total 18 patients aged 4–6 years old. **D** Total 16 patients aged 10–15 years old. **E** Total 7 patients aged higher than 15 years to adults. Note that the scales of the vertical and horizontal axes are different among the figures

number of total lymphocytes in WBC was also calculated as a low value (data not shown). Reduction in the number of lymphocytes was observed for a broad spectrum of lymphocyte subsets including $CD4^+$T, $CD8^+$T and B cells in all age groups. The number of monocytes was also reduced in a similar fashion (data not shown). However, the number of neutrophils was not altered significantly for uncomplicated cases. Therefore, it is concluded that severe panlymphopenia was induced in measles patients transiently at the acute stage in all age groups.

It should be noted that severity of the lymphopenia was age-dependent, and the mode of drift in the number of each lymphocyte subset was reproducibly observed within the same age group. For the age group of 1–3 years old children, with which measles occurred most frequently, the total number of lymphocytes, 340 cells per μl on day 1 of illness, turned to increase on day 6 and recovered to the normal value within two weeks (Fig. 1B). The kinetics of $CD4^+$T and $CD8^+$T cell numbers was also in parallel to the mode of total lymphocytes. However, the number of B cells remained low until as late as 5 weeks after onset of rash (Fig. 1B-d), but it was recovered finally by 15 weeks. The reduction in the number of monocytes in measles patients of this age group was as low as approximately 15% of the healthy controls on day 1. This reduction was then in turn recovered to 41% on day 5, 46% on day 6, and reached the normal level on day 7 (data not shown).

For measles cases of 4–6 years of age, recovery from the severe panlymphopenia was delayed to an extent (Fig. 1C). Even though the lymphocyte number showed a slight increase on day 6, severe lymphopenia lasted as late as day 12. Complete recovery was found by day 30 (data not shown). This tendency appeared more significantly with the age group of 10–15 years (Fig. 1D). The average numbers of total lymphocytes, $CD4^+$T, $CD8^+$T, and B cells were only 120, 44, 48, and 120 cells/μl, respectively, on day 2. They began to increase on day 9, with an exception for B cells, and the final recovery occurred as late as 5 weeks after onset of rash. Increase in the number of $CD8^+$T cells could be due to proliferation of measles virus-specific cytotoxic T lymphocytes (CTL).

In adolescent and adult patients older than 15 years of age, a more profound lymphopenic status was induced which lasted much longer; only 126 cells/μl for total lymphocytes, 68 for $CD4^+$T cells, 19 for $CD8^+$T cells and 25 for B cells, respectively, on day 10 (Fig. 1E). Recovery from the lymphopenia was extensively prolonged for more than 6 weeks. Most of the patients in this age group showed severe symptoms, such as high fever, pneumonia, hematemesis, melena, and neurological signs suggesting encephalitis, rather than typical skin rash, and thereby diagnosis as measles was often hardly made in the acute stage.

On the other hand, severe and prolonged lymphopenia was also evident for infant patients under 12 months of age, where recovery hardly took place as long as two months (Fig. 1A). Data indicating the time of final recovery was not available for this age group. It was noted that all the newborn babies without maternal antibodies against measles virus were suffered from extremely severe illness with prolonged lymphopenia not restored at all, leading to a fatal outcome.

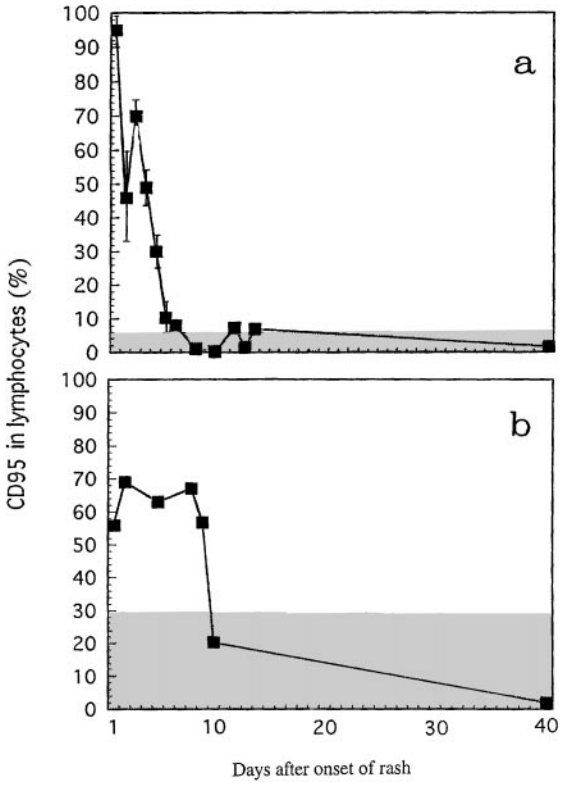

Fig. 2. Expression of CD95(Fas) on the surface of lymphocytes. Ratios of CD95-expressing cells to total lymphocytes are shown in percentage from 35 measles patients aged 1–3 years old (**a**), and 4 patients aged 10 years old to adults (**b**). Grey zones indicate the normal level in age-matched healthy controls

Apoptosis of uninfected lymphocytes

Since measles virus-infected cells remained only a minor population in PBMC at any time during the course of illness, the extensive decrease in the number of lymphocytes could not be explained by the death of viral infected cells. We therefore examined whether or not apoptotic cytolysis of uninfected PBMC occurred with measles patients in analogy of HIV-1 infections [10]. For this, cell surface expression of apoptosis-related molecules on PBMC were quantified according to the course of infection.

Relative ratios of the number of CD95(Fas) positive cells to that of the total surviving lymphocytes from the measles patients of the age groups of 1–3 years old (35 cases) and those older than 10 years old to adults are shown in Fig. 2. For the former age group, almost all of the surviving cells were demonstrated to express CD95(Fas) on day 1 and the high proportion of CD95 expressing cells was retained for 3 to 5 days. This ratio, however, was reduced rapidly by day 6 (Fig. 2a). During incubation period before onset of rash, TRAIL-R and TRAIL, in addition to CD95, were highly expressed, and they disappeared just after the disease became apparent (data not shown). On the other hand, for the age group of 10 years old to adults, the ratio as high as 50–70% was maintained longer up to day 10, and then suddenly decreased to the normal level around day 10 (Fig. 2b). The mode of TRAIL and TRAIL-R expression was essentially identical to those of the former group (data not shown). The period of expressing CD95 and TRAIL-

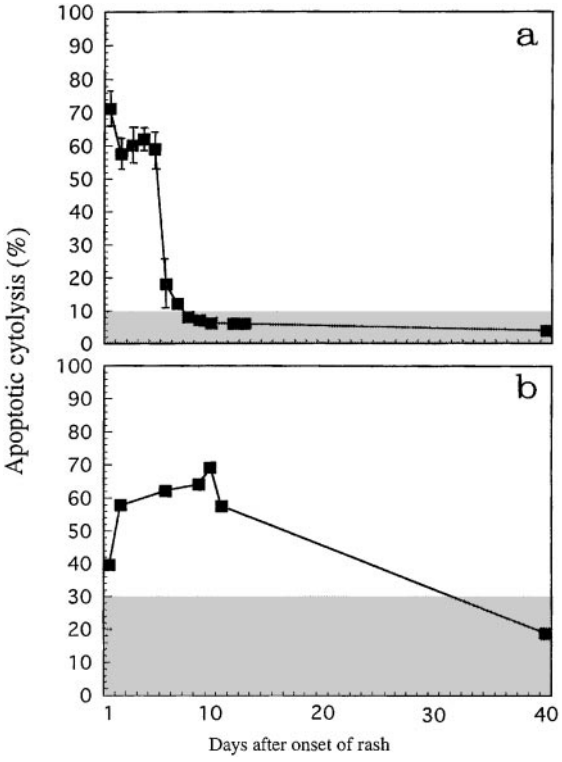

Fig. 3. Apoptosis inducing activity of PBMC. PBMC obtained from measles patients at the indicated days after onset of rash were incubated in vitro for 24 h and fragmentation of the chromosomal DNA was quantified by flowcytometry as a marker of apoptosis. Total 35 cases of measles patients aged 1–3 years old (**a**), and from 4 cases aged 10 years old to adults (**b**). Figures are expressed in percentage. Grey zones indicate the normal levels in age-matched healthy controls

related molecules was similarly extended for the age group of infant babies (data not shown).

The PBMCs isolated from measles patients were cultured in vitro for 24 hr, and the recovered cells were analyzed for the phase in cell cycle by flowcytometry (Fig. 3). In the patients aged 1–3 years old, 60–70% of the surviving cells in peripheral blood became apoptotic accompanied by the G1 arrest of cell cycle and DNA fragmentation of the chromosome (Fig. 3a). This high ratio was retained for 5 days and suddenly dropped to the normal level on day 6. In contrast, in the patients aged older than 10 years to adults, this pattern was significantly prolonged: ratio of apoptotic cells was also as high as 60 to 70% in total PBMC and this continued from day 2 to at least day 10 (Fig. 3b). These results strongly suggested that most of non-infected lymphocytes in the acute stage of measles patients were in the condition of apoptosis through the TRAIL system and CD95(Fas) molecules, and that duration of the apoptotic phase was age-dependent and correlated to the severity of lymphopenia.

Increase of NK cells

The proportions of NK cells to total living lymphocytes are presented in Fig. 4. For the group of 1 to 3 years of age, total population of NK cells expressing CD16/CD56 and T-NK cells additionally expressing CD3 appeared on the next day of the onset of rash. They were increased up to 80% in the surviv-

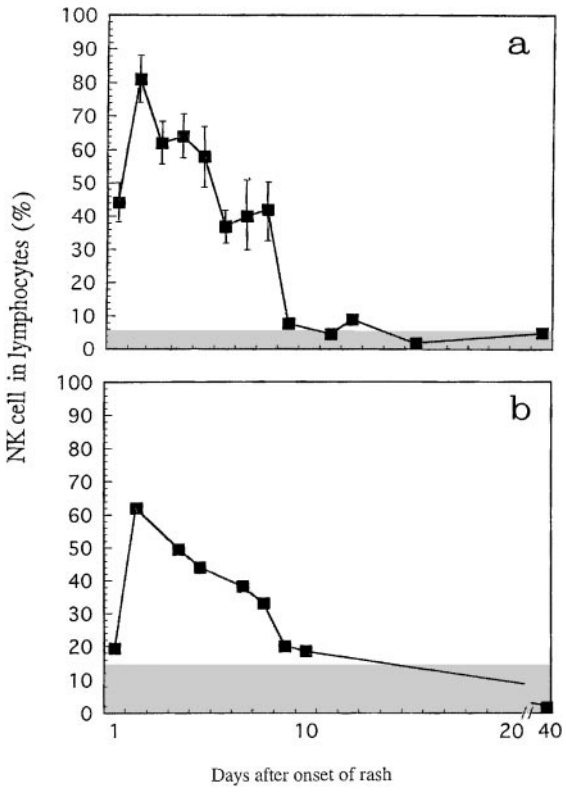

Fig. 4. Ratios of NK cells to total surviving PBMC obtained from 73 cases of measles patients aged 1–3 years old (**a**), and from 23 cases aged 10 years old to adults (**b**). Grey zones indicate the normal levels in age-matched healthy controls

ing PBMC, which was retained until day 9 along the stage of lymphopenia, and rapidly dropped down thereafter (Fig. 4a). A similar kinetic pattern was obtained also for the age group of 10 years old to adults (Fig. 4b). A highest ratio was 60%, which gradually decreased to the normal level as early as day 9 far before the recovery from lymphopenia (see Fig. 1D, E). In the other age groups, essentially identical results were obtained (data not shown).

Measles antibody responses

Both IgG and IgM antibody titers were determined for all the patients by the use of plasma. For all age groups, IgG antibodies began to rise on days 3 to 4 after onset of rash, and became to considerably high titers with 3 weeks, while IgM antibody could be readily detected on day 1 for most cases and peak titers were obtained as early as day 10 (data not shown). These results indicated that serum IgG and IgM antibodies to measles virus antigens were produced normally in spite of the prolonged suppression of B cells and irrespective of the severity of lymphopenia (see Fig. 1).

Discussion

Measles patients of all age groups were found to be in a transient, severe panlymphopenic state after onset of rash, where a broad spectrum of various subsets

of lymphocyte, including $CD4^+$T, $CD8^+$T and B cells and monocytes, were involved (Fig. 1). This supports a previous report indicating a moderate decrease in helper and cytotoxic/suppressor T cell numbers during two weeks after onset of measles [11]. The absolute number of $CD4^+$T cells became much lower than 500 cells/μl, a critical level for AIDS development. However, different from HIV infections, where a progressive, selective decrease in $CD4^+$T cell number occurs, a transient decrease in all cell subsets is a unique feature for measles. Accordingly, unlike HIV infection, $CD4^+$T/$CD8^+$T ratio did not necessarily reflect the severity of measles virus-induced immune suppression as described previously [11].

Although the remarkable decrease in B cell number was not restored long after recovery of the other subsets, serum antibody responses to the measles viral antigens occurred normally. The antibodies might be produced by B cells present in bone marrow and the spleen.

In some textbooks of pediatrics and pediatric infectious diseases, measles virus infection is described as characterized by the increase or relative increase in the number of lymphocytes [19], whereas others indicate its decrease [2, 4, 6, 11, 12, 33]. The present investigation clearly showed that a remarkable panlymphopenia with a decrease in both the absolute and relative numbers of total lymphocytes occurred in the acute stage of measles patients of a wide range of the age.

It should be noted that duration of the measles-associated lymphopenia was age-dependent. At the time of rash onset, severe panlymphopenia had already occurred almost equally with all age groups of the patients. The age group of 1–3 years old recovered from the lymphopenia most rapidly within 6 days (Fig. 1B). However, the recovery was significantly delayed according to increase in the age of patients. With measles patients in the age group of 15 years or older, the symptoms were generally severer and critical. In addition, infants before the first birthday also tended to show prolonged lymphopenia (Fig. 1A). It is well known that symptoms of measles are severer and long lasting when infection occurs in suckling infants as well as adolescents and adults. It should therefore be stressed that the mode of lymphopenia was quite consistent with the age-dependent severity of measles. A relationship might exist between maturation of host immune system and the death of non-infected cells by measles virus infection. Such a severe lymphopenia, similar to that of the terminal stage of AIDS, may provoke opportunistic infections with exogenous infective agents or re-activation of latently infecting agents in these high risk groups of measles.

In PBMC of measles patients, virus-infected cells remained as a minor population, approximately 0.01% of total lymphocytes, which disappeared just after the onset of rash for ordinary measles cases [9]. Actually, however, approximately 90% of lymphocytes had disappeared from the peripheral blood. Similar to the observation that thousands times more non-infected lymphocytes were destroyed than HIV-infected cells in AIDS patients [10], it was strongly suggested that the lymphopenia in measles patients was caused mostly by cytolysis of non-infected cells.

As a possible mechanism underlying the death of a huge amount of non-infected cells within a short period, we found that in all age groups, more than 90% of the surviving lymphocytes were positive for CD95(Fas), an apoptosis-associated molecule, from the day of rash onset to day 3, which decreased rapidly thereafter and became normal within one week (Fig. 2). TRAIL and TRAIL-R [13] were highly expressed on a wide range of cell subsets before onset of rash. When these PBMC were cultured in vitro, a potent induction of apoptosis was actually observed. These results lead to the conclusion that the extended death of non-infected cells was principally caused by apoptosis. In the age group of 1–3 years old, high proportion of CD95 expressing cells was retained for 5 days, which was then lowered rapidly in a reversely parallel manner to the recovery of the number of lymphocytes. In the age group of 10 years or older, on the other hand, CD95 expression lasted up to day 9, and recovered to the normal level after day 10. However, lymphopenia persisted for 45 days in this age group. Therefore, this delay in the recovery from lymphopenia cannot be explained only by apoptosis. The long-lasting immunosuppression in the aged and infant patients could be caused by a lack in supply of lymphocytes from bone marrow, in which suppressed proliferation of lymphocytes due to G1 arrest by the contact with measles virus or virus-infected cells might be involved [8, 20, 28]. Approximately 40-fold more CD34 positive stem cells were detected in the peripheral bloods from severe cases of infants and adults than in healthy controls (data not shown), suggesting that maturation of bone marrow cells was also impaired. It is therefore necessary to examine hematopoietic organs, such as bone marrow and thymus. For this purpose, we will perform measles virus infection experiments in monkeys as a suitable model for human measles [17].

The difference in the duration of the highly apoptotic phase between the two age groups, 1–3 years old and 10 years old to adults (Fig. 3) was 5 days, which was correlated with the mode of CD95(Fas) expression on uninfected cells (Fig. 2). Since measles virus-infected cells were no longer detected in PBMC after onset of rash, viral factors would not be involved directly in the difference. This difference might cause the delay in the recovery from lymphopenia in the older patients.

Opportunistic infections as a result of the transient immune suppression are usually not so serious in ordinary measles patients as in AIDS patients. Therefore, some host defense mechanism(s) is expected to be in action in measles patients. We found an enhanced expression of CD16/CD56 molecules, a surface marker of NK cells, in the surviving PBMC of measles patients (Fig. 4). Just after onset of rash, the number of NK cells was markedly increased, and in turn, they were decreased within 7 days. It was therefore suggested that the NK cells were activated and compensating for the immune suppression resulted from the extended deaths of non-infected immune cells. This was supported by the result that INF-γ was also increased in the NK cells (data not shown). However, the mode of NK cell proliferation was similar among all age groups (Fig. 4), although for the older groups, the severe lymphopenia persisted for a long period, even after the recovery of NK cells to the normal level (Fig. 1). Taking together, it is suggested

that NK cells played a roll in compensating for the lymphocyte deficient in the age group of 1–3 years old, whereas in the age group of 10 years or older, such compensation by NK cells could not be retained until the lymphopenia was restored. This might explain, at least partly, why the immunosuppression is severer in aged groups.

All measles viruses isolated from the present cases were of genotypes D3 and D5 [22], and there was no particular relationship between the genotype and the data presented (data not shown). The question whether or not the results obtained in this paper can be generalized to all other genotypes of wild-type virus is left to be answered.

A critical question has been raised as to whether it is proper to immunize AIDS children, especially in developing countries, with live attenuated measles vaccines [1]. It is necessary to reexamine its safety and efficacy to promote the Expanded Programme on Immunization (EPI) by WHO. We are also investigating the immune state of vaccinees after inoculation with live measles vaccines, compared with wild measles patients.

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