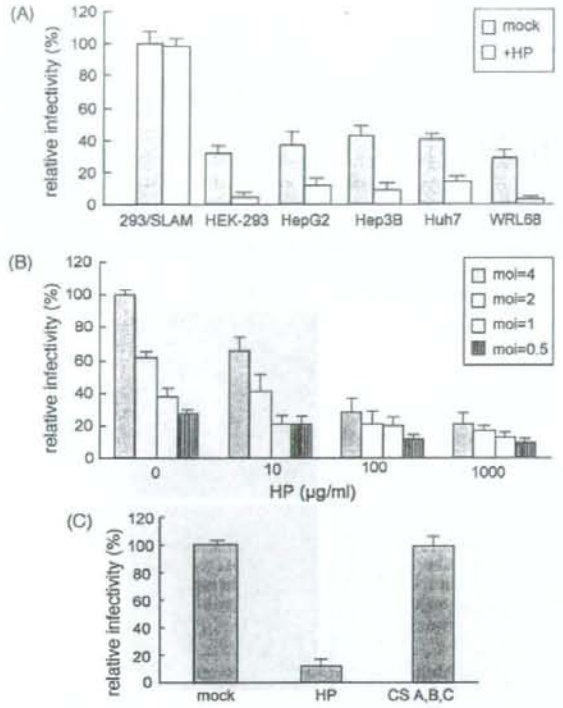

Fig. 3. Inhibition of rMV-EGFP infection by soluble HP. 293/SLAM, HEK-293, and human liver cells (HepG2, Hep3B, Huh7, and WRL68) were infected with rMV-EGFP at an MOI of 2 $TCID_{50}$/cell in the presence of soluble HP at a concentration of 100 μg/ml (A). Effect of the concentration of HP and MOI of the virus on infection of HEK-293 cells (B). Effect of the treatment with 100 μg/ml CS on infection of HEK-293 cells (C). The viral infectivity was measured at 40 h after the infection by FACS analysis.

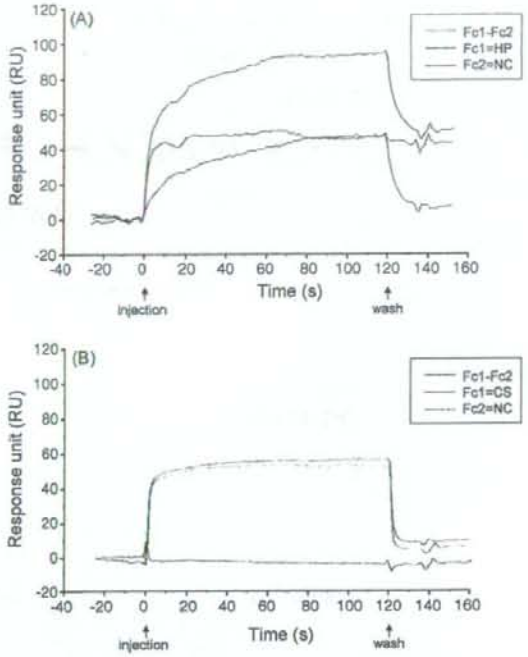

Fig. 4. Interactions between HP and MV analyzed by performing an SPR assay. HP (A) or CS (B) was covalently immobilized onto the SPR analysis. Interaction with the HP or CS were examined in real time by using 10 μg/ml purified MV. Associations among ligands occurred for 120 s following injection of the relevant analyte (Fc1, sensorgram obtained from the HP-coated sensor chip; Fc2, sensorgram obtained from the uncoated sensor chip; Fc1–Fc2, net value of Fc1).

inhibitory effect of HP was also confirmed in the experiments using different MOIs of the virus (Fig. 3B).

Two major types of glycosaminoglycan chains are found in animal cells, namely HS and chondroitin sulphate (CS) chains. To investigate whether the CS is capable of inhibiting the infection, HEK-293 cells were infected with rMV-EGFP in the presence of CS A, B, and C at a concentration of 100 μg/ml, respectively, and the infectivity of rMV-EGFP was analyzed by FACS analysis. We observed that CS A, B, and C did not significantly inhibit the rMV-EGFP infection in HEK-293 cells (Fig. 3C).

3.4. Interaction between HP and MV analyzed by performing SPR measurements

The direct interactions between MV and HP were analyzed based on the SPR (Fig. 4A). Increasing concentrations of purified MV were introduced over the HP surface on an Fc1-coated biosensor. The binding and subsequent dissociation of the MV was monitored in real time by measuring changes in the SPR in terms of response units (RU). Although MV particles bound non-specifically to the uncoated sensor chip surface during the binding phase (Fc2), coating of the sensor chip with HP dramatically increased the binding (Fc1) (Fig. 4A). Furthermore, most of the MV particles bound to the uncoated sensor chip were washed away from the chip surface (Fc2), while approximately 60% of the MV particles remained bound to the HP-coated sensor chip after washing (Fc1). When CS was coated on the Fc1 sensor chip, there was no interaction with MV (Fig. 4B). Thus, these BIAcore experiments revealed the specific interactions between the MV particles and HP *in vitro*.

3.5. HP binding envelope glycoproteins

To determine the viral proteins responsible for HP binding, affinity chromatography was performed using HP- and BSA-agarose beads. First, the concentrated supernatant of the rMV-EGFP-infected B95a cells was subjected to HP affinity chromatography. The bound materials were eluted with 2 M NaCl and analyzed by performing SDS–PAGE and western blotting with an anti-N polyclonal antibody. Although rMV-EGFP virions could not be recovered from the BSA-agarose (Fig. 5A, lane 5), they were eluted from the HP-agarose (Fig. 5A, lane 3). Thus, recombinant MV directly binds to HP at a physiological salt concentration.

Next, the *in vitro* HP binding of solubilized viral membrane glycoproteins, namely, the H and F proteins, was assessed by using HP-agarose beads. The H and F viral glycoproteins were expressed individually in HEK-293 cells by transfecting expression plasmids for these proteins. The detergent-solubilized lysates of transfected cells were subjected to HP affinity chromatography. The F protein in the resulting fractions was identified by immunoblot using an MAb against the F protein, while the H protein was identified by using an anti-myc MAb. The H protein (approximately 75 kDa) was eluted at high salt concentrations (500 mM to 1 M), while the F protein was not recovered even at the highest salt concentration (Fig. 5B), indicating the lack of interaction between HP and the F protein. These results suggest that the H protein, but not the F protein, is responsible for the binding of MV particles to HP.

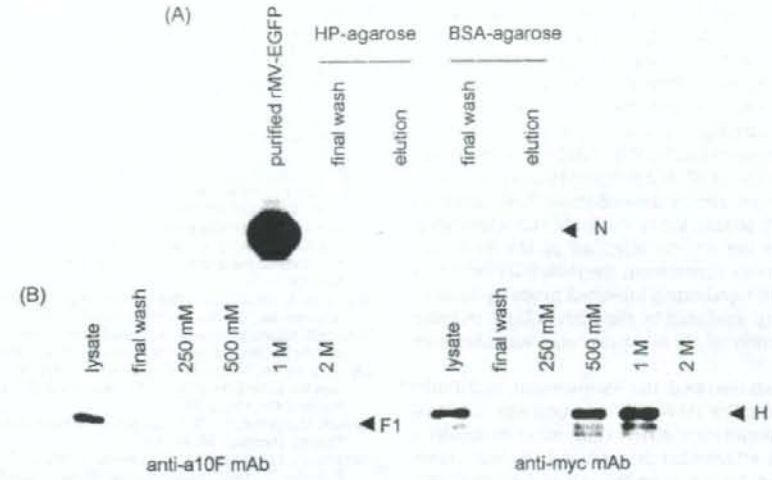

Fig. 5. HP affinity chromatography of virus particles and MV glycoproteins. (A) HP affinity chromatography of rMV-EGFP. Total MV proteins prior to before chromatography (lane 1), the final wash fraction (lane 2) and bound proteins (lane 3) eluted from the HP column, the final wash fraction obtained from the BSA-agarose beads (lane 4), and the elution fraction obtained from the BSA-agarose beads (lane 5) were subjected to western blotting with a polyclonal antibody against the N protein of MV. (B) HP affinity chromatography by using the extract of HEK-293 cells transfected with expression plasmids encoding either the F (left panel) or H gene (right panel) of MV. The proteins were eluted with increasing concentrations of NaCl in a stepwise manner. Lane 1: lysate of HEK-293 cells transfected with the plasmid expressing the corresponding MV gene; lane 2: final wash fraction; lanes 3–6: elution fractions. The NaCl concentrations that were used for elution are indicated at the top of the lanes. The fractions were analyzed by performing SDS–PAGE and detected by western blotting with the anti-MV-F or anti-myc MAb.

4. Discussion

We established and applied the reverse genetics system to wt MV in order to generate a recombinant virus carrying the EGFP marker gene, i.e. an EGFP-expressing recombinant virus. Our initial studies revealed that MV can infect some SLAM-negative cells, and this was consistent with the results obtained in our previous study on CDV (Fujita et al., 2007). Since attachment to a cell surface receptor is a critical step in viral infection and entry into the cell, some important molecules are suggested to be involved in the MV infection of SLAM-negative cells.

In this study, we demonstrated that MV can replicate in various liver cell lines (Fig. 3). However, its infectivity towards the human liver cell lines was considerably lower than that towards B95a and 293/SLAM cells that express SLAM as a receptor.

Griffin have suggested that MV spreads to a wide range of organs including skin, conjunctivae, kidneys, lungs, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver (Griffin, 2007). Furthermore, they reported that the mechanism of MV replication in the liver, particularly in the bile duct epithelium, is common in all age groups; however, clinically evident hepatitis is most frequent in adults (Griffin, 2007). The MV infection spreads to many epithelial surfaces, and this may directly cause gastrointestinal symptoms. Recently, it has been reported that the DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) plays an important role in MV infection of DCs (de Witte et al., 2006). Nevertheless, in contrast to SLAM and CD46, DC-SIGN does not support MV entry, but only functions as an attachment receptor for MV to enhance the SLAM/CD46-mediated infection of DCs. These studies suggest that the broad range of infections caused by MV in SLAM-negative cells may involve interactions with (as yet) unidentified receptor(s).

HP and HS belong to a class of carbohydrates designated as glycosaminoglycans, which are unbranched polymers of repeating disaccharide units. They are found as proteoglycans covalently linked to membrane proteins on the surface of most mammalian cells. They are classified as CS, dermatan sulphate (DS), HP/HS, keratan sulphate (KS), and hyaluronic acid (HA). HP is produced by mast cells, and more than 85% of its glucosamine residues are *N*-sulphated (Jacobsson and Lindahl, 1980). On the other hand, HS is produced by almost all cell types, and only 40–60% of its glucosamine residues are *N*-sulphated. The most prominent physicochemical property of glycosaminoglycans is that they carry a large and varying number of negative charges that are conferred onto the molecule by sulphate residues in most glycosaminoglycan types (Lindahl and Kjellén, 1991). The attachment of a number of viruses to the cell surface is known to involve proteoglycans (Jackson et al., 1991).

Baron reported that HS is important for RPV (RBOK strain) infection (Baron, 2005). It has been shown that wild-type RPV requires CD150 (SLAM) as a receptor, while the cell culture-adapted vaccine strain has acquired the ability to use HS as an alternative receptor. We tested the effect of HP by using the RPV-Lv strain and observed that interactions with HS observed in RPV infection were consistent with this previous report, although the Lv strain is a lapinized (i.e. attenuated by serial passage in rabbits) RPV strain (data not shown). In addition, we previously reported that an HP-like molecule is an important factor for CDV infection and that wt CDV also uses this molecule for infecting SLAM-negative cells (Fujita et al., 2007). In our present study, we showed that an HP-like molecule is also implicated in the entry of MV into cells by competitive inhibition assay using HP and rMV-EGFP (Fig. 3). The rMV-EGFP infection of SLAM-negative HEK-293 cells and liver cell lines was greatly inhibited by treatment with soluble HP. Furthermore, the SPR assay clarified that purified virions interacted with soluble HP; and HP affinity chromatography revealed the binding of the envelope H glycoprotein to immobilized HP (Figs. 4 and 5). Therefore, HP/HS commonly play an important role in the infection of morbilliviruses including MV, CDV, and RPV, and thus HP/HS or a related molecule could be a target for inhibition of morbilliviruses in non-lymphatic organs.

Our observation that MV (HL strain) infection was inhibited by HP is interesting because it has been reported that soluble HP does not inhibit the infection of Vero cells with the Edmonston vac-

cine strain of MV (Feldman et al., 2000). This MV strain can use CD46 as well as SLAM as cellular receptors (Naniche et al., 1993; Dörig et al., 1993) and these receptors are expressed in a wide range of human cells, except for erythrocytes. Vero cells express the high-affinity receptor CD46; therefore, the effect of soluble HP on MV attachment may be inconspicuous. In contrast, wild-type MV strains isolated from the marmoset B cell line B95a or from human B cell lines are unable to use CD46 as a receptor (Ono et al., 2001). Since we used recombinant viruses derived from cDNA clones of wild-type MV strain (HL strain), we could study the implication of HP-like molecules in the natural infection of MV in SLAM-negative human cells. In our experiment, the rMV-EGFP infection of 293/SLAM cells was not significantly inhibited, probably because this infection was mainly mediated by the high-affinity receptor SLAM and the contribution of HP-like molecules was therefore small.

In summary, we demonstrated the involvement of HP-like molecules in MV infection. The rMV-EGFP infection was not completely blocked by the treatment with HP. Thus, other molecule(s) must be involved in the attachment process, and the interaction between MV and HP-like molecules on the cell surface may promote the binding of MV envelope glycoproteins to a receptor that has not (yet) been identified.

Acknowledgements

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Inhibition of host protein synthesis in B95a cells infected with the HL strain of measles virus

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Abstract

The shut-off of host protein synthesis in virus-infected cells is one of the important mechanisms for viral replication. In this report, we showed that the HL strain of measles virus (MeV-HL) as well as other field isolates, which were isolated from human blood lymphocytes using B95a cells, induce the shut-off in B95a cells. Since the Edmonston strain of MeV failed to induce the shut-off in B95a cells, the ability to induce the shut-off was considered to be dependent on virus strains. Although, the modification of eukaryotic translation initiation factors (eIF) including eIF4G, eIF4E, and 4E-BP1 was reported for shut-off by various viruses, the involvement of these eIFs was not observed in MeV-HL-infected B95a cells. Instead, the accumulation of phosphorylated eIF2α was found to coincide to the decrease of host protein synthesis, suggesting the involvement of phosphorylation of eIF2α in inhibition of translation as one of the mechanisms of the shut-off.

Keywords: Measles virus; Shut-off; eIF2α

Résumé

La suppression de la synthèse protéique de la cellule hôte au cours de l'infection est un des mécanismes majeurs de la réplication virale. Dans cette étude nous avons montré que la souche HL du virus de la rougeole (MeV-HL) ainsi que d'autres souches sauvages du virus, isolées dans

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des cellules B95a à partir de lymphocytes sanguins humains, induisent ce type de suppression dans les cellules B95a. Comme la souche Edmonston du virus de la rougeole est incapable d'induire cette suppression dans les cellules B95a, cette propriété a été considérée comme dépendante de la souche virale. Bien qu'il ait été observé une extinction de l'expression des facteurs d'initiation de la traduction eucaryote (eIF) dont eIF4G, eIF4E et 4E-BP1 par de nombreux virus, il n'a pas été vu d'implication de ces facteurs dans les cellules B95a infectées par MeV-HL. Par contre, dans ces cellules on a montré que l'accumulation de la forme phosphorylée de eIF2a est concomitante à la diminution de la synthèse protéique, suggérant l'inhibition de la traduction par phosphorylation de eIF2a dans pourrait être un des mécanismes de la suppression de la synthèse protéique.

Mots clés : virus de la rougeole ; eIF2α ; suppression de la synthèse protéique

1. Introduction

One of the most striking changes observed in the cells infected with certain viruses is the almost complete inhibition of the translation of host mRNAs in the presence of effective translation of viral mRNAs [1]. Such inhibition of host protein synthesis (shut-off) have been reported in the infection with picornaviruses, adenovirus, influenza virus and vesicular stomatitis virus (VSV) and considered to occur at the stage of host translational level, as cellular mRNAs are recovered as an intact and functionally active form from the virus-infected cells [2–5]. Modification of eukaryotic initiation factors (eIFs) including the subunit of eIF4F complex (e.g., eIF4G and eIF4E) is observed in these virus-infected cells [6], and the modification of eIF4F by the viral infection resulting in the inhibition of cap-dependent translation is proposed as one of the mechanisms for shut-off.

Measles virus (MeV) belongs to the genus *Morbillivirus* within the family Paramyxoviridae, the genome of which is a single-stranded RNA with negative polarity. The MeV mRNA has a cap structure at 5′ end of the mRNA and is thought to be translated in a cap-dependent manner [7]. The Edmonston strain of MeV (MeV-Ed) has been reported not to induce the host shut-off of host protein synthesis [8,9]. Although most information on MeV–cell interaction has been obtained from the studies on MeV-Ed in epithelial or epithelial-like cells such as CV-1, Hela and Vero cells, accumulating evidence obtained by the use of lymphoblastoid B95a cells has suggested that MeV circulating in human is heterogenous and MeV-Ed represent minor subpopulation of the virus selected during long passage in cell cultures [10].

In the present study, we examined the effect of the HL strain of MeV (MeV-HL), which was isolated using B95a cells from a measles patient and maintains virulence in monkeys [10], on the host protein synthesis in B95a cells and found that MeV-HL induces marked shut-off of host protein synthesis. As the mRNA level of host proteins was not altered in MeV-HL-infected B95a cells, we focused on possible modification of translation factors involved in the cap-dependent translation initiation to clarify the mechanisms involved in the induction of the shut-off of host protein synthesis by MeV-HL infection.

2. Materials and methods

2.1. Cell and viruses

B95a cells [11] were grown in RPMI1640 supplemented with 5% fetal calf serum (FCS). As a typical virus isolated from measles patient, MeV-HL and two other field isolates, 9106 and 9301 strains [12] were used. MeV-Ed that was passed twice in B95a cells was also used. Virus infectivity titers were determined in B95a cells and expressed as a 50% tissue culture infectious dose ($TCID_{50}$). For examining shut-off of host protein synthesis, a monolayer culture of B95a cells was infected with MeV with a multiplicity of infection (MOI) of 1 $TCID_{50}$.

2.2. Metabolic labeling of cells

B95a cells were mock-infected or infected with MeV and then labeled with [^{35}S] EXPRESS (PerkinElmer, MA, USA) in methionine- and cystein-free RPMI1640 with 2% FCS for 1 h at 0, 11, 17, 23 and 35 h post-infection (hpi). Cells were lysed in lysis buffer C (125 mM NaCl, 20 mM Tris–HCl pH 8.0, 0.5% NP-40). Cell lysates were centrifuged and the supernatants were collected. Proteins were electrophoresed in SDS-PAGE gels, and ^{35}S-labeled proteins were visualized with autoradiography using X-ray film or quantitated with a phosphoimager plate in BAS 2000 (Fujifilm, Tokyo, Japan).

2.3. Real-time RT-PCR

Real-time RT-PCR was used to determine the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and 18S rRNA in accordance with the method described by Sato et al. [13].

2.4. Antibodies

Rabbit polyclonal antibodies against eIF4G and eIF2α, goat polyclonal antibodies against phospho-eIF4E, 4E-BP1 and β-actin and mouse monoclonal antibody against eIF4E were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Rabbit polyclonal antibody against phospho-eIF2α was purchased from Cell Signaling Technology (MA, USA). Horseradish peroxidase-conjugated secondary antibodies against rabbit, goat or mouse immunoglobulin were purchased from DAKO (Glostrup, Denmark).

2.5. Western blotting assay

Mock- or MeV-HL-infected B95a cells were lysed with lysis buffer C containing 1 mM PMSF, 1 mM benzamidine, 1 μg/ml aprotinine, 100 μM NaF and 1 μM Na_3VO_4. Equal amounts of protein extracts were subjected to SDS-PAGE and the proteins were transferred onto Hybond-N(+) nitrocellulose membrane (GE Healthcare UK Ltd., Buckinghamshire, UK). Detection of 4E-BP1 was performed as described previously [14]. Western blotting assay was performed with each antibody according to the recommendations of

manufacturer. Bands were visualized using an ECL plus detection reagent (GE Healthcare UK Ltd.). The intensity of bands was quantitated by scanning with LAS-1000 mini and Imagegauge software (Fujifilm).

2.6. Establishment of cell lines stably expressing eIF2α

Human eIF2α cDNA was obtained with PCR using the sense primer (5′-GCGGGAATCACACACATACCTCAGAA-3′) and antisense primer (5′-TCAAGTCTAG-GATTTACAGCCAGGAAGCGC-3′) with reverse transcription (RT) products from the mRNA of HeLa cells and was then subcloned into pCR2.1-TOPO vector (Invitrogen, CA, USA). Phosphorylation site at serine 51 of eIF2α cDNA was mutated to alanine (S51A) using a PCR-based mutagenesis strategy with Pfu turbo polymerase (Stratagene, CA, USA) to obtain the cDNA of S51A mutant eIF2α. The eIF2α cDNA was inserted into a pCMV-myc expressing vector (Clontech, CA, USA). The myc-tagged eIF2α (wild type or S51A mutant) expression vector (1.5 μg) and 0.5 μg of pCDNA3.1 (Invitrogen) were co-transfected to B95a cells with DMRIE-C reagent (Invitrogen) according to the recommendations of manufacturer. After incubation for 24 h, the cells were replated to 150-mm dishes and cultured in RPMI1640 with 5% FCS and 500 μg/ml G418 (bioactive; Invitrogen). G418-resistant colonies were selected approximately 2 weeks later. Expression of myc-tagged proteins was confirmed by Western blotting assay using a monoclonal antibody against myc tag (Clontech).

3. Results

3.1. Effect of several strains of MeV on host protein synthesis in B95a cells

Effect of MeV-HL-infection on protein synthesis was shown in Fig. 1a. The rate of host protein synthesis was determined by quantitation of the total radioactivity of one lane except four bands of N, P, M and H derived from MeV-HL. The ratio of host protein synthesis in MeV-HL-infected B95a cells to that in mock-infected cells was shown in Fig. 1b. The relative rate of viral protein synthesis to host protein synthesis, which was determined by sum of the radioactivity of four viral proteins and that of host proteins, was also shown in Fig. 1b. A marked decrease of host protein synthesis was observed between 18 and 36 hpi. On the other hand, relative viral protein synthesis to host protein synthesis increased and reached a peak at 24 hpi. This result indicates that MeV-HL induces shut-off of host protein synthesis in B95a cells.

Subsequently, other field isolates of MeV, 9106 strain and 9301 strain were also examined for their ability to induce the shut-off (Fig. 2a right). The autoradiograph gel was stained with Coomassie brilliant blue before drying the gel to confirm that total protein levels are equal (Fig. 2a left). As shown in Fig. 2a, 9106 and 9301 strains also induced the inhibition of host protein synthesis at 24 hpi similar to MeV-HL. The effect of the MeV-Ed, which was reported not to induce shut-off in CV-1 or Hela cells, was tested in B95a cells. Host protein synthesis in MeV-Ed-infected B95a cells was not inhibited until 24 hpi when the inhibition was clearly observed in MeV-HL-infected cells (Fig. 2b). These results

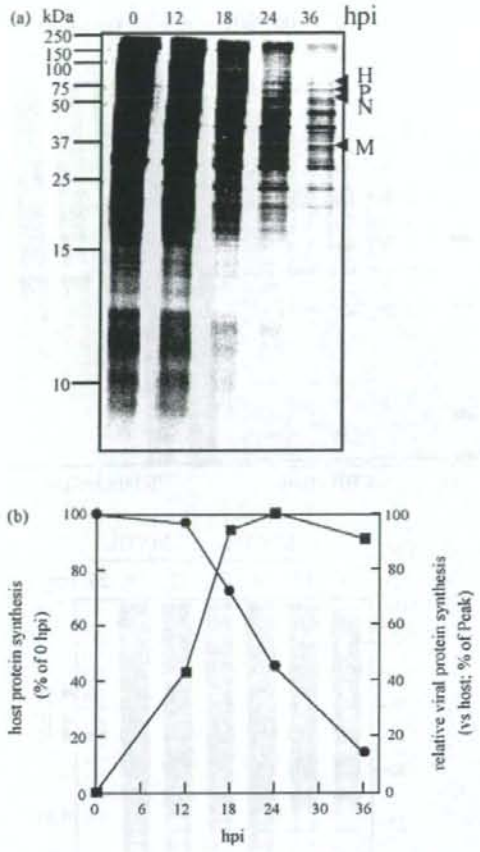
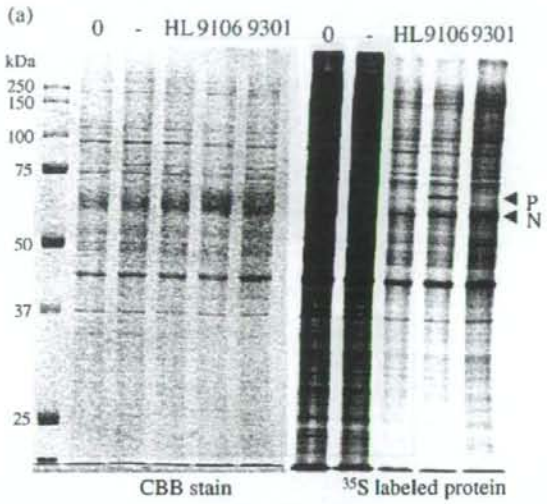


Fig. 1. Protein synthesis in B95a cells infected with MeV-HL. (a) B95a cells infected with MeV-HL were labeled with a mixture of [^{35}S] methionine/cysteine for 1 h and collected at the indicated time. Labeled proteins were separated by 12% SDS-PAGE gel. The proteins derived from MeV-HL are indicated to the right of the image. (b) Quantitation of host protein synthesis (closed circle) and relative viral protein synthesis to host protein synthesis (closed square). The rates of protein synthesis were determined from images as described in the text.

indicate that field isolated MeVs, which maintain their virulence, have an ability to induce shut-off of host protein synthesis, whereas only the MeV-Ed does not.

3.2. *Effect of MeV-HL infection on GAPDH mRNA*

The expression level of the GAPDH gene, one of house keeping genes, was measured as a representative to determine whether MeV-HL-induced inhibition of host protein synthesis occurs at the transcription stage (Fig. 3). The relative expression level of GAPDH



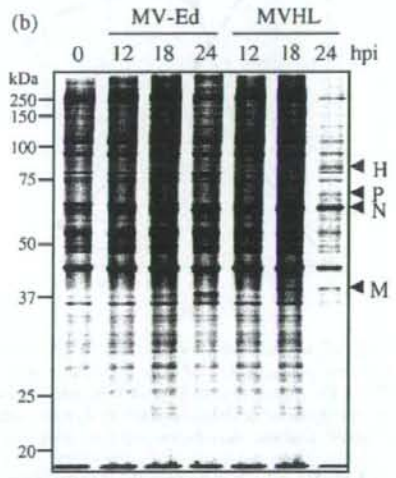


Fig. 2. The shut-off effect of other strain of MeV (a) Protein synthesis at 24 hpi in B95a cells infected with field isolates of MeV was examined similar to Fig. 1a (right panel) (0: 0 hpi; - :mock-infected B95a cells). To confirm the equivalence of protein, the autoradiograph gel was stained with Coomassie brilliant blue (left panel). (b) The protein synthesis in MeV-Ed- or MeV-HL-infected B95a cells was examined similar to Fig. 1a.

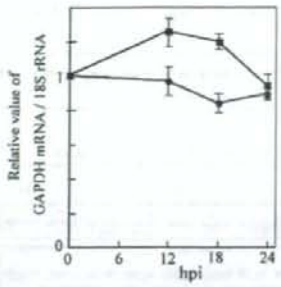


Fig. 3. The mRNA levels of GAPDH in B95a cells infected with MeV-HL. The expression levels of GAPDH mRNA in mock- (closed circle) or MeV-HL- (closed square) infected B95a cells were determined using one-step real-time RT-PCR and shown as means of three experiments.

mRNA to 18S rRNA was not suppressed by MeV-HL infection. Since the transcription level of GAPDH did not decrease during the period of shut-off, the inhibition of host protein synthesis in MeV-HL-infected B95a cells is suggested to occur at a post-transcription stage.

3.3. Modification of eIF4G, eIF4E and 4E-BP1 by MeV-HL infection

Previous reports on other viruses indicated that cap-binding proteins such as eIF4G, eIF4E, and 4E-BP1 are major targets for the virus-induced shut-off of host protein synthesis [6]. Picornavirus (except for cardiovirus) cleaved the eIF4G, which is one of the subunits of cap-binding complex eIF4F, by viral protease, $2A^{pro}$ [15–17]. This cleavage results in inhibition of binding of eIF4F to cap of host mRNA. In adenovirus- or influenza virus-infected cells dephosphorylation of eIF4E, which is a cap-binding protein, is observed [18–20]. Phosphorylation of eIF4E increases its affinity for the cap of mRNA [21]. Therefore, dephosphorylation of eIF4E by viral infection results in decrease of the affinity for the cap and may inhibit cap-dependent translation. The eIF4E is also regulated by eIF4E-binding protein-1 (4E-BP1). Encephalomyocarditis virus (EMCV), poliovirus and VSV dephosphorylate 4E-BP1 [22,23]. Dephosphorylated 4E-BP1 binds to eIF4E strongly, resulting in the suppression of cap-dependent translation. Considering these functions of cap-binding proteins, we first examined the characteristics of these three proteins in B95a cells at intervals after inoculation with MeV-HL. As shown in Fig. 4a, eIF4G was not cleaved throughout the course of MeV-HL-infection. Moreover, dephosphorylation was not observed for eIF4E and 4E-BP1 until 36 hpi (Fig. 4b and c). These results indicate that eIF4G and eIF4E are not involved in MeV-HL-induced shut-off of host protein synthesis as their function appear to be intact.

3.4. Accumulation of phosphorylated eIF2α in MeV-HL-infected B95a cells

Given that the modification of eIF4F was not detected in MeV-HL-infected B95a cells, we then focused on phosphorylation of eIF2α. It was reported that the interferon-inducible

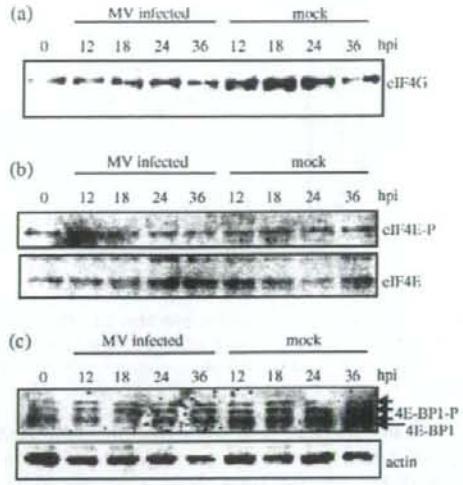


Fig. 4. Modification of the components of the eIF4F complex in MeV-HL-infected B95a cells. Expression levels of eIF4G and the phosphorylated states of eIF4E and 4E-BP1 in mock- or MeV-HL-infected B95a cells were determined by Western blotting assay. (a) Cell lysates were subjected to 6% SDS-PAGE and the proteins were transferred onto nitrocellulose membrane. The eIF4G was detected by Western blotting assay using rabbit antibody against eIF4G. (b) Detection of phosphorylated eIF4E by Western blotting assay using antibodies against phospho-eIF4E at serine 209 (upper panel) or eIF4E (lower panel) antibody. (c) The phosphorylation state of 4E-BP1 in the mock- or MeV-HL-infected B95a cells was examined by Western blotting assay using goat antibody against 4E-BP1. The quantity of protein was normalized to that of β-actin determined by goat antibody against β-actin.

PKR, known as a kinase that phosphorylate eIF2α at serine 51 [24], is activated by dsRNA during the infection with RNA viruses and involved as a host defense in preventing the translation of viral transcripts, concomitantly with the inhibition of host mRNA translation [25]. Considering such function of eIF2α, we analyzed the phosphorylation state of eIF2α by Western blotting assay with an antibody against phospho-eIF2α or eIF2α (Fig. 5). The ratio of phospho-eIF2α in MeV-HL-infected B95a cells increased after 12 hpi and reached a maximum (3.9-fold increase) at 18 hpi, although the effect was lower than that observed in the control with thapsigargin that induces eIF2α phosphorylation through ER-stress [26]. Thereafter, the ratio was sustained until 36 hpi. Phosphorylation of eIF2α occurred at a relatively early stage of infection, prior to the clear inhibition of host protein synthesis. The acceleration of host shut-off was accompanied by an increase in phosphorylation of eIF2α.

3.5. Suppression of MeV-HL-induced phosphorylation of eIF2α in B95a cells stably expressing S51A mutated human eIF2α

Involvement of phosphorylation of eIF2α in shut-off of host protein synthesis in MeV-HL-infected B95a cells was examined using B95a cells that stably express

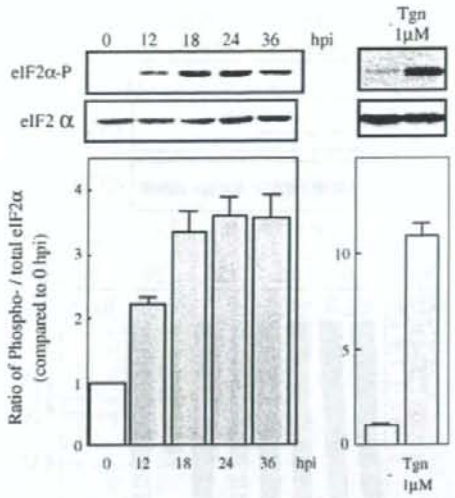


Fig. 5. Phosphorylation of eIF2α in MeV-HL-infected B95a cells. Lysates of mock- or MeV-HL-infected B95a cells were analyzed by Western blotting assay using antibodies against eIF2α-P (phosphorylated form at serine 51) or eIF2α on the same membrane (left, top). Quantitation of the relative amounts of phospho-eIF2α and the total eIF2α was measured and the ratio of phosphorylated eIF2α to total eIF2α (vs. 0 hpi) is shown as a bar graph (left, bottom). As a control experiment, B95a cells were treated with 1 μM thapsigargin for 1 h and shown as the same way as left column (right). The values are means ± standard errors of triplicate determinations.

eIF2α mutant, of which phosphorylation site serine 51, was replaced to alanine (B95a-2αS51A) and is able to inhibit the phosphorylation of endogenous eIF2α [27]. As a control experiment, the B95a cells that stably express wild type of eIF2α (B95a-2αWT) were used. The phosphorylation rate of total eIF2α in B95a-2αWT cells apparently increased at 18 hpi (Fig. 6a), whereas that in B95a-2αS51A cells was significantly inhibited. Shut-off of host protein synthesis was noted from 12 hpi in B95a-2αWT cells similar to the parental B95a cells. In B95a-2αS51A cells, shut-off of host protein synthesis was suppressed until 18 hpi (Fig. 6b and c) and the rate of host protein synthesis was higher than that of B95a-2αWT cells throughout the test period. These results indicate that the phosphorylation of eIF2α involved in shut-off of host protein synthesis in MeV-HL-infected B95a cells.

4. Discussion

In the present study, we showed that MeV-HL induces the shut-off of host protein synthesis in B95a cells. This shut-off is not specific feature of MeV-HL because other field isolates, 9106 and 9301 strain, also induce the shut-off in B95a cells. On the other hand, MeV-Ed that has been reported not to induce the shut-off in epithelial or epithelial-like cells did not induce the shut-off of host protein synthesis in B95a cells as well. Therefore, the inability of MeV-Ed to induce shut-off is suggested to be a characteristic of this strain

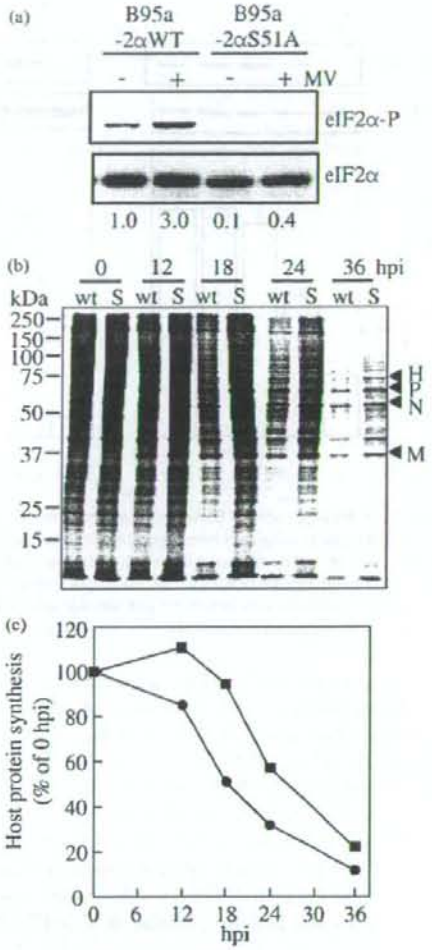


Fig. 6. MeV-HL-induced shut-off in eIF2αWT and S51A expressing cells. (a) eIF2α was detected by Western blotting assay using antibodies against phospho-eIF2α (upper panel), or eIF2α (lower panel) on the same membrane. The ratio of phosphorylated eIF2α (vs. mock infected B95a-2αWT) is shown under each lane. (b) Protein synthesis in MeV-HL-infected B95a-2αWT (wt) and B95a-2αS51A (S) cells was examined similar to Fig. 1a. Viral proteins are indicated to the right of the image. (c) The rates of host protein synthesis in B95a-2αWT cells (closed circle) or B95a-2αS51A (closed square) were determined from Fig. 6b by quantitation similar to Fig. 1b.

and independent of cell type. Similarly to MeV, Smith et al. reported that ability of reovirus to induce the shut-off of host protein synthesis is dependent of the viral strain [28].

The shut-off of host protein synthesis by virus infection was reported to be caused by a number of mechanisms such as inhibition of transcription, degradation of host mRNA and inhibition of translation. As the level of GAPDH mRNA was unaltered in MeV-HL-infected B95a cells, the shut-off by MeV-HL is suggested to be caused by inhibition of translation.

The shut-off of host translation is caused mainly by inhibition of the cap-dependent mechanism [6]. Contrary to many other virus-infected cells in which the components of the eIF4F complex including eIF4G, eIF4E and 4E-BP1 are involved in cap-dependent translation, they were not modified by MeV-HL infection. Therefore, the cap-binding activity of eIF4F complex appears to be intact. Instead, phosphorylation of eIF2α in MeV-HL-infected B95a cells was noted (Fig. 5). The phosphorylation rate of eIF2α correlated with the inhibition of host protein synthesis after MV infection. In addition, in B95a-2αS51A cells that stably expressed the eIF2α-S51A mutant, the shut-off phenomenon appeared to be suppressed compared with those in B95a and B95a-2αWT cells (Fig. 6). Therefore, phosphorylation of eIF2α is suggested as one of the mechanisms particularly at the early stage for the induction of host shut-off by MeV-HL infection.

Conner and Lyles reported that phosphorylation of eIF2α in VSV-infected cells suppressed viral translation rather than host translation [22]. In the case of MeV-HL infection, the suppression effect on host proteins was obviously much greater than that on viral proteins (Figs. 1a and 6b). MeV-HL mRNA may be more resistant to the effect of phosphorylated eIF2α than cellular mRNA. The mechanisms of the selective synthesis of viral protein in the shutoff stage of MeV-HL-infected cells are currently under investigation.

Recently, we also reported that the N protein of MeV-HL inhibits host translation by the binding to eIF3-p40 [13]. In our report, the inhibitory effect of the N protein is partial and inhibitory rate reaches a plateau at approximately 50–60%. On the other hand, MeV-HL-infection suppressed about 90% of the host translation (Fig. 1b). Experiment using eIF2αS51A mutant in this study, in which the inhibition of eIF2α phosphorylation observed in18 hpi lasted 24 hpi (data not shown) showed that the shut-off was inhibited at 18 hpi but became partial after 24 hpi (Fig. 6c). The expression level of the N protein increases rapidly after 18 hpi and reaches a peak at 24 hpi (data not shown). Taken together, we hypothesize that in MeV-HL-infected B95a cells the accumulation of phosphorylated eIF2α probably resulting from the replication of viral genome occurs at a relatively early stage of infection initiating the shut-off and then binding of increased N protein binds to eIF3-p40 and enhance the shut-off of host translation at later stage of infection.

Acknowledgement

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日消外会誌　41（2）：229～234，2008年

症例報告

脾辺縁帯リンパ腫の1切除例

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内藤　嘉紀　　久米　　徹[1]　　内藤　雅康[1]
橋口　道俊[2]　　岡村　　孝[2]　　江里口直文[3]
内藤　壽則[3]　　大島　孝一　　矢野　博久

症例は64歳の女性で，熱発，左腰痛を主訴に受診．4年前よりB型慢性肝炎の指摘あり．腹部CTで脾内腫瘍を指摘され，PETでは脾臓に異常集積を認めた．以上より，脾臓原発悪性リンパ腫を疑い組織診断および腫瘍減量目的に膵尾部合併脾臓摘出術が施行された．脾臓は19×15cmと著明な腫大を認め，割面では多発性に白色結節状の腫瘍性病変を認めた．組織学的には，円形から類円形の核を有する中型異型細胞が結節様構造を呈しながら浸潤増殖し，免疫染色では，CD20が陽性であった．以上より，脾辺縁帯リンパ腫（B cell type）と最終診断した．術後早期に全身リンパ節腫脹が出現し，血液内科に緊急入院．入院当日よりTHP-COP療法を開始，全身症状は改善傾向を認め自宅退院となり，手術後13か月現在，外来定期通院中である．本症例は，手術後の急性増悪に対して肝炎の増悪なく化学療法導入に成功した症例である．

はじめに

脾辺縁帯リンパ腫（splenic marginal zone lymphoma：以下，SMZL）は，脾臓において辺縁から白脾髄を取り囲むように進展し，濾胞を消失させ，髄内において小型リンパ球が増殖するB細胞由来の悪性リンパ腫であり，WHOの分類ではmarginal zone lymphomaの一亜型として定義されている[1]．Papadakiら[2]の報告においても，脾辺縁帯のB細胞由来であると示唆され，増殖形態は結節性増殖を示すものが最も多いとされている．また，SMZLは病期の進行が緩徐であるとされており，治療選択は以前まで摘脾が第1選択とされているが，近年化学療法にCD20抗体を追加する方法など多く検討がなされている[3][4]．今回，我々はB型慢性肝炎患者に発症したSMZLに対して脾臓摘出術を行い，術後急速に全身リンパ節腫脹を来した症例を経験したので報告する．

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症　例

患者：64歳，女性

主訴：熱発，左腰痛

既往歴：4年前にB型慢性肝炎を指摘．

家族歴：特記事項なし．

現病歴：平成18年3月頃より発熱（弛張熱型），左腰痛を認め当院受診．腹部超音波検査にて脾腫，脾内腫瘍および腹部リンパ節腫脹を認め血液内科紹介受診となった．

入院時現症：身長154cm，体重54kg，血圧124/80mm/Hg，脈拍76回/分，体温38℃，表在部リンパ節腫脹は認めない．腹部は平坦であるが，脾臓は触知可能であった．

入院時血液検査所見：白血球が異常高値を認めたが，血小板については正常範囲内であった．また，LDHおよびIL2-Rの異常高値が認められた．HBs抗原は陽性であった（**Table 1**）．

腹部CT所見：動脈相および門脈相で脾腫および脾内腫瘍を認めた．また，脾門部を中心とする腹部リンパ節腫脹がみられた．他の臓器に腫瘍性病変は認めなかった（**Fig. 1**）．

Table 1　Laboratory date on admission

WBC	18,700 /mm³	BUN	19.1 mg/dl	HBs Ag	(+)
Stab	5 %	Cre	0.62 mg/dl	HBV-DNA	4.7 LGE/ml
Seb	80 %	Na	144 mEq/l	HCV-Ab	(−)
Baso	0 %	Cl	108 mEq/l	VDRL	(−)
Mono	0 %	K	3.8 mEq/l	TPHA	(−)
Lym	8 %				
RBC	387×10⁴ /mm³	CA19-9	8 U/ml	IL2-R	33,848 U/ml
Hb	11.9 g/dl	CEA	1.1 ng/ml		
Ht	35.6 %				
Plt	14.1×10⁴ /mm³	FBS	75 mg/dl		
AST	46 U/l				
ALT	24 U/l	CRP	(4+)		
LDH	1,503 U/l				
ALP	344 U/l				
γ-GTP	37 U/l				
T-Bil	1.2 mg/dl				

Fig. 1　Enhanced abdominal CT showed a nodular lesion in the spleen.

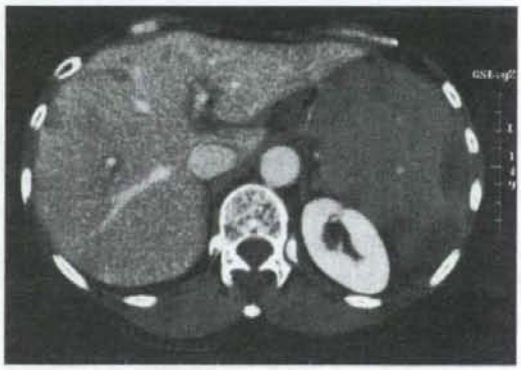
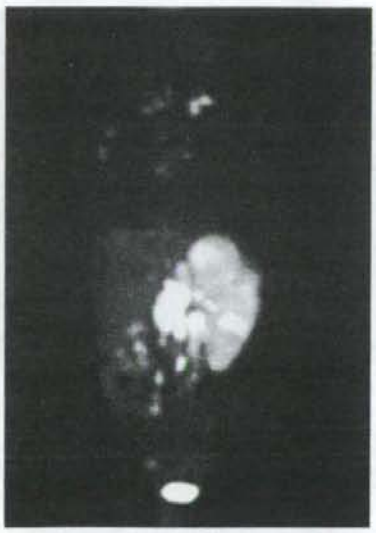


Fig. 2　PET demonstrated FDG uptake of nodular lesion of the spleen (FDG-SUV max 5.6).



PET所見：脾臓および左腸管膜領域に異常集積を認めた(FDG-SUV max 5.6)．また，頸部リンパ節や腋窩リンパ節の異常集積を認めた(Fig. 2)．

以上の所見より，脾臓原発悪性リンパ腫 Stage IV を考えた．治療については，腫瘍減量，組織診断目的に脾臓摘出術を施行した後に化学療法導入予定とした．

手術所見：上腹部正中切開にて開腹した．脾臓は著明な腫大を認めた．腹水はみられなかった．脾門部を中心に腹腔内リンパ節が腫脹しており，脾臓のみの摘出は困難であったため，膵尾部合併脾臓摘出術とした．

病理組織学的検査：脾臓は19×15cmと著明な腫大を認め，割面では多発性に白色結節状の腫瘍性病変を認めた(Fig. 3)．組織学的には，円形から類円形の核を有する中型異型細胞が結節様構造を呈しながら浸潤増殖していた(Fig. 4)．免疫染色では，CD20(+)，CD45RO(−)，CD10(−)であった(Fig. 5)．また，Flowcytometric analysisではCD19(+)，CD20(+)，κ>>λ，CD25(+)であった．以上より，Splenic marginal zone lym-

Fig. 3 Spleen measuring was 19×15cm. The cutting surface was showed that tumor in the spleen was multiple nodular lesions, white in color (arrow head).

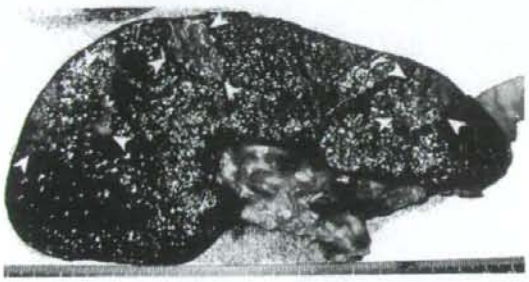
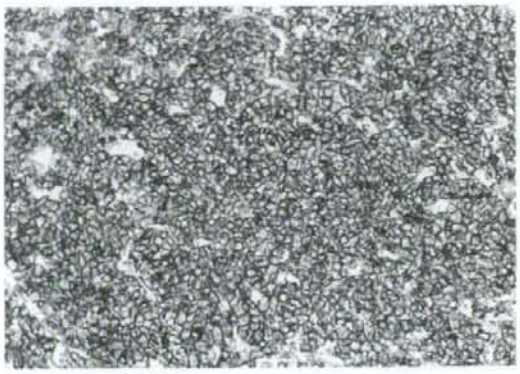


Fig. 4 The tumor was characterised by micronodular infiltration of the spleen (HE stain ×12.5).

phoma (B cell type) と最終診断した.

術後経過：術翌日に左肺に胸水が出現し，胸腔ドレナージ術を施行し，1,200ml の排液を認めたが，胸水細胞診では異型細胞の出現はみられなかった．胸水の改善はないものの，全身状態は比較的安定していたため術後 6 日目からラミブジン 100mg を内服開始となった．術後 14 日目頃より頸部および腋窩リンパ節の痛みを伴う腫脹および呼吸苦が出現した．また，白血球の異常高値も続いていることから，悪性リンパ腫の骨髄浸潤が考えられ術後 24 日目に血液内科緊急入院となった．入院直後に胸水穿刺を施行，異型リンパ球の出現を認めたことから SMZL の急性増悪を考え，家族

Fig. 5 The tumor cells were positive for CD20 (CD20 ×100).

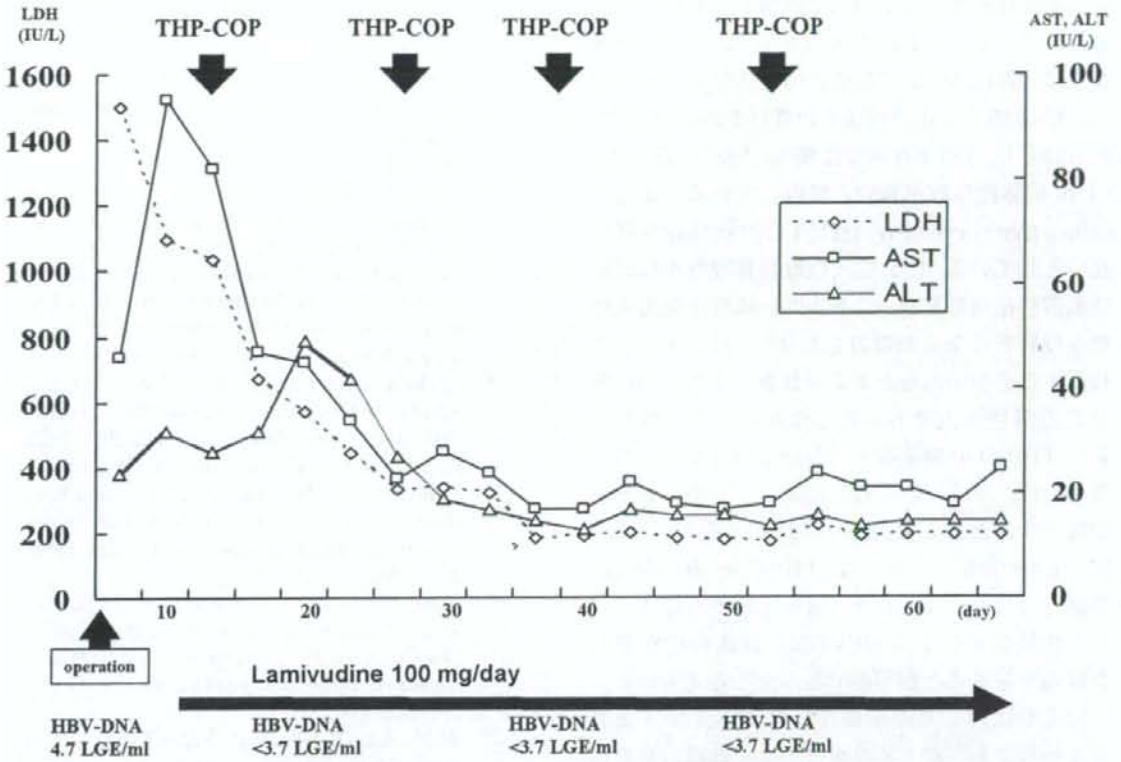


に対して十分なインフォームドコンセントを行い，同意のうえで入院当日より THP-COP 療法(塩酸グラニセトロン 3mg，塩酸ピラルビシン 50mg，ビンクリスチン 1.4mg，プレドニゾロン 100mg)4 コースを導入した．導入後，頸部リンパ節腫脹は改善傾向を示し，呼吸状態は安定した．また，初期には肝機能異常は認めたものの，術後早期からのラミブジン投与により HBV-DNA 4.7LGE/ml (正常値 3.7LGE/ml 未満) であったのが 3.7LGE/ml 以下と正常範囲まで低下した (Fig. 6)．THP-COP 療法導入後 7 日目に骨髄穿刺を施行したが，明らかな異型細胞の出現はみられなかった．THP-COP 療法 4 コース終了時の PET では術前指摘されていた全身のリンパ節の異常集積は指摘されなかった．手術後 13 か月現在，外来定期通院中である．

考　察

SMZL は，脾臓の辺縁帯 B 細胞由来の低悪性度 B リンパ腫とされ，我が国では全リンパ腫症例の 1% にも満たないと報告されている[5]．また，医学中央雑誌で「悪性リンパ腫」,「Splenic marginal zone lymphoma」をキーワードとして 1983 年から 2007 年までについて検索したところ，自験例を含めて 9 例が報告されているのみで，まれな疾患群として報告されている．近年では，肝疾患と悪性リンパ腫との関係についての検討も報告されており，1994 年 Ferri ら[6]の報告では，B-cell. NHL の 50 症例中 17 症例に HCV 抗体陽性であったと

Fig. 6　Clinical course



している．SMZL についての HCV の関連については，Arcaini ら[7]は HCV の血清陽性率を伴う SMZL は 19% であったとし，Interferon-α との関連についても近年検討されている[8][9]．また，血清 HBs 陽性を伴う SMZL の報告はあるものの[10]，B 型慢性肝炎との関連については報告が少なく，両者についての因果関係については不明である．

Chacon ら[4]の報告では，主症状として脾腫や全身症状が約半数以上でみられているが，腹痛は 33% と頻度的には低く，脾臓や骨髄の精査で診断されることが多いとされている．また，86.6% の症例で Stage IV であり，無痛性の疾患群であるため早期に発見されることが少ないと考えられる．今回，我々が経験した症例についても，病期が進んできたため自覚症状が出現し発見となったと推察される．

SMZL に対しての治療方針は，以前までは組織診断を目的とした脾臓摘出術を第 1 選択とされてきた[11]．本症例は明らかな表在リンパ節の腫脹がみられず手術以外の組織採取が困難であったことから，組織診断のために手術を施行することも必要であるといえる．一方で，手術翌日から胸水や全身のリンパ節腫脹が出現し，術後 24 日目には異型リンパ球が出現するという急激な進展経過を辿っている．脾臓摘出後の影響についての報告については，Thieblemont ら[12]は脾臓摘出術後においても骨髄浸潤する可能性を示唆し，多変量解析においても脾臓摘出術は独立した予後因子とはなっていないと報告している．また，西森ら[13]の報告でも，術後早期に急激な腹膜播種を生じ死亡していることから，骨髄浸潤のみでなく全身性に進展する可能性があると言える．本症例における急激な進展経過は手術侵襲による影響があった可能性もあり，組織診断を主な目的とする脾臓摘出術においても術後経過に対しては十分な観察が必要である．