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Influenza Virus Infection is not Affected by Serum Amyloid P Component

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Abstract

Background: Binding of serum amyloid P component (SAP) to its ligands, including bacteria, chromatin and amyloid fibrils, protects them from degradation, is anti-opsonic and anti-immunogenic. SAP thereby enhances the virulence of pathogenic bacteria to which it binds. However SAP also contributes to host resistance against bacteria to which it does not bind. Human SAP has been reported to bind to the influenza virus and inhibit viral invasion of cells in tissue culture. We therefore investigated a possible role of SAP in either host resistance or viral virulence during influenza infection *in vivo*.

Materials and Methods: The clinical course of mouse adapted influenza virus infection, the host antibody response, and viral replication, were compared in wild type mice, mice with targeted deletion of the SAP gene, and mice transgenic for human SAP. The effects of reconstitution of SAP deficient mice with pure human SAP, and of a drug that specifically blocks SAP binding *in vivo*, were also studied. Binding of mouse and human SAP to immobilized influenza virus was compared.

Results: The presence, absence, or availability for binding of SAP *in vivo* had no significant or consistent effect on the course or outcome of influenza infection, or on either viral replication or the anti-viral antibody response. Mouse SAP bound much less avidly than human SAP to influenza virus.

Conclusions: In marked contrast to the dramatic effects of SAP deficiency on host resistance to different bacterial infections, mouse SAP apparently plays no significant role during infection of mice with influenza virus. Human SAP binds much more avidly than mouse SAP to the virus, but also had no effect on any of the parameters measured and is therefore unlikely to be involved in human influenza infection.

Introduction

Serum amyloid P component (SAP), a normal plasma protein of the pentraxin family (1), binds specifically to amyloid fibrils, to certain anionic glycans, to DNA, chromatin and apoptotic cells, and to some bacteria (2). SAP bound to all these ligands acts as an anti-opsonin, protecting them from degradation *in vitro* and *in vivo* (3,4). This contributes to pathogenesis of amyloidosis (5). In contrast, SAP has an important normal function in maintaining tolerance to DNA and chromatin, as SAP deficient mice spontaneously develop florid anti-nuclear autoimmunity (6). With respect to innate immunity against bacteria, SAP is protective in infection with organisms to which it does not bind, such as smooth Gram negative species (4,7). However for organisms to which it does bind, such as *S. pyogenes* and rough strains of *E. coli,* SAP enhances virulence by protecting the bacteria against phagocytosis, and SAP deficient mice are much more resistant than wild type animals to these infections (4). Human SAP also binds to the influenza virus hemagglutinin and inhibits invasion of cells *in vitro*, whilst preincubation of the virus with human SAP *in vitro* or intra-nasal administration of human SAP to mice, have been reported to prevent infection *in vivo* (8,9). These observations suggest that SAP might play a role in innate immunity to influenza infection. On the other hand the protective anti-opsonic effect of SAP binding with respect to bacteria, and the anti-immunogenic effect of SAP with respect to chromatin autoantigens suggested that SAP might rather contribute to influenza pathogenesis. We have therefore investigated the clinical course and immune response to mouse adapted strains of influenza virus in SAP deficient compared to wild type mice, as

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well as in human SAP transgenic animals, and mice treated with our novel SAP-inhibitory drug (7).

Materials and Methods

Viruses

Influenza virus strains A/Puerto Rico/8/34 (H1N1) (PR8), A/Shanghai/24/90 (H3N2) (Shg/24) and A/Wilson Smith/33 (H1N1) (WS/33) propagated in fertile hens' eggs, were from the stocks of the National Institute for Biological Standards and Control, UK, and from Roche Discovery Welwyn, UK.

Mice and SAP

All experiments were conducted in C57BL/6 mice, comparing wild type animals with SAP knockouts (5) and mice transgenic for human SAP (10). Both strains of genetically modified mice had been back crossed onto the C57BL/6 background for more than 10 generations. Human SAP was isolated and purified as previously described (11).

Infection of Mice

Mice were intra-nasally infected with influenza viruses (20 μl per mouse containing 50 MID_{50} or more in different experiments), while under light anesthesia (12). Nasal washes in PBSA were taken on days 3–7 after infection and samples were titrated for influenza virus on Madin-Darby Canine Kidney (MDCK) cells using standard procedures (13). Virus titers ($TCID_{50}$/ml) were calculated by the Spearman Kärber method (14). In other experiments infected mice were weighed daily and their survival monitored, or groups were killed at the times shown for determination of anti-influenza virus antibody titers in serum and broncho-alveolar lavage fluid, and of virus titers in the lung. Following the last time point shown in the Figures reporting survival experiments, all remaining mice were vigorous and healthy with no signs of disease and were therefore culled 24 h later.

Assay of Influenza Virus A/PR/8/34 from the Lungs of Infected Mice

Lungs removed from A/PR/8/34 infected mice were homogenized for one minute at 20500 rpm (Ultra Turrax T-25 homogenizer, IKA Labortechnik) in 1 ml Eagles MEM medium, supplemented with 0.14% w/v BSA fraction V, 2 mM L-glutamine, 100 U penicillin/100 μg streptomycin. Homogenates were clarified by centrifugation at 2000 rpm for 5 min, and infectious influenza virus was titrated on MDCK cells as reported (15).

Binding of SAP to Immobilized A/PR/8/34 Virus

Microtiter plates (Costar high binding EIA/RIA strip plate, type 1, Corning, USA) were coated overnight at 4°C with 50 μl/well of a highly purified A/PR/8/34 preparation at 0.0125 mg/ml in 0.1 M Na_2CO_3/0.1 M $NaHCO_3$, pH 9.2 (coupling buffer), and then washed three times with coupling buffer. Wells were blocked by incubation for 30 min at room temperature with 50 μl coupling buffer containing 2% w/v BSA, followed by washing with coupling buffer alone and then preincubation with 10 mM Tris, 140 mM NaCl, 2 mM $CaCl_2$, pH 8.0 (TC buffer) containing 4% w/v BSA and 0.05% v/v Tween 20 (TCBT buffer) for 2 min prior to assay. After removal of this equilibrating buffer, to each well were added TCBT buffer, TC, and ^{125}I-human SAP (specific activity 3.1 MBq/nmol) or ^{125}I-mouse SAP (specific activity 3.7 MBq/nmol) in 10 mM Tris, 140 mM NaCl, pH 8.0 (TN buffer), to provide 100,000 cpm per well in 50 μl with final concentrations of 4% w/v BSA, 2 mM $CaCl_2$ and 0.05% v/v Tween 20. Control wells contained the same reagents but with a final concentration of 10 mM EDTA. The plates were then incubated at room temp for 2 h before washing each well three times with 200 μl volumes of TCBT, or the same buffer in which the calcium was replaced with 10 mM EDTA, as appropriate. Finally individual wells were counted after drying for 1 h at room temperature. Each determination was carried out in triplicate.

Assay of Antibody to A/PR/8/34

Wells coated with A/PR/8/34 virus as above were equilibrated for 2 min with 50 μl PBS containing 10 mM EDTA, 1% w/v BSA, 0.2% v/v Tween 20, 0.1% NaN_3, pH 7.4 (PEBT buffer). Sera to be assayed were diluted appropriately in PEBT and 50 μl volumes dispensed to wells in triplicate for incubation at 37°C for 1 h before washing three times with PEBT (200 μl). Bound antibody was then detected using ^{125}I-labeled sheep anti-whole mouse immunoglobulin antibody, 50,000 cpm in 50 μl of PEBT. After incubations at 37°C for 1h, wells were washed three times with PEBT (200 μl), dried at room temperature for 1 h and then counted. The assay was calibrated using dilutions of a single high titer mouse antiserum to which a value of 1000 antibody units was assigned.

Results

Influenza Virus Infection in Wild Type and SAP Deficient Mice

Wild type C57BL/6 mice mounted a vigorous acute phase response following intra-nasal infection with different strains of mouse adapted human influenza virus. Circulating SAP values rose from a baseline of 2–5 mg/l typical of this strain (16) to 20–30 mg/l on day 5 and 60–110 mg/l on days 7 and 10. However, there was no consistent difference between normal wild type mice and SAP knockout mice, regardless of the severity of infection, with respect to the clinical course or outcome (Figs. 1 and 2), virus shedding in nasal washes (Table 1), virus titers in the lung (Table 2), or anti-viral antibody titers in the blood or bronchoalveolar lavage fluid (Table 2).

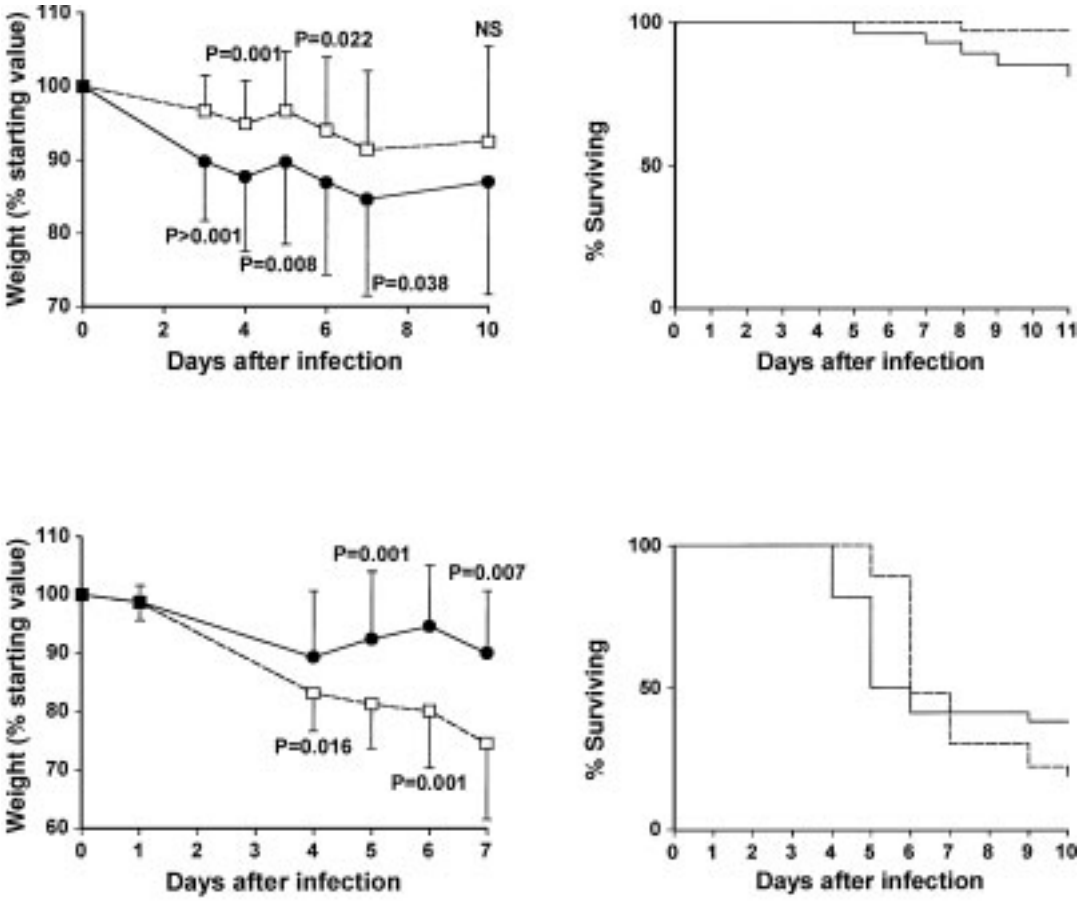

Fig. 1. Influenza infection in C57BL/6 wild type and SAP knockout mice. Upper panel, body weight (left) and survival (right) following intra-nasal infection on day 0 with the A/PR/8/34 viral strain, in 27 female wild type mice (● and solid line) aged 15–16 weeks, mean (SD) initial body weight 24.0 (1.6) g, and 31 female SAP knockout mice (□ and broken line) aged 13–20 weeks, mean (SD) initial body weight 27.3 (1.8) g. Lower panel, body weight (left) and survival (right) following intra-nasal infection on day 0 with the A/WS/33 ATCC VR1520 viral strain, in 34 female wild type mice (● and solid line) aged 10–13 weeks, mean (SD) initial body weight 20.5 (1.9) g, and 27 female SAP knockout mice (□ and broken line) aged 8–25 weeks, mean (SD) initial body weight 25.0 (2.7) g. Each point (left panels) represents the mean (SD) of all surviving animals in each group at each time point; significant differences between groups by t-test are shown by P values. In the milder infection (upper panel) the wild type mice showed greater percentage weight loss, but in the more severe infection (lower panel) the wild type animals showed less weight loss. In both experiments the SAP knockout mice had a wider age range, owing to their breeding pattern and the need to have substantial sized groups, and also had a greater mean initial weight. However there was no difference in survival between SAP knockout and wild type mice in either case, indicating that initial weight had no systematic effect on morbidity or outcome.

Influenza Virus Infection in SAP Deficient and Human SAP Transgenic Mice

Mouse SAP binds much less avidly to amyloid fibrils, and to other known SAP ligands, such as phosphoethanolamine (11,17), than does human SAP (18). This was true also for binding of mouse and human SAP to immobilized influenza virus, by a factor of five or more (Fig. 3). However following intra-nasal infection with influenza virus, there was no difference between SAP knockout mice and SAP knockouts that were transgenic for human SAP, in either the clinical course or outcome (Fig. 4). The human SAP transgenics have a mean circulating concentration of human SAP of around 80 mg/l, which is 2–3 fold higher than the normal value in man (19), and thus provide a robust test for a significant role of SAP in influenza infection. Furthermore, the antibody responses to subcutaneous immunization with human clinical influenza virus vaccine was the same in human SAP transgenic mice on the wild type background, human SAP transgenic mice on the mouse SAP knockout background, and SAP knockout mice (results not shown).

Effect of Passive Administration of Human SAP, and Inhibition of SAP Binding In Vivo on Influenza Infection

Reconstitution of SAP knockout mice with a very large dose of pure human SAP, 5 mg given by intraperitoneal injection just before intra-nasal infection with influenza virus and again after 24 h, had no

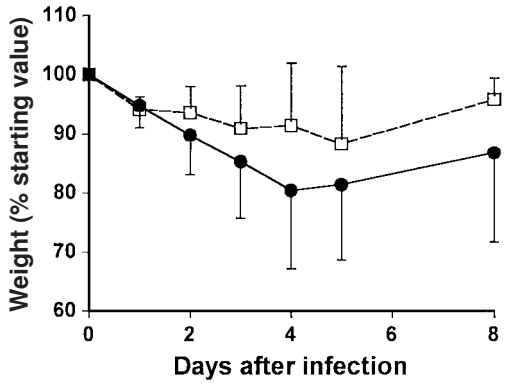

Fig. 2. Influenza infection in C57BL/6 wild type and SAP knockout mice. Body weight following intra-nasal infection on day 0 with the A/PR/8/34 viral strain in 12 female wild type mice (●), aged 9–11 weeks, mean (SD) initial body weight 21.0 (2.0) g, and 12 female SAP knockout mice (□) aged 9–11 weeks, mean (SD) initial body weight 23.0 (1.8) g. Four mice from each group were killed on days 3, 5 and 8 for measurement of antibody response and viral titers, as shown in Table 2. Each point represents the mean (SD) of all surviving animals in each group at each time point, and there was no significant difference between the groups.

effect on outcome. At day 10 there were 9 survivors out of 29 mice in the control group of SAP knockouts treated with buffer alone, and 13 survivors out of 29 in the group receiving human SAP. The plasma half life of human SAP in mice is about 3 h (20) and control uninfected mice receiving these huge amounts of human SAP had very high circulating concentrations that still exceeded the normal human plasma SAP value (21) of about 30 mg/l at 48 h. Availability in the body of abundant human SAP during the first days thus had no effect on the course of influenza virus infection.

Our potent inhibitor of SAP binding, (R)-1-[6-[(R)-2-Carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2-carboxylic acid (22), completely abrogated the inhibition by human SAP of influenza virus induced hemagglutination *in vitro*, and had an IC50 of ~2μM for inhibition of binding of radiolabelled human SAP to immobilized A/PR/8/34 virus. However, treatment of wild type mice with the drug had no effect on morbidity or outcome following influenza virus infection (results not shown). This contrasts sharply with the action of the drug in significantly ameliorating the course of lethal infection of mice with bacteria to which SAP binds (4), and thus provides a robust test for a possible role of endogenous mouse SAP in influenza infection.

Discussion

The observation that SAP binds to the influenza virus, inhibits hemagglutination and invasion of

Table 1. Influenza virus shedding during sub-lethal infection in wild type C57BL/6 and SAP knockout mice

	Virus Titer in Nasal Washings (TC (ID_{50}/ml))					
Mouse no.	Day 3	Day 4	Day 5	Day 6	Day 7	Mean
			Gp 1 SAP knockouts			
1	4.3	ND	ND	ND	ND	
2	3.5	ND	ND	ND	ND	
3	3.7	3.4	2.2	2.0	0.5	**2.36**
4	3.6	1.6	1.0	3.0	1.8	**2.20**
5	3.0	2.6	0.5	2.8	0.5	**1.88**
6	4.4	2.8	1.9	1.1	1.9	**2.42**
7	3.7	1.7	3.6	2.2	0.5	**2.34**
Mean	**3.74**	**2.42**	**1.84**	**2.22**	**1.04**	**2.24**
			Gp 2 C57BL/6 wild type			
1	2.2	2.9	2.8	1.9	1.8	**2.32**
2	2.8	1.5	2.8	2.2	2.7	**2.40**
3	3.5	2.8	0.5	2.7	0.5	**2.00**
4	2.3	3.0	2.0	2.7	1.7	**2.34**
5	2.8	2.1	2.5	1.3	2.6	**2.26**
6	3.7	2.8	3.0	1.2	0.5	**2.24**
7	3.4	2.6	2.6	1.2	1.7	**2.30**
8	3.4	3.3	2.0	1.7	1.6	**2.40**
9	2.2	2.7	1.8	1.6	1.6	**1.98**
10	2.1	1.7	2.9	1.8	2.3	**2.24**
Mean	**2.84**	**2.54**	**2.29**	**1.83**	**1.7**	**2.25**

Mice were infected intra-nasally on day 0 with the A/Shanghai/24/90 strain of influenza virus. Virus shedding was measured by titration of nasal washings taken daily as shown.

Table 2. Antibody response and viral titers during influenza virus infection in C57BL/6 wild type and SAP knockout mice

	Serum Antibody [Mean (SD)]		BAL Antibody [Mean (SD)]		Lung Viral Titer [$TCID_{50}$, Individual Values]	
Day	Wild Type	SAP ko	Wild Type	SAP ko	Wild Type	SAP ko
3	59 (8)	94 (30)	25 (7)	17 (8)	3.75, 4.75, 4.75, NA	<0.75, <0.75, 2.5, 3.5
5	485 (194)	311(138)	13 (4)	17 (9)	3.75, 3.75, 4.5, 4.5	3.5, 4.5, 4.75, 5.0
8	4016 (777)	4350 (254)	300 (155)	58 (21)	1.25, 1.25, 1.5, 4.0	1.0, 1.5, 1.75, 2.75

Mice were infected intra-nasally on day 0 with the A/PR/8/34 strain of influenza virus, and batches of 4 animals were killed on days 3,5 and 8 to provide serum and bronchoalveolar fluid for anti-influenza virus antibody titration, and whole lung homogenates for virus titration. Total mouse anti-influenza virus antibody was estimated by solid phase immunoradiometric assay calibrated with a single standard high titer mouse antiserum to which a value of 1000 antibody units was assigned. There were no spontaneous deaths and the weights of all surviving animals on each day are shown in Fig. 2.

cells *in vitro*, suggested that SAP might contribute to innate defenses against this important pathogen (8). Preincubation of influenza virus with human SAP, and intra-nasal administration of human SAP, also apparently reduced the virulence of influenza infection in mice (9). On the other hand our findings in SAP deficient mice, in relation to bacterial infection (4) and in relation to anti-nuclear autoimmunity (6), raised the possibility that binding of SAP under physiological conditions *in vivo* may contribute to virulence of the influenza virus by reducing phagocytosis of the virus, and possibly also its immunogenicity. This was of particular interest in view of our recent development of a potent, non-toxic, low molecular weight compound that selectively blocks binding of SAP to its ligands *in vivo* (7). However the present extensive series of experiments showed no consistent significant effects of the presence, absence or availability of mouse or human SAP on morbidity, outcome, viral replication or antibody responses following intra-nasal infection of mice with mouse adapted influenza virus, using three different virus strains, and regardless of the infecting dose.

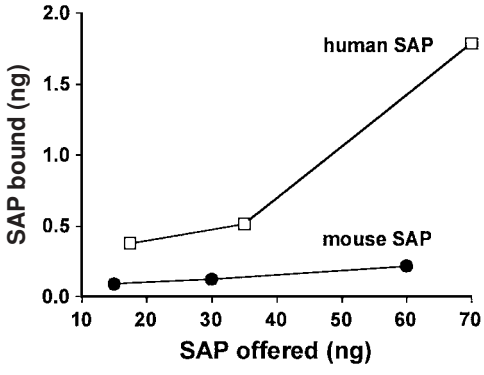

Fig. 3. Calcium-dependent binding of ^{125}I-labeled mouse and ^{125}I-labeled human SAP to immobilized A/PR/8/34 influenza virus. Each point is the mean of triplicate measurements. There was no detectable binding of SAP in the presence of EDTA.

The pentraxin family of plasma proteins, including SAP, have been very stably conserved in evolution (23–26), suggesting that they have important biological functions. SAP is the single normal plasma protein that specifically undergoes calcium-dependent binding to DNA and chromatin (27,28), and SAP binds specifically to apoptotic cells both *in vitro* and *in vivo* (29). Binding of SAP stabilizes chromatin, retarding its degradation *in vitro* and *in vivo*, and the functional importance of these effects is reflected by the spontaneous development of marked anti-nuclear autoimmunity in SAP deficient mice (6). Furthermore immunization with extrinsic chromatin induces greater antibody responses in SAP knockout mice than in wild type animals (6). In addition to its binding to chromatin, SAP also binds to some bacteria (30) and inhibits their subsequent phagocytosis and destruction by neutrophils, both *in vitro* and *in vivo* (4). This is reflected in the markedly enhanced resistance of SAP deficient mice to otherwise lethal infection with such organisms (4). Passive administration of human SAP to SAP knockout mice reconstitutes wild type susceptibility, whilst (R)-1-[6-[(R)-2-Carboxy-pyrrolidin-1-yl]-6-oxo-hexnoyl] pyrrolidine-2-carboxylic acid, the drug that inhibits SAP binding, confers enhanced resistance on wild type animals (4). In contrast, SAP knockouts are more susceptible than wild type mice during infection with bacteria to which SAP does not bind, indicating that SAP also has host defense functions (4,7). For example, SAP may protect against toxic products of microbial and/or host origin released during infection.

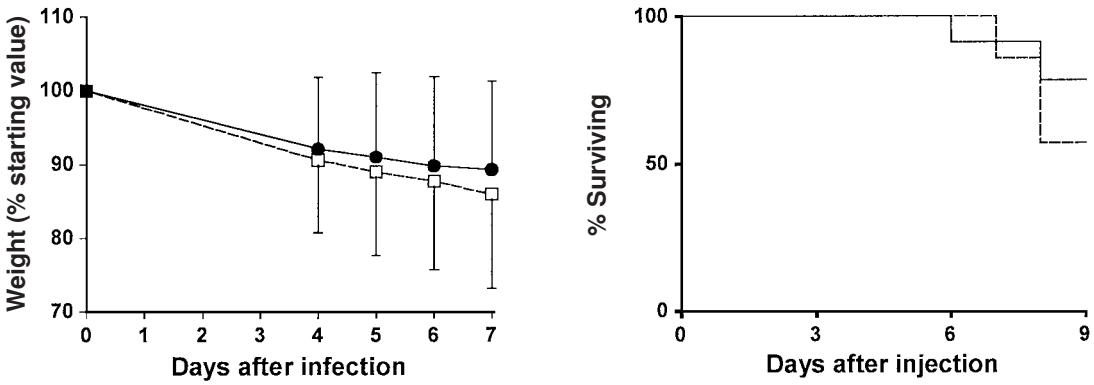

Fig. 4. Influenza infection in C57BL/6 SAP knockout mice and C57BL/6 SAP knockouts transgenic for human SAP. Body weight (left) and survival (right) following intra-nasal infection on day 0 with the A/PR/8/34 viral strain, in 23 female human SAP transgenic mice (● and solid line) aged 17–39 weeks, mean (SD) 29.0 (8.7) weeks, mean (SD) initial body weight 31.9 (3.7) g, and 23 female SAP knockout mice (□ and broken line) aged 17–39 weeks, mean (SD) 31.0 (6.3) weeks, mean (SD) initial body weight 30.6 (3.2) g. Each point (left) represents the mean (SD) of all surviving animals in each group at each time point. There was no significant difference in weight loss or survival between the groups.

However, despite binding of SAP to influenza virus particles (8), availability of SAP *in vivo* had no apparent effect on influenza infection or the host response to this virus. Mice without SAP handled the infection normally and SAP is thus not a necessary component of host defense against influenza virus in this species. We show here that mouse SAP binds only weakly to influenza virus, but even human SAP, which binds more avidly, had no effect either in increasing resistance to infection or in promoting viral virulence, despite being expressed at high levels in transgenic mice or administered parenterally in high doses. It remains possible that, in contrast to the situation with respect to pyogenic bacteria (4), human SAP may have no biological action on influenza virus infection in mice because it may not be recognized by necessary molecular or cellular components of murine host defence mechanisms. However, unlike the reported effects of SAP *in vitro* (8) or following intra-nasal administration (9), it is clear that in mice the systemic presence of either human or mouse SAP has no significant influence on the replication or effects of influenza virus, or the host response *in vivo*. This is likely to be true also in man and the inhibitor of SAP binding, (R)-1-[6-[(R)-2-Carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid, thus probably will not have a role in treatment of influenza virus infection.

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