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Effect of siRNA mediated suppression of signaling lymphocyte activation molecule on replication of peste des petits ruminants virus *in vitro*

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ABSTRACT

Signaling lymphocyte activation molecule (SLAM) expression was inhibited in B95a cell line using siRNA and the effect of SLAM inhibition on peste des petits ruminants virus (PPRV) replication and infectivity titre was studied. SLAM suppression was assessed using real-time PCR and flow cytometry to confirm suppression at the m-RNA and protein levels, respectively. Three chemically synthesized siRNAs were transfected individually using oligofectamine into B95a cell line. This resulted in SLAM suppression from 48 to 454-folds, in comparison to the untransfected B95a cell line. When the SLAM suppressed B95a cell line was infected with PPRV, replication was reduced by 12–143-folds and virus titre was reduced from $\log_{10}$ 1.09 to 2.28. siRNA 3 showed the most potent inhibition of SLAM expression both at m-RNA and protein levels. This also caused the maximum reduction of virus replication and virus titre. A 100-fold reduction in PPRV titres was seen in anti-SLAM antibody neutralized B95a cell line. This further confirms that SLAM is one of the (co) receptors for PPRV. However, the presence of other putative virus receptor(s) is/are not ruled out.

1. Introduction

The way in which cells respond to dsRNA by silencing homologous genes has revealed a new approach to study the function of many unexplored and existing genes. RNA interference (RNAi) technology can be employed to unleash the dormant potential of sequenced genomes, to identify targets for drug designing, to silence a disease-causing mutant allele specifically, in *ex vivo* manipulation of stem cells, in delaying ageing process, *etc.* It can also be exploited as a powerful tool to prevent virus multiplication, and has already been proven in inhibiting replication and spread of many viruses (Hu et al., 2002; Mohapatra et al., 2005; Mallanna et al., 2005).

Peste des petits ruminants (PPR) is a viral disease of goats and sheep with a widespread distribution across sub-Saharan Africa, the Arabian peninsula and the Indian subcontinent (Nanda et al., 1996). The causative agent peste des petits ruminants virus (PPRV) is classified in the *Morbillivirus* genus of the family *Paramyxoviridae*. This genus also includes rinderpest virus (RPV), canine distemper virus (CDV), human measles virus (MV) and viruses of marine mammals, phocine distemper virus of seals and the cetacean morbillivirus isolated from dolphins and porpoises (Tatsuo et al., 2001; Dhar et al., 2002).

Cellular receptors are one of the major determinants of the host range and tissue tropism of viruses. Signaling lymphocyte activation molecule (SLAM) or CD150 molecules have been reported as receptor for MV (Tatsuo et al., 2001), CDV (Tatsuo et al., 2001; Seki et al., 2003) and RPV (Tatsuo et al., 2001). RNAi could silence SLAM expression and inhibit the MV infection in B95a cells (Hu et al., 2005). SLAM is a 70 kDa glycoprotein belonging to the CD2 subset of the immunoglobulin (Ig) superfamily and is expressed on the surface of a proportion of primary B cells, Epstein-Barr virus (EBV) transformed B cells (B95a), activated T cells, memory T cells, T cell clones and immature thymocytes (Tangye et al., 2000). The present study was aimed at determining the effect of SLAM suppression in B95a cell line on PPRV replication.

2. Materials and methods

2.1. Cell line

B95a, an adherent cell line procured from Indian Veterinary Research Institute, Mukteshwar, India and maintained in our

Abbreviations: PPRV, peste des petits ruminants virus; siRNA, small interfering RNA; SLAM, signaling lymphocyte activation molecule.

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Table 1
Primers and probe sequences used in real-time PCR of B95a SLAM, beta actin and PPRV 'M' gene with TaqMan chemistry

Name	Sequence (5′ → 3′)	Nucleotide position[a]
B95a SLAM FP	CACTGTGAGCAACCCCATCA	638–657
Probe	**FAM** AGCTCCCAGGACTTCATTCCATGGC **TAMRA**	663–687
B95a SLAM RP	AGGGTTCTTGCCTGCATCTG	689–708
PPRV 'M' FP	CAACCTAGTCCCGCTTGATACTC	3814–3836
Probe	**FAM** CACAACCCTGAATCTC **MGB**	3841–3856
PPRV 'M' RP	GGCACACTATAGTAACCATTGTCTGAA	3876–3903
β-Actin FP	GGCTGTGCTATCCCTGTAC	463–481
Probe	**FAM** CTGGCCGTACCACTG **MGB**	486–500
β-Actin RP	CCGGAGTCCATCACGATGC	501–519

[a] Accession No. of B95a SLAM—AF257239; Accession No. of PPRV 'M' gene—AY560591; Accession no. of β-actin—AK308277.

laboratory was used for small interfering (si) RNA transfections and growth and assay of PPRV.

2.2. Real-time PCR with TaqMan probe for SLAM and PPRV 'M' gene

Total RNA was extracted from B95a cell line either infected with PPRV alone or after transfections with siRNAs using TRIzol (Invitrogen, USA) and cDNA was synthesized using the High capacity cDNA archive kit (Applied Biosystems Inc., USA). β-Actin was used as an endogenous control for real-time PCR. Separate master-mixes were prepared for endogenous control and target gene (SLAM or PPRV 'M' gene) using TaqMan Universal PCR master mix (Applied Biosystems Inc., USA, 2×, 10 μl), TaqMan primers and probe (1 μl, 20×) and 100 ng cDNA adjusted in 9 μl of DEPC water to give a total reaction volume of 20 μl.

Each reaction was prepared in triplicate. A no-template control (NTC) was prepared using only DEPC water (instead of cDNA) as above. The plate was centrifuged in the cooling (4 °C) plate centrifuge at 560 rpm for 3–5 min to rid the mix of any air bubbles. Then the plate was kept inside the real-time PCR machine (Applied Biosystems Inc., USA, Model 7500). The wells were located according to the NTC, Target and endogenous control using software of the machine. The program was run using the following universal cycling condition consisting of one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The Ct values were recorded for both the target and endogenous controls. The data was accepted only when the NTC had no amplification. The primers and probes used for the endogenous control and target genes are shown in Table 1.

2.3. siRNAs for B95a SLAM

siRNAs were designed using the online software tool available on Ambion website www.ambion.com and 3 different siRNAs were selected in different regions of target gene (Table 2) on the basis of the guidelines published by Ui-Tei et al. (2004) for chemical synthesis.

Table 2
siRNA for SLAM designed and chemically synthesized for transfection into B95a cells

SLAM siRNA	Position of target gene (B95a SLAM)	Target sequence (5′-3′)
SLAM siRNA 1	214–234	AACAGTGTCGAGAACAAAATA
SLAM siRNA 2	689–709	AAGAACCCTCAGAGACAAATA
SLAM siRNA 3	789–809	AAGAGGTAAAACGGACCATTA

2.4. siRNA transfection of B95a cells using oligofectamine and PPRV infection of SLAM-inhibited B95a cells

Transfection optimization was done with different concentrations of transfecting reagent, Oligofectamine (Invitrogen, USA), different concentration of siRNA and different cell densities. siRNAs were transfected into B95a cells using the method described by Volinia et al. (2006) and Ovchrenko et al. (2005). Briefly, 2.5 μl of oligofectamine (Invitrogen, USA) was added into 50 μl of Opti-MEM (Invitrogen, USA) and mixed gently and incubated for 5 min at room temperature. Then 120 pmol of each siRNA (6 μl from 20 μM stock) was added into 50 μl of Opti-MEM, mixed gently and kept for 5 min incubation. Both siRNA and oligofectamine complexes were mixed and kept for incubation at 37 °C for 45 min. Meanwhile B95a cells were trypsinized and cells were counted using haemocytometer, 4×10^5 cell concentration was used for siRNA transfections. siRNA and oligofectamine complexes (total volume 100 μl) per well were added to a 12 well plate and again 100 μl of Opti-MEM was added to the wells. 4×10^5 cells were added to each wells and antibiotic free growth medium (5% FCS) was added to make the final volume up to 500 μl. Cells was incubated under a 5% CO_2 at 37 °C for 48 h. After 48 h incubation, media was changed and again 50 pmol of siRNA added to the cells (50 pmol of siRNA in 1 μl of oligofectamine). Again cells were incubated under a 5% CO_2 at 37 °C for next 24 h. 72 h after post original siRNA transfection, one set of cells were used for RNA extraction to study inhibition of B95a SLAM using real-time PCR. Another set of cells was infected with $10^3 TCID_{50}$ of PPRV after 72 h of initial siRNA transfection and cytopathic effect (CPE) observed. After 48 h post virus infection, cells were frozen-thawed and used for virus titration (Dhinakar Raj et al., 2000) and another set of cells used for RNA extraction to study effects of SLAM inhibition on virus replication in terms of PPRV 'M' gene expression. As a negative control, chemically synthesized siRNA designed for Newcastle disease virus (NDV) that was part of another study was used.

2.5. Expression and inhibition study of B95a SLAM using flowcytometry

Flow cytometry was used to assess reduction in SLAM expression following siRNA transfections of B95a cells using the anti human SLAM-FITC conjugate (eBiosciences, USA) that cross-reacted with B95a SLAM. Briefly, 72 h post siRNA transfection B95a cells were trypsinized, cells were counted and suspended at a concentration of 1×10^6 per ml of medium. The cells were pelleted at 550 rpm for 5 min. Then anti-human SLAM FITC was added to the cell pellet @ 30 μl/million cells and incubated at 4 °C for 45 min. Then the cells were washed with FACS buffer (PBS + 3% horse serum and 0.01% sodium azide) and re-suspended in 500 μl of FACS buffer (Becton Dickinson, USA) for flow cytometry analysis in FACS Calibur machine (Becton and Dickinson, USA) using Cell Quest software. Ten thousand cells were counted for SLAM expression in normal cells while only 5000 cells were counted in siRNA transfected cells. As a negative control, siRNA specific for Newcastle disease virus was used. SLAM expression was assessed in three different samples, each time in triplicate. The mean percentages of B95a cells expressing SLAM were recorded in flow cytometry.

2.6. Real-time PCR results analysis

The Ct values were recorded for each gene expression assayed in real-time PCR. All the Ct values are mean of triplicate samples tested. At least three independent samples were tested on each occasion. The ΔCt values indicate the difference in the Ct values between the target gene and the endogenous gene. The ΔΔCt value

Table 3
Expression and siRNA-mediated inhibition study of SLAM in B95a cells using real-time PCR with TaqMan probe

Treatment	Mean (±S.D.) Ct values for β-actin (endogenous control)	Mean (±S.D.) Ct values for SLAM (target gene)	Mean ΔCt (gene of interest Ct-endogenous Ct)	ΔΔCt[a]	$2^{-\Delta\Delta Ct}$ (fold decrease in SLAM expression)
B95a cell line (untreated)	19.891[b] ± 0.089	18.129 ± 0.007	−1.762	0	1
siRNA 1	19.807 ± 0.028	23.646 ± 0.016	3.839	5.601	48.536
siRNA 2	19.773 ± 0.077	25.608 ± 0.014	5.835	7.597	193.609
siRNA 3	19.684 ± 0.047	26.750 ± 0.009	7.066	8.828	454.457
Control B95a (Mock siRNA)	20.137 ± 0.085	19.43 ± 0.066	−0.707	1.055	2.078

No. of testings = 3. Each sample was tested in triplicate.
[a] ΔΔCt = ΔCt of sample − ΔCt of calibrator (SLAM expression in untreated B95a cell line); fold change = $2^{-\Delta\Delta Ct}$.
[b] Ct values are mean of triplicate samples tested.

indicates the difference between the ΔCt of sample and the ΔCt of calibrator. The calibrator has been chosen as the SLAM expression in untransfected B95a cell line. The folds change in the gene expression is calculated as $2^{-\Delta\Delta}$Ct. Correlation coefficients were calculated between these folds changes using MS Excel package.

2.7. Infection inhibition assay

To confirm that SLAM acts as a receptor for PPRV, infection inhibition assay was performed following the alpha neutralization method (constant antibody and variable virus) that is used in conventional virology. Briefly, 2×10^4 B95a cells were grown in 96-wells plates overnight under 5% CO_2. The cells were then incubated at 37 °C for 1 h with medium containing anti-human SLAM antibody (eBiosciences, USA) @ 2 μg per well (constant antibody). One hour after treatment with anti-SLAM antibody, cells were infected (three wells each) with 10-fold dilution of B95a cell adapted PPRV (variable virus). Appropriate controls treated with irrelevant antibody (against a poultry virus, infectious bronchitis virus, available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai) were also included. After 24 and 48 h of incubation, the CPE was observed between antibody neutralized and unneutralized cells. The presence of PPRV in the wells was also confirmed using the haemagglutination assay with chicken red blood cells (Dhinakar Raj et al., 2000) for calculation of virus titres. The neutralization index was calculated as the difference in PPRV titres in SLAM antibody neutralized B95a cells and unneutralized cells.

3. Results

One of the requirements of using the relative quantification method is that the PCR efficiency of both the target and endogenous control should be similar. The slope of the standard curves determines the PCR efficiencies. For SLAM and beta actin, the slope of the standard curve, generated using different dilutions (concentrations) of the cDNA were −3.34996 and −3.2583, respectively. The calculated PCR efficiencies $\{(10^{-1/\text{slope}}) - 1 \times 100\}$ for SLAM and beta actin were 98.8% and 102.7%, respectively, indicating that their comparison using relative quantification was acceptable. The PCR efficiency of the PPRV 'M' gene was also 105%.

SLAM expression in siRNA transfected B95a cell line is shown in Table 3. All the siRNAs resulted in decrease in SLAM expression resulting in increase in Ct values in real-time PCR over untreated cells. The increase in Ct values ranged from 5 to 8 cycles, while the endogenous control (beta actin) Ct values were in the range of 19–20 cycles only. The decrease in SLAM expression ranged from 48.54- to 454.46-folds. Maximum decrease was seen with siRNA 3 transfected cells. Negative control siRNA transfection showed negligible decrease in SLAM expression.

To further confirm, the reduction in SLAM expression at the protein level, flow cytometry was performed on siRNA transfected B95a cells using anti-human SLAM-FITC conjugate. The results of flow cytometry analysis are depicted in Table 4 and Fig. 1. 77.56 percent of normal B95a cells expressed SLAM. Taking this as 100 percent expression, siRNA transfection decreased SLAM expression ranging from 31.02% to 68.64%. Negative control siRNA inhibited up to 13.28% level.

Correlating with the real-time PCR results, siRNA 3 showed the highest decrease in SLAM expression at the protein level also.

The siRNA transfected cells showed less pronounced CPE following PPRV infection. By 24 h post-infection (PI), normal (no siRNA transfection) PPRV infected cells exhibited ballooning and syncytium formation while siRNA transfected cells showed only clumping and rounding at that time point.

Table 5 shows the effect of SLAM inhibition on PPRV replication and virus titres in B95a cell line. Decrease in SLAM expression resulting in increase in Ct values in real-time PCR for PPRV 'M' gene expression. The increase in Ct values ranged from 5 to 8 cycles, while the endogenous control (beta actin) Ct values were in the range of 19–20 cycles only. The folds decrease in virus replication (expression of PPRV 'M' gene assessed by TaqMan chemistry) varied from 12.84 to 143.71 for the different siRNAs tested. This was also reflected as decrease in infective virus titres that ranged from $\log_{10}$ 1.09 to 2.28 (12–190 times). The siRNA3 transfected cells showed maximum decrease in virus replication (143.71-folds) and in virus titre ($\log_{10}$ 2.28 or 190 times).

The virus titres in anti-SLAM antibody neutralized B95a cells were calculated to be $10^{5.5}$ $TCID_{50}$ while in the SLAM neutralized cells it was reduced 100-folds to $10^{3.5}$ $TCID_{50}$ based on haemagglutination test as indicator for presence of virus. The neutralization index was 2.0. The CPE was also delayed in the SLAM blocked cells. The SLAM unneutralized cells exhibited cell rounding by 24 h post-infection (Fig. 2b) and syncytium formation by 48 h PI (Fig. 2d). However, in the SLAM blocked cells no changes were seen by 24 h PI (Fig. 2a) while cell rounding was evident by 48 h PI (Fig. 2c).

4. Discussion

Cellular receptors are one of the major determinants of the host range and tissue tropism of viruses. Different viruses uses different receptors such acetylcholine for rabies virus (Lentz et al., 1983),

Table 4
Expression of SLAM in B95a cell line and its inhibition after siRNA transfection assessed using flow cytometry with anti-human SLAM-FITC conjugate

Treatment	Percentage of cells expressing SLAM (mean[a] ± S.D.)	SLAM expression (%)
Control B95a (untreated)	77.56 ± 0.389	100
siRNA 1	53.50 ± 1.13	68.98
siRNA 2	40.01 ± 1.00	51.59
siRNA 3	24.32 ± 0.588	31.36
Negative control (NDV siRNA)	67.26 ± 2.89	86.72

No. of samples tested = 3. Each sample was tested in triplicate.
[a] Mean of three results.

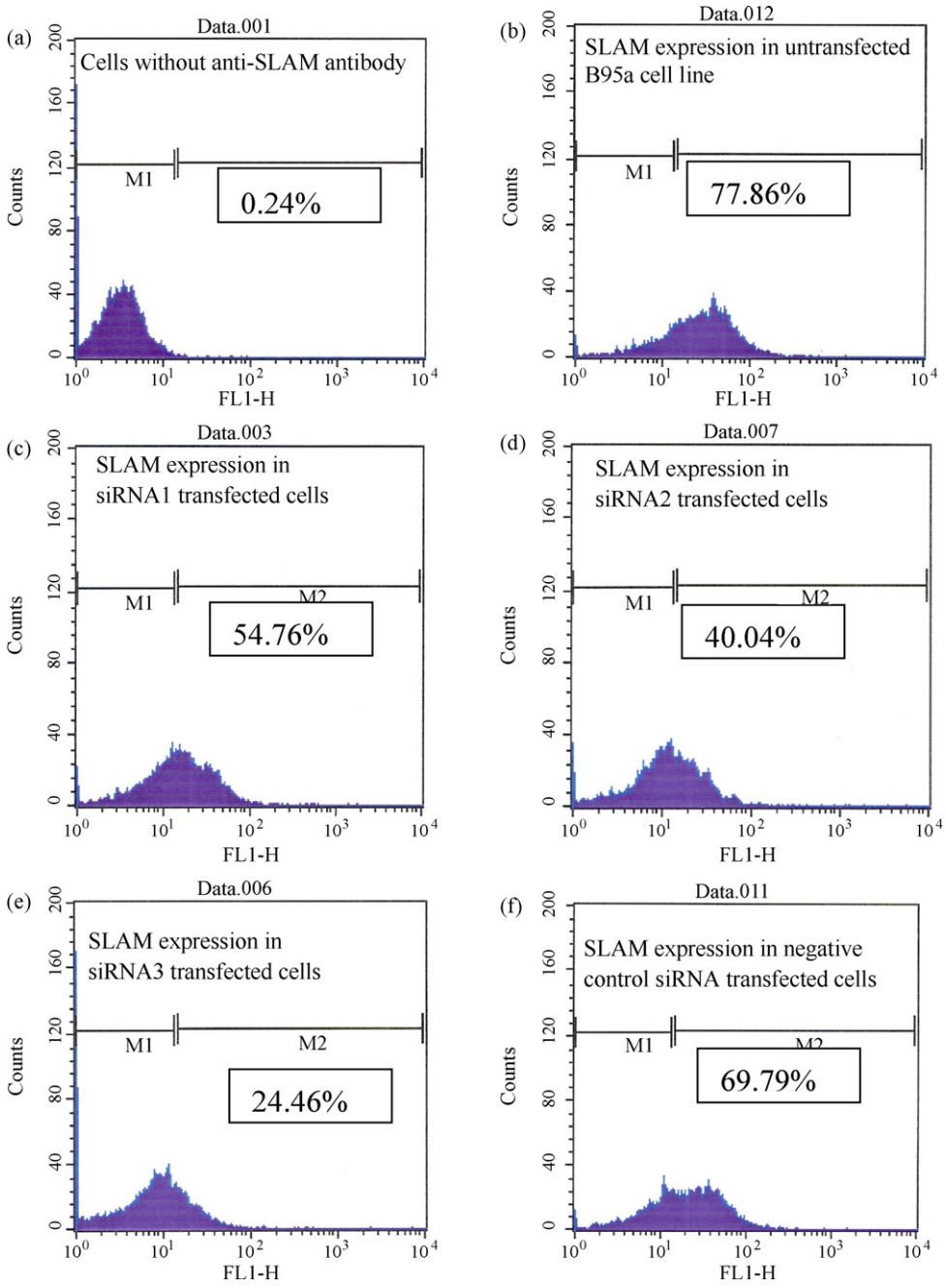

Fig. 1. Flow cytometry analysis of SLAM expression in normal and siRNA transfected B95a cell line stained using anti-human SLAM-FITC conjugate (*data from one representative sample is shown*). The percentages indicate the percentage of B95a cells expressing SLAM.

human aminopeptidase N (hAPN) for human corona virus (Breslin et al., 2003) and sialic acid for influenza A virus (Suzuki et al., 2000).

SLAM has been shown to be a receptor for MV (Tatsuo et al., 2001), CDV (Seki et al., 2003) and RPV (Baron, 2005), all belonging to the *Morbillivirus* genus. However, with respect to another virus, PPRV, also belonging to the same genus there is only indirect evidence that SLAM could be one of the receptors. Sreenivasa et al. (2006) have shown that PPRV virus grew to higher titres in B95a cells, which express SLAM when compared to Vero cells, which does not express SLAM. However the fact that PPRV causes haemmaglutination of pig and chicken RBCs (Monoharan et al., 2005) may suggest that sialic acid residues could act as PPR viral receptor. Under this background, proposed study was undertaken to find out whether SLAM acts as a receptor for PPRV.

RNAi technology clearly has significant potential for analyzing critical gene functions and for identifying and testing the new target for diseases. A large number of gene functions have been resolved in recent past by using siRNA technology and many more are being attempted in laboratories all over the world (Zou et al., 2002; Moskalenko et al., 2002; Bakker et al., 2002). Hence in this study, this approach was tried for elucidate the specific role of SLAM during PPRV replication *in vitro*.

B95a cell line was used since this cell line constitutively expressed SLAM. Since this study involved identification of receptor for virus, it was essential that virus infection be done at the time when potential virus receptor expression was highly suppressed. This only would ensure that virus would be inhibited during its entry in to the susceptible cells. In studies that involve suppression

Table 5
Effect of siRNA-mediated SLAM inhibition on PPRV replication assessed using real-time PCR for PPRV 'M' gene (TaqMan assay) and PPRV infectivity titres

Treatment	Mean (±S.D.) Ct values for β-actin (endogenous control)	Mean (±S.D.) Ct values for PPRV M gene (target gene)	Mean ΔCt (gene of interest Ct-endogenous Ct)	ΔΔCt[a]	$2^{-\Delta\Delta Ct}$ (fold decrease in PPRV M gene expression)	Virus titre (log 10)	Decrease in virus titre (log 10)
Control B95a (untreated)	19.541 ± 0.027	14.803 ± 0.009	−4.738	0	1	6.69	
siRNA 1	20.44 ± 0.026	19.384 ± 0.03	−1.056	3.682	12.835	5.60	1.09 (12-folds)
siRNA 2	19.864 ± 0.055	21.952 ± 0.047	2.088	6.826	113.457	4.80	1.89 (77-folds)
siRNA 3	19.664 ± 0.013	22.093 ± 0.019	2.429	7.167	143.708	4.41	2.28 (190-folds)
B95a cells + NDV siRNA (irrelevant siRNA)	19.41 ± 0.017	15.09 ± 0.010	−4.320	0.418	0.748	6.33	0.36 (2.29-folds)

No. of samples = 3. Each sample was tested in triplicate.
* Ct values are mean of triplicate samples tested.
[a] ΔΔCt = ΔCt of sample − ΔCt of calibrator (PPRV M gene expression in untreated B95a cell line); fold change = $2^{-\Delta\Delta Ct}$.

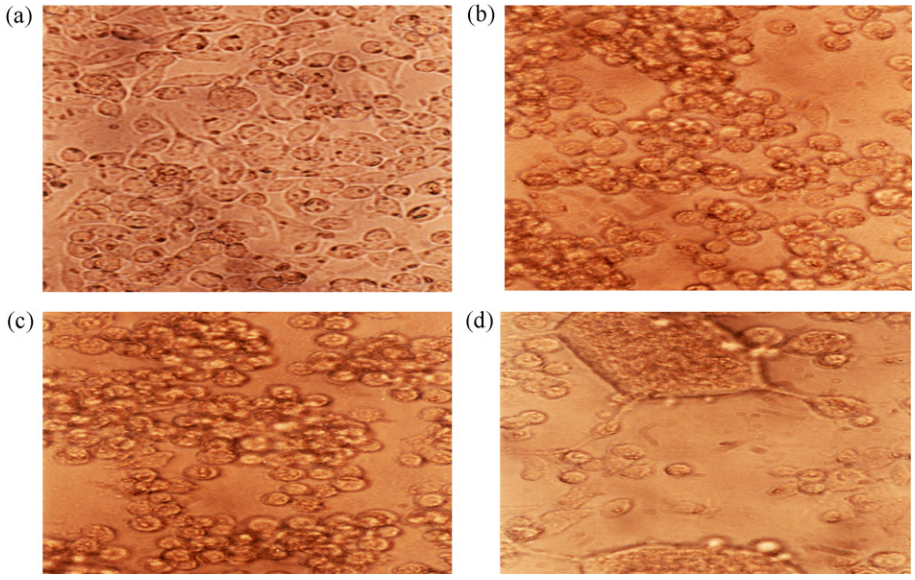

Fig. 2. (a–d) Effect of SLAM inhibition in B95a cells by anti-human SLAM antibody on PPRV induced CPE at 24 and 48 h post-infection. (a) PPRV infected B95a cells neutralized with SLAM antibody 24 h PI. Note normal appearance of cells. (b) PPRV infected B95a cells unneutralized with SLAM antibody 24 h PI. Note cell rounding. (c) PPRV infected B95a cells neutralized with SLAM antibody 48 h PI. Note cell rounding. (d) PPRV infected B95a cells unneutralized with SLAM antibody 48 h PI. Note giant cell formation.

of viral gene it is possible that siRNA and virus are given together and in some cases after few hours interval, so that the siRNA can directly inhibit virus replication (and not its entry) (Hu et al., 2002; Mohapatra et al., 2005; Li and Ding, 2001).

siRNA transfected B95a cells that had lowered levels of SLAM expression were used for PPRV infection. If SLAM was the receptor required for PPRV virus entry, virus should replicate at lowered levels in the SLAM suppressed cells. This effect was seen as delayed CPE and decreased virus titre and virus replication assessed by real-time PCR. However, virus replication was not completely inhibited by siRNA. Thus it seems likely that SLAM was used as a receptor of PPRV and when SLAM levels were suppressed, virus entry was reduced, virus CPE delayed and virus replication and titres were lowered.

The levels of fold-decrease in SLAM expression was highly correlated with decrease in virus titres in different siRNA treated cells, with a correlation coefficient of 0.908. Similarly, although the decrease in PPRV M gene and SLAM expression was also highly correlated (correlation coefficient of 0.941), the magnitude of decrease was more in the case of SLAM rather than in PPRV M gene expression or virus titres. This could be probably because SLAM is constitutively expressed in B95a cells while PPRV M gene expression could vary based on virus replication status.

Decrease in infective virus titre in SLAM suppressed cells ranged from $\log_{10}$ 1.09 to 2.28. This may be due to the fact that SLAM levels, although was reduced, it was not totally abolished. Residual presence of SLAM could have been used by PPRV for its entry. Another possibility could be the possible usage of other receptors by PPRV. In case of MV, in addition to SLAM or CD150, CD46 has also been shown to play an important role in virus entry (Dorig et al., 1993; Naniche et al., 1993).

Further when SLAM was blocked using antibody, the virus titres were decreased 100-folds. This gives unequivocal proof that SLAM is one of the (co) receptors for PPRV, since it could also be possible that inhibition of SLAM expression by siRNA inhibited some other cellular function(s), which may be affect virus replication and virus production. However, the presence of other receptors cannot be ruled out since virus titres were not completely blocked and PPRV can also be isolated and cultivated in cell lines such as Vero, which does not express SLAM.

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