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Pneumopathogenicity in Mice of a Sendai Virus Mutant, TSrev-58, Is Accompanied by In Vitro Activation with Trypsin

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The pneumopathogenicity of a trypsin-sensitive revertant of Sendai virus, TSrev-58, which was derived from a trypsin-resistant mutant, TR-5, was examined in mice. In comparison with TR-5, the revertant had a single amino acid substitution at residue 116 (Ile→Arg) on F protein, which was the cleavage site, and had the same trypsin sensitivity as the wild-type virus. However, TSrev-58 still had a single amino acid difference from the wild-type virus at residue 109 (Asn→Asp) (M. Itoh, H. Shibuta, and M. Homma, J. Gen. Virol. 68:2939–2943, 1987). Nevertheless, the present study revealed that TSrev-58 had the same pneumopathogenicity in mice as the wild-type virus. This result indicates that the activating protease of Sendai virus present in the lungs of mice is quite similar to trypsin and also that the in vitro trypsin sensitivity of Sendai virus can be a good marker of pneumopathogenicity in mice.

The fusion glycoprotein of Sendai virus (F protein) mediates the entry of the viral genome into target cells by fusing the viral envelope with the plasma membrane (3, 5, 11). For F protein to function, its cleavage into F_1 and F_2 subunits by a trypsinlike protease is essential (1–3, 5, 6, 9–12). Since Sendai viruses grown in many established lines of tissue culture cells possess an uncleaved form of F and lack the ability of envelope fusion, they remain inactive and never show infectivity until they become activated by proteolytic cleavage of the F protein. A view has been presented, on the basis of the above facts, that the organ tropism of Sendai virus can be determined not only by the distribution of cellular receptors, but also by the presence of a protease(s) that cleaves F protein (4, 9, 14). We found that wild-type Sendai virus exclusively caused pneumonia in mice as a result of multiple-cycle replication in the bronchial and bronchiolar epithelia (14, 15). On the other hand, a trypsin-resistant mutant of Sendai virus (TR-2), which could be proteolytically activated by chymotrypsin but not by trypsin, could replicate only in a single cycle in these epithelia, causing very limited pathological changes (14). From these observations, we suggested that a trypsinlike protease(s) is present in the bronchial and bronchiolar epithelia of mice which causes the cleavage-activation of F protein and is responsible for pneumopathogenicity (14). Recently, we isolated a trypsin-sensitive revertant, TSrev-58, from another TR mutant, TR-5. This revertant was shown to have the same trypsin sensitivity as the wild-type virus. The amino acid sequences of the wild-type Sendai virus and TR mutants, TR-2 and TR-5, predicted from their nucleotide base sequences, indicated that there were two amino acid substitutions in the TR mutants at residues 109 (Asn→Asp) and 116 (Arg→Ile), the latter being the cleavage site of the wild-type virus by trypsin (8). This was compatible with the observation made with the pa-c1 mutant (7). On the other hand, with TSrev-58, only a single amino acid reversion at residue 116 (Ile→Arg) was found, leaving Asp as before at residue 109. From these results, we concluded that the trypsin sensitivity of Sendai virus could be changed by a single amino acid substitution at the cleavage site of F protein (8). We previously reported that the activating protease(s) present in the lung of mice was very similar, though not identical, to trypsin. The protease activity was inhibited by tosyllysylchloromethylketone, soybean trypsin inhibitor, leupeptin, and antipain, but not by phenylmethylsulfonyl fluoride; all of these are known to be potent trypsin inhibitors (15).

In the present study, we examined whether TSrev-58, which regained sensitivity to trypsin in vitro, also restored sensitivity to the protease(s) in the lung of mice and subsequently caused pneumopathogenicity.

The wild type of the Fushimi strain of Sendai virus, TR-5, and TSrev-58 were used for the experiments. The isolation procedures for TR-5 from the wild-type virus and TSrev-58 from TR-5 were as described previously (8, 14). All viruses were prepared in LLC-MK_2 cells, an established line of rhesus monkey kidney cells, and were activated in vitro by either trypsin or chymotrypsin as described previously (8, 14). Three-week-old male mice of the ICR/CRJ (CD-1) strain (Charles River Japan Inc.) were inoculated intranasally with 25 μl of virus under mild ether anesthesia and investigated daily for body weight, clinical signs, lung lesions, and virus titers in lung homogenates (14). Infectivity was measured by the plaque assay method on cultures of LLC-MK_2 cells. To detect both active and inactive viruses, trypsin was present in the agar overlay throughout the incubation period at a concentration of 3 μg/ml (13). On the other hand, to detect active virus, the double agar overlay method described by Zhirnov et al. (16) was used, in which trypsin was included only in the second agar overlay, which was made 24 h after the first agar overlay. Histological examination and immunoperoxidase staining for detection of Sendai virus antigens were done as described previously (14).

Infection of mice with TSrev-58. Serial 10-fold dilutions of active TSrev-58 were inoculated into mice. The animals were measured daily for body weight (Fig. 1), which was shown to be a good indication of the pneumopathogenicity of Sendai virus (14). The virus at 12.8 hemagglutinating units (HAU) per ml caused a decrease in the rate of body weight gain on day 2 after inoculation, and body weight loss began to be apparent on day 5. Signs of respiratory infection, such as breathing crepitation, cyanosis, and dyspnea, were ob-

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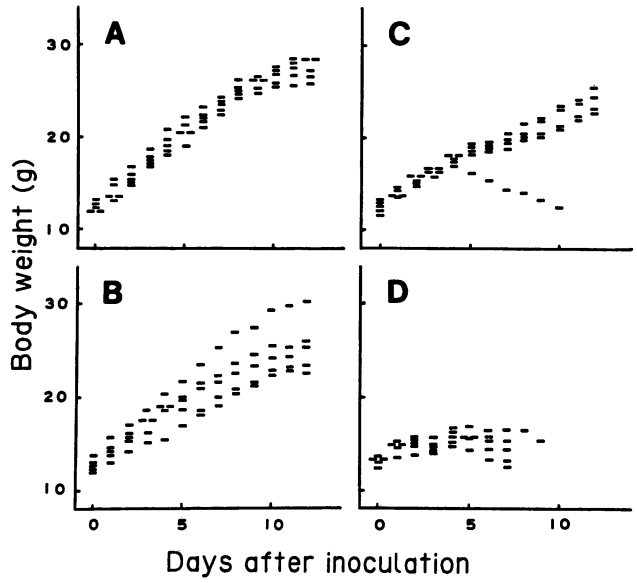

FIG. 1. Time course of body weight change of mice after inoculation with active TSrev-58. TSrev-58 grown in LLC-MK_2 cells was used after activation in vitro with trypsin (1 μg/ml) for 10 min at 37°C. After activation in vitro, the ratio of infectivity to hemagglutinating titer was 9.4×10^5 PFU/HAU, and infectivity was increased 6,000-fold in comparison with inactive virus. Specific-pathogen-free, 3-week-old ICR male mice were inoculated intranasally with 25 μl of 10-fold dilutions of each virus sample at the indicated concentrations. Five mice were used for each dilution. The amounts of virus in the inocula were (A) 0.0128 HAU/ml, (B) 0.128 HAU/ml, (C) 1.28 HAU/ml, and (D) 12.8 HAU/ml.

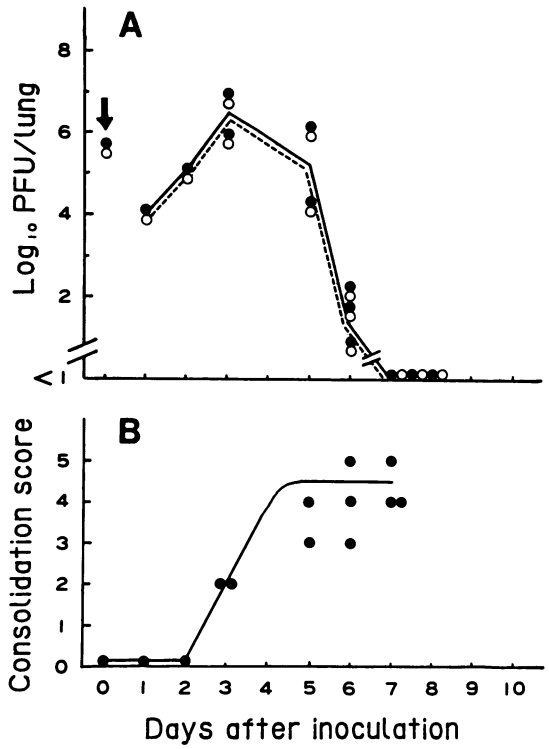

FIG. 2. Time course of viral replication in mouse lung and lung consolidation after inoculation with TSrev-58. Each mouse was inoculated intranasally with 25 μl of active TSrev-58 at 12.8 HAU/ml. Mice were sacrificed on the days indicated, and 10% lung homogenates were prepared for determination of virus titer. The amount of whole virus (●) and activated virus (○) in the lung (A) was assayed by the method of Zhirnov et al. (16). The arrow indicates the amount of virus in the inoculum. The lung lesion (B) was graded from 1 to 4 according to the surface consolidation area, and one point was added when the mouse died (14).

served in parallel during the course of infection, and all of the mice died within 10 days. However, inactive virus caused neither body weight loss nor signs of respiratory disease (data not shown). The above time courses of body weight change and the clinical features of mice inoculated with active and inactive TSrev-58 were the same as those found for mice inoculated with the respective viruses of the wild type (14). The mice inoculated with active TR-5, from which TSrev-58 was derived, did not develop any sign of lung disease (data not shown), as was the case with TR-2 (14).

Replication of TSrev-58 in the lungs of mice. To examine whether the progeny virus of TSrev-58 in the lungs of mice was produced as activated virus, the infectivity of lung homogenates from different times of infection was measured by the two methods as described above (Fig. 2). Infectivity began to increase 2 days after inoculation and reached the maximum titer on day 3 to 5. It was evident that the progeny virus was able to be activated in the lung, since no significant difference was found between infectivity titers measured by the two different methods described above. Lung consolidation appeared on day 3, and the consolidation score reached the maximum after 5 to 7 days. In contrast to TSrev-58, TR-5 activated by chymotrypsin could replicate only in a single cycle and caused only negligible pathological changes in the lung (data not shown). These results indicated that the pneumopathogenicity of TSrev-58 was apparently related to the multiple-step viral replication in the lung, as was the case with the wild-type virus (14).

Histology and virological examination on lung sections. From immunohistological examinations of mice infected with active TSrev-58, destruction of bronchial and bronchiolar epithelia, round cell infiltration, and venous congestion were apparent, and viral antigens were detected exclusively in the bronchial and bronchiolar epithelia. Figure 3 shows the accumulation of viral antigens and degenerative changes such as cellular and nuclear swelling and presence of sloughy cells in the bronchial epithelium. These histological changes were characteristic of broncho-bronchiolitis and indicated that the target cells of TSrev-58 were the bronchial and bronchiolar epithelia, findings comparable to those obtained with the wild-type virus (14).

The overall results show that TSrev-58 regained pneumopathogenicity in mice as a result of multiple-cycle replication in the bronchial and bronchiolar epithelia. TSrev-58 restored the trypsin sensitivity of F protein in vitro owing to the amino acid change from Ile to Arg at residue 116 (8). This amino acid reversion at the cleavage site was also accompanied by restoration of sensitivity to the activating enzyme in the lung of mice. The substitution of Asp for Asn at residue 109 remained unchanged, however, in TSrev-58 (8), and it did not influence sensitivity to the above enzyme. Therefore, multiple-step replication of the virus in the lung and the subsequent lung pathology should depend on the single amino acid of the cleavage site at residue 116. The virus-activating protease in the lung of mice was shown not to be identical with trypsin (15). Although its entity is not definite yet, the present result revealed its similarity to trypsin. Accordingly, we now tentatively conclude that in vitro

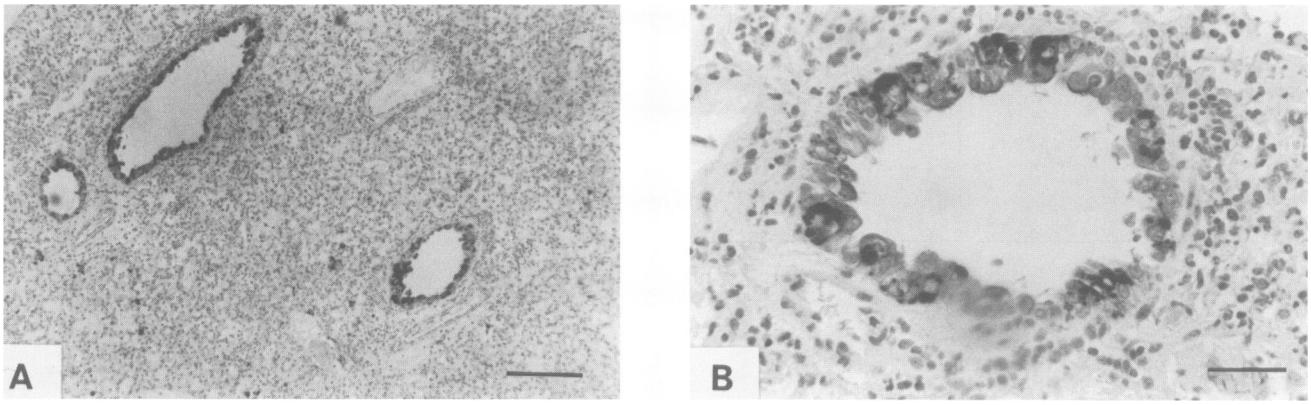

FIG. 3. Immunoperoxidase staining for Sendai virus antigens on mouse lung sections. Paraffin-embedded lung sections were prepared on day 5 after intranasal inoculation with active TSrev-58 at 12.8 HAU/ml. Peroxidase staining was done with rabbit anti-Sendai virus serum as the primary antibody (14). Nuclei were counterstained with hematoxylin. Magnification: ×40 (A); ×200 (B). Bars, 100 μm (A); 20 μm (B).

trypsin sensitivity of Sendai virus can be a good marker of its pneumopathogenicity.

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