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Serum amyloid P component inhibits influenza A virus infections: in vitro and in vivo studies

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Abstract

Serum amyloid P component (SAP) binds in vitro Ca^{2+}-dependently to several ligands including oligosaccharides with terminal mannose and galactose. We have earlier reported that SAP binds to human influenza A virus strains, inhibiting hemagglutinin (HA) activity and virus infectivity in vitro. These studies were extended to comprise five mouse-adapted influenza A strains, two swine influenza A strains, a mink influenza A virus, a ferret influenza A reassortant virus, a influenza B virus and a parainfluenza 3 virus. The HA activity of all these viruses was inhibited by SAP. Western blotting showed that SAP bound to HA trimers, monomers and HA1 and HA2 subunits of influenza A virus. Binding studies indicated that galactose, mannose and fucose moieties contributed to the SAP reacting site(s). Intranasal administration of human SAP to mice induced no demonstrable toxic reactions, and circulating antibodies against SAP were not detected. Preincubation of virus (A/Japan/57) with SAP prevented primary infection of mice and development of antiviral antibodies. After a single intranasal administration of SAP (40 μg) 1 h before primary infection with virus ($2LD_{50}$), nine out of 10 mice survived on day 10 and these mice approached normal body weight, whereas control mice (one out of five surviving on day 10) died. The data provide evidence of the potential of intranasally administered SAP for prophylactic treatment of influenza A virus infections in humans.

Keywords: Antiviral effect; Serum amyloid P component; Influenza A virus

1. Introduction

Serum amyloid P component (SAP) is a serum glycoprotein belonging to the pentraxin family of

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proteins. The serum concentration of human SAP is 24–32 μg/ml (Nelson et al., 1991). SAP consists of five non-covalently associated, identical protomers (MW 25,462 Da) (Emsley et al., 1994) each with one intrachain disulfide bond. The SAP protomers have two Ca^{2+}-binding sites and show Ca^{2+}-dependent binding in vitro to certain oligosaccharides, glycosaminoglycans, chromatin and DNA (Feizi, 2000).

The hemagglutinin molecules of influenza A virions express complex as well as oligo-mannoside types of oligosaccharides; we have earlier (Andersen et al., 1997) reported that SAP binds to these oligosaccharides, thereby inhibiting hemagglutination and the infectivity of the virus. These studies have now been extended to comprise additional influenza A serotypes, influenza B and parainfluenza virus as well as in vivo studies. This report will focus on the prophylactic effect of SAP on infection induced in mice by influenza A virus.

2. Materials and methods

2.1. Antibodies

Rabbit IgG antibodies against human SAP, normal rabbit IgG, alkaline phosphatase (AP) conjugated goat *anti*-rabbit IgG and AP rabbit *anti*-mouse IgG were from Dako A/S (Glostrup, Denmark). $F(ab')_2$ fragments were produced by pepsin digestion of *anti*-SAP IgG. Horse-radish peroxidase conjugated goat *anti*-mouse IgM and IgG were from Southern Biotechnology Assoc. Inc. (Birmingham, AL). Polyclonal rabbit antibodies against influenza A hemagglutinin were from Lee Biomolecular Research Laboratory. (San Diego, CA). Monoclonal *anti*-hemagglutinin antibodies (MC3A10-18, *anti*-H3) were from WHO Collaborating Center for Virus Reference and Research, National Influenza Center (Lyon, France) and monoclonal *anti*-HLA-DR (TAL 1B5) from Dako A/S (Glostrup, Denmark).

2.2. Reagents

Bovine serum albumin (BSA), human serum albumin (HSA), alkaline phosphatase-conjugated avidin (AP-avidin), *ortho*-phenylenediamine, 1,4-dithio-L-threitol (DTT), Nitro blue Tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Sigma-Aldrich (St. Louis, MO). *Para*-nitrophenyl-phosphate (PNPP) was from Boehringer Mannheim (Mannheim, Germany) and DNA-agarose and Mono Q column from Amersham Pharmacia Biotech (Uppsala, Sweden). Softigen® was from Lyfjathroun (Reykjavik, Iceland). Polysorp and Maxisorp ELISA plates were from Nunc (Roskilde, Denmark), polyvinylchloride plates (EnzyPlates) from Propilen G.M. (Pécs, Hungary) and polyvinylidene difluoride membranes (Immobilon-P) from Millipore (Bedford, UK). Novex gels were from Novex (San Diego, CA).

2.3. Virus strains

Nobi-Equenza Vet Subunit vaccine (split virions) containing the influenza A strains: A/eq1/Prag/56 (H7N7), A/eq2/Miami/1/63 (H2N2), A/eq2/Fontainebleau/79 (H2N2) (Intervet AB, Frölunda, Sweden). Fluzone split virion influenza A vaccine: A/Texas/36/91 (H1N1), A/Johannesburg/33/94 (H3N2), B/Harbin/7/94 (Connaught Lab. Inc., Swiftwater, PA, USA). Mouse-adapted influenza A virus strains: A/Japan/57 (H2N2); A/Aichi/2/68 (H3N2); A/HongKong/107/71 (H3N2); A/Singapore/1/57 (H2N2) and A/PR/8/34 (H1N1). Influenza A/mink/Sweden/84 (H10N4) from Dr L. Englund, National Veterinary Institute, Uppsala, Sweden, ferret influenza A/England/939/69 (H3N2)-A/Puerto Rico/8/34 (H1N1) reassortant from Dr C. Sweet, University of Birmingham, Birmingham, England; influenza A swine/New Jersey/76 and A/swine/DK/Bavaria/2/77 from Dr B. Klingeborn, National Veterinary Institute, Uppsala, Sweden. Influenza B virus (B/HongKong/5/72) (ATCC, Manassas, VI, USA), and influenza B virus (two Beijing/184/93-like clinical isolates) from Dr P.C. Grauballe, Statens Serum Institut, Denmark. Parainfluenza 3 virus U 23 B 8/79 from Dr. B. Morein, National Veterinary Institute, Uppsala, Sweden.

2.4. Mice

Female BALB/c mice, 8–10 weeks old, pur-

chased from LATI (Gödöllö, Hungary), were used in the infection studies. Outbred male NMRI mice, 8–10 weeks old (Biomedical Laboratory, SDU-Odense University, Odense, Denmark) were used in all other in vivo tests.

2.5. Purification of SAP

SAP was purified from human serum by affinity chromatography on DNA-agarose and ion exchange chromatography (Mono Q column) as described previously (Dhawan et al., 1990; Nybo et al., 1999). The purity was analyzed by SDS-PAGE and silver staining, immunoblotting and electron microscopy. SAP was quantitated as earlier described (Juul Sørensen et al., 1995).

2.6. Fractionation of influenza A virus subunits

Nobi-Equenza Subunit vaccine was fractionated by ion-exchange chromatography on a Mono Q column using the Pharmacia FPLC System. The start buffer was 10 mM Tris and elution was performed with 0–1 M NaCl gradient in 10 mM Tris. The flow rate was 1 ml/min and 1 ml fractions were collected. Peak fractions were tested by SDS-PAGE, Western blotting, ELISA and for virus hemagglutination (HA).

2.7. Hemagglutination inhibition (HI) assay

Assays for inhibition of HA were carried out as previously described (Andersen et al., 1997).

2.8. SDS-PAGE and Western blotting

SDS-PAGE analyses of influenza A split virions (12.5 μl/lane) and HA-containing fractions were performed on 14% Novex agarose gels with Tris–glycine-SDS as running buffer under reducing (60 mM DTT) or non-reducing conditions. Proteins were electroblotted onto Immobilon-P membranes which were blocked with TBS-HSA 0.1% for 15 min and incubated for 30 min with SAP (20 μg/ml) in TBS-Ca or -EDTA. The strips were washed and blocked with 0.15% HSA in TBS-Ca or -EDTA and incubated with biotinylated *anti*-SAP (1 μg/ml TBS-Tw) at 4 °C overnight. After three washes the reaction was developed with AP–avidin (1:3000) and NBT-BCIP. Controls were performed without addition of SAP or by omitting the antibodies. Blots were also incubated with *anti*-H3 antibodies followed by AP conjugated rabbit *anti*-mouse IgG and substrate. Other blots were stained for total protein by the colloidal gold method.

2.9. ELISA for SAP binding to influenza virus

The 96-well microplates (Maxisorp) were incubated overnight at 4 °C with 100 μl Nobi Equenza vaccine, HA-containing fractions of the same vaccine or influenza B virus (B/HongKong/5/72) all diluted 1:15 in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). After three washes in TBS-Tw, the wells were quenched with TBS-0.1% Tween 20, pH 7.4, for 30 min at 37 °C. SAP (0.1–24 μg/ml), with or without additives, was diluted in TBS-Tw–Ca, and 100 μl was added to the wells and incubated for 90 min at 20 °C. The wells were washed four times with TBS-Ca, and 100 μl of biotinylated *anti*-SAP (1 μg/ml) was added and incubated for 45 min at 20 °C. After three washes with TBS-Ca, AP–avidin was added and the plates were incubated for 1 h at 20 °C. The plates were developed with PNPP (1 mg/ml) in 9.7% diethanolamine, pH 9.6, and the optical density was read at 405 nm.

2.10. ELISA for antibodies against influenza virus

Sera of mice were analyzed for antibodies against A/Japan/57 influenza virus. The 96-well microplates (EnzyPlates) were coated with 50 μl of the virus (10 HA units/ml) in PBS for 1 h at 37 °C. Saturation of the plates, and dilution of the sera and of the developing reagents was performed with PBS-0.05% Tween 20, pH 7.4, (PBS-Tw). Excess of reagents and antibodies was washed out six times with PBS-Tw. Bound antibodies were detected by incubation for 1 h at 37 °C with horse-radish peroxidase-conjugated goat *anti*-mouse IgM and IgG. The plates were

developed with *ortho*-phenylenediamine and H_2O_2, the substrate reaction was stopped with 4 M H_2SO_4 and the optical density was read at 492 nm.

2.11. Competitive ELISA for measurement of antibodies against SAP

The 96-well Polysorp plates were coated with 100 μl $F(ab)_2$ antibodies (1 μg/ml) against SAP diluted in 50 mM sodium carbonate, pH 9.6, and incubated overnight at 4 °C. After three washes in TBS-Tw the wells were quenched with TBS-0.1% Tween 20, pH 7.4, for 30 min at 20 °C. SAP (4 μg/ml) and dilutions of mouse serum were incubated for 1 h at 20 °C, and 100 μl samples were then applied to the wells and incubated for 2 h at 20 °C. After washing the wells were incubated with biotinylated *anti*-SAP, and developed as described above.

2.12. Infection of mice with influenza virus

Infection of female BALB/c mice was performed by intranasal administration of 30 μl influenza A/Japan/57 given as drops after anaesthetization with chetamin. The effect of SAP was assessed either by preincubation of virus (final dilution 1:1000, $2LD_{50}$) with SAP (800 μg/ml 150 mM NaCl and 1.2 mM $CaCl_2$ (NaCl–Ca)) overnight at 4 °C before infection or by intranasal administration of SAP (40 in 30 μl NaCl–Ca) and additives 1 h before infection ($2LD_{50}$).

In the first type of experiment five mice were infected intranasally with 30 μl of the SAP-virus mixture and five mice with virus ($2LD_{50}$) in NaCl as control. Blood samples were drawn on the 9th and 21st day after infection.

The second type of experiment included five groups of mice, each group consisting of five mice. Two groups received 40 μg SAP in NaCl–Ca and addition of either methylcellulose (1.5% v/v) or Softigen® (4%) intranasally 1 h before viral exposure. Two other groups received only NaCl–Ca and either methylcellulose (1.5%) or Softigen® (4%) 1 h before viral infection. A fifth group of mice received no treatment prior to viral exposure. The body weight and mortality of the mice were recorded daily for 11 days after viral exposure.

3. Results

3.1. In vitro studies

3.1.1. SAP inhibition of influenza and parainfluenza virus hemagglutination

We have earlier reported (Andersen et al., 1997) that SAP inhibited HA activity of eight human and two equine influenza A virus strains. This part of the study was extended to comprise also five mouse-adapted influenza A virus strains, two swine influenza virus strains, one mink influenza A virus strain, one influenza A reassortant virus, one influenza B virus and one parainfluenza 3 virus strain. The HA activity of all these virus strains could be inhibited by SAP. However, the lowest SAP concentration required to inhibit the HA reaction showed virus- and strain-related differences, as illustrated in Table 1.

Table 1
Inhibition of influenza and parainfluenza virus hemagglutination

Virus strain	SAP concentration (μg/ml) required for inhibition
Swine infl. A, New Jersey/76	32
Swine infl. A, Bavaria 2/77	64
Mink infl. A, Sweden/84	16
Ferret infl. A, reassortant 1	30
Infl. B, Beijing /184/93, 28451	6
Infl. B, Beijing /184/93, 29016	12
Parainfl. JM 8/79	6

1A/Puerto Rico /8/34 (HINI)—A/England/939/69 (H3N2).

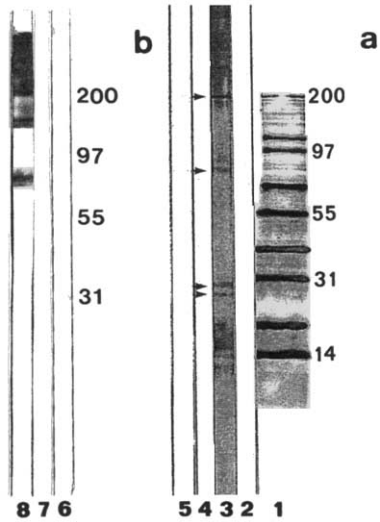

Fig. 1. Western blot of SDS-PAGE of split virions showing binding of SAP (a) and *anti*-HA antibodies (b). MW marker (lane 1), buffer with 2 mM Ca^{2+} (lane 2), SAP with 2 mM Ca^{2+} (lane 3, four bands indicated by arrow heads), SAP with 10 mM EDTA (lane 4), SAP with 2 mM Ca^{2+} but without *anti*-SAP (lane 5), *anti*-HLA–DR (lane 6), *anti*-HA antibodies (lanes 7 and 8). Reducing conditions lanes 1–7, non-reducing conditions lane 8. Vertical numbers, kDa from MW marker.

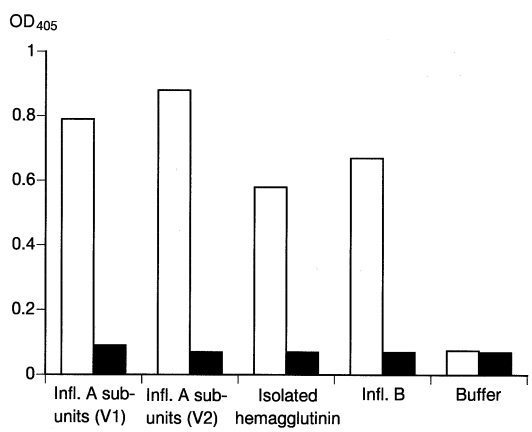

Fig. 2. Ca^{2+}-dependent binding of SAP to influenza A split virions, isolated influenza A hemagglutinins (top 3 from Mono Q column) and influenza B virus determined by ELISA. SAP with 2 mM Ca^{2+} (□), SAP with 10 mM EDTA (■). V1, Fluzone vaccine; V2, Nobi-Equenza vaccine.

3.1.2. SAP-binding to influenza split virions analysed by SDS-PAGE and Western blotting

SDS-PAGE and silver staining of the vaccine showed a strong component at about 75 kDa and weaker bands at about 26, 150 and 220 kDa (non-reduced). Reducing conditions showed major bands at 26, 28 and 56 kDa (not shown). On Western blots the monoclonal antibody *anti*-H3 reacted with components of about 75, 150 and 220 kDa and some material of higher MW (non-reduced) and strongly with the 26, 28 and 56 kDa peptides and weakly with components between 100 and 200 kDa (reduced) (Fig. 1b). SAP bound to the 26, 28 and 75 kDa peptides as well as to a component of 200–220 kDa, some higher MW material and breakdown products (13–15 kDa) (Fig. 1a). A weaker binding to the 56 kDa peptide was occasionally also seen.

3.1.3. SAP-binding to fractionated influenza A virus and to influenza B virus

The elution curve from the Mono Q column of Nobi-Equenza subunit vaccine showed 3–4 peaks, one of which (top 3) showed strong reactivity with *anti*-influenza A hemagglutinin antibodies in ELISA. SDS-PAGE and Western blot of top 3 showed Ca^{2+}-dependent binding of SAP to 28 and 75 kDa peptides (not shown). The ELISA confirmed that this HA-containing fraction bound SAP (Fig. 2) and that the binding was partly inhibited by preincubation of SAP (2 μg/ml) with D-mannose, L-fucose, and D-galactose (16 and 32 mM) for 30 min at 20 °C in the presence of 2 mM Ca^{2+} (not shown). SAP was found to bind Ca^{2+}-dependently also to influenza B (HongKong/5/72) virus (Fig. 2).

3.1.4. Effect of different compounds on the binding of SAP to influenza A virus

Serum albumin at physiological concentrations is known to reduce the propensity of purified SAP to aggregate (Hutchinson et al., 2000). To examine whether BSA interfered with SAP's binding to influenza A virus hemagglutinin, BSA was added to SAP at increasing concentrations before determining the binding. The ELISA results showed a minor reduction in SAP binding when the BSA concentration exceeded 10 mg/ml (not shown). It was decided to routinely incorporate 1% serum albumin in the SAP preparations.

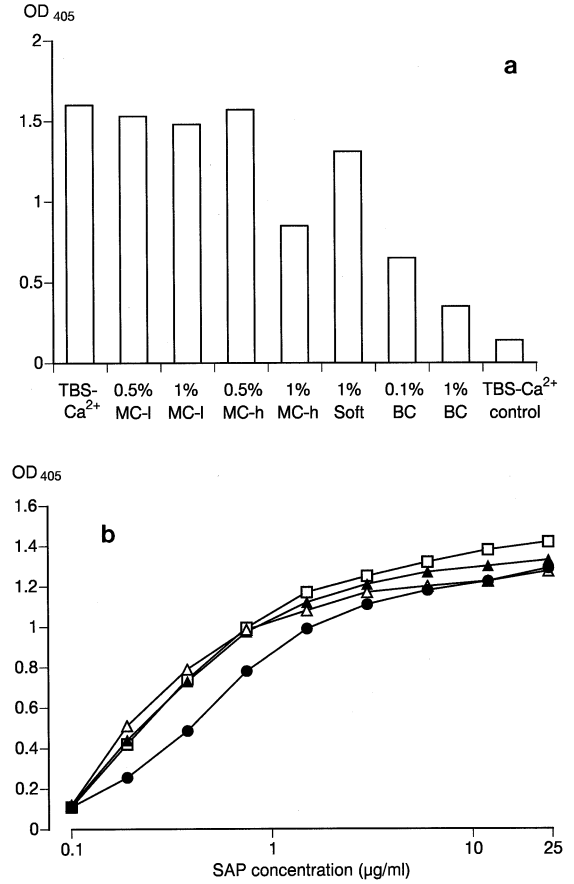

Fig. 3. (a) The influence of methylcellulose of low (MC-l) and high viscosity (MC-h), Softigen® (Soft) and benzalkonium chloride (BC) on SAP's binding to influenza A hemagglutinins. TBS-Ca^{2+}, SAP without additives; TBS-Ca^{2+} control, background in absence of SAP; (b) binding of SAP to influenza A split virions (Nobi Equenza). SAP preparation 1 (□), SAP preparation 2 (△), SAP preparation 2 in the presence of 1% MC-l (●) and SAP preparation 2 in the presence of 4% Softigen® (▲).

In preparation for the in vivo studies the influence of methylcellulose, benzalkonium chloride, and Softigen® on the binding of SAP to influenza A virus hemagglutinin was also examined. Results obtained when varying concentrations of the compounds were added to SAP (20 μg/ml) before determining the SAP binding by ELISA are shown in Fig. 3a. The most pronounced inhibition of SAP binding was seen with methylcellulose of high viscosity (1%) and with benzalkonium chloride. One percent methylcellulose, low viscosity, and 4% Softigen® were selected for further studies. Fig. 3b shows the binding of SAP at increasing concentrations to influenza A virus and in the presence of these additives, as determined by ELISA.

3.2. In vivo studies

3.2.1. Toxicity in mice

Six outbread NMRI male mice were each given 40 μl of SAP (100 μg/ml) in NaCl–Ca as intranasal drops three times with 3 h interval. The treatment was repeated on day 2. Six control mice received NaCl–Ca only.

The mice were observed daily and no sneezing or coughing was observed. The mice were killed on days 2 and 5 (three SAP treated and three controls each day). Visual inspection of the nasal mucosa, pleura and lungs showed a normal appearance for all mice. There was no local irritation observed in the upper respiratory tract observed in the experiments (Section 3.2.2) reported below.

3.2.2. SAP administered as nasal drops is not immunogenic

To investigate whether intranasally administered SAP (8 μg twice with 4 h interval on day 1 and 8 μg twice with 4 h interval on day 2) would penetrate the mucosal membrane and generate antibodies against human SAP, sera were collected 14 (three mice) and 20 (three mice) days after treatment and tested by a competitive ELISA. Control sera were obtained from six mice receiving NaCl as nasal drops. Polyclonal antibodies against SAP were used as positive control. No antibodies against SAP were detected in the sera from the mice (not shown).

3.2.3. Effect of different compounds on SAP's retention on the nasal mucosa

To investigate the effect of different compounds on the mucociliary clearance of SAP after intranasal administration, benzalkonium chloride, methylcellulose (high and low viscosity) and Softigen® were added to SAP labelled with I^{125} by the Jodo-Gen® method and administered as nose drops (15 μl/nostril) to mice. The mice were killed

after 1–4 h, and the radioactivity in transections (3 mm) of the nasal cavity and pharynx as well as in lungs and stomach was determined. The compounds retarded transport of SAP from the nasal mucosa in a concentration-dependent manner and the effect was most pronounced using 2.1% benzalkonium chloride (not shown). However, due to the etching effect of benzalkonium chloride and its inhibition of SAP's binding to virus (Fig. 3a), this compound was omitted in further studies. Based on these findings and the results of the SAP-binding studies, it was decided to incorporate 1% serum albumin and either 1–1.5% methylcellulose, low viscosity, or 4% Softigen® in the SAP preparations to be administered intranasally.

3.2.4. Influence of nasal secretion, broncho-alveolar lavage and saliva on the binding of SAP to influenza A virus

Dilutions of human nasal secretion were added to SAP (3 μg/ml) and preincubated for 30 min at 37 °C before testing the binding of SAP to influenza A hemagglutinin by ELISA. Nasal secretion caused a modest concentration-dependent inhibition of SAP binding. The highest concentration (1:2 dilution) of the secretion caused 20–25% inhibition of SAP binding (not shown). Human broncho-alveolar lavage (BAL) and saliva (both 1:2) were also preincubated for 30 min at 37 °C with SAP (600 μg/ml) before testing SAP's HI activity. Neither BAL nor saliva reduced SAP's HI activity at the concentration tested.

3.2.5. Effect of SAP on influenza A virus infection

One group of five mice was infected intranasally with $2LD_{50}$ of A/Japan/57 virus preincubated with SAP (800 μg/ml (see Section 2). Another group of five mice was infected with the virus only. The results (Fig. 4a) showed that preincubation of virus with SAP completely prevented the infection. The body weight of mice not receiving SAP had decreased, on average, by 36% on day 9, and three mice in this group died. Blood samples were collected on days 9 and 21 postinfection and tested for antibodies against the influenza virus strain used. In the sera of mice infected with virus preincubated with SAP no virus specific antibodies of either IgM or IgG class were detected (Fig. 4b). A secondary infection of the surviving mice (two in the group initially exposed to virus only and five in the group given virus and SAP) with $2LD_{50}$ of influenza A/Japan/57 virus, also indicated that an antiviral immune response had not developed in the mice infected initially with virus incubated with SAP (Fig. 4a).

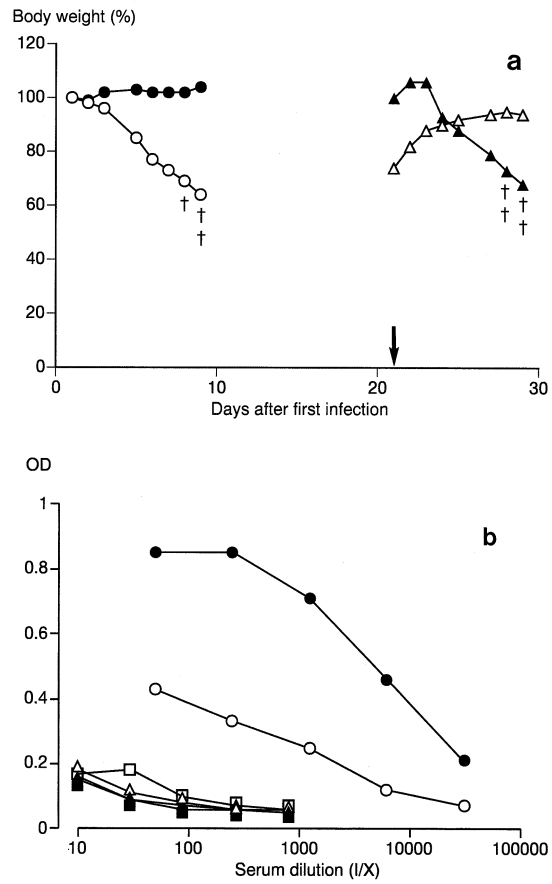

Fig. 4. (a) Body weight and mortality of mice infected with influenza A Japan/57 virus only (○) or virus preincubated with SAP (●). The mice receiving virus and SAP developed no infection and challenge with the virus (arrow) indicated that they had not developed immunity (▲) in contrast to the mice in the other group (△). Dead mice +; (b) antiviral antibodies were not detected in mice receiving SAP and virus. Administration of virus only; bleedings 21 (●) and 9 days (○) after infection. Administration of SAP and virus; bleedings 21 (▲) and 9 days (△) after infection. Non-infected animals; bleedings 21 (■) and 9 days (□) after infection.

Another experiment comprised five groups of mice with five mice per group. Two groups of mice received SAP (40 μg/mouse) in NaCl–Ca and either 1.5% methylcellulose or 4% Softigen® intranasally 1 h before viral (A/Japan/57, $2LD_{50}$) exposure. Two other groups received either 1.5% methylcellulose or 4% Softigen® only 1 h before infection and the fifth group received no treatment prior to viral exposure. The two groups receiving SAP and either methylcellulose or Softigen® showed a mild infection typically lasting 4–6 days, and at the 11th day after infection most of the animals were approaching normal body weight. Only one out of the 10 animals in these two groups had died within 10 days after viral exposure. By contrast, mice, which did not receive SAP before viral exposure lost significant amounts of body weight and four of the five animals had died within 10 days of viral exposure (Fig. 5). Pretreatment with either methylcellulose or Softigen® alone had a modest inhibitory effect on the viral infection. Administration of Softigen® (4%) caused weight loss on days 1–3 indicating some toxicity. It is being investigated whether this toxicity can be eliminated by reducing the Softigen® concentration. Although the antibody response was not measured, the mild infection following SAP treatment would be expected to induce a local immune response.

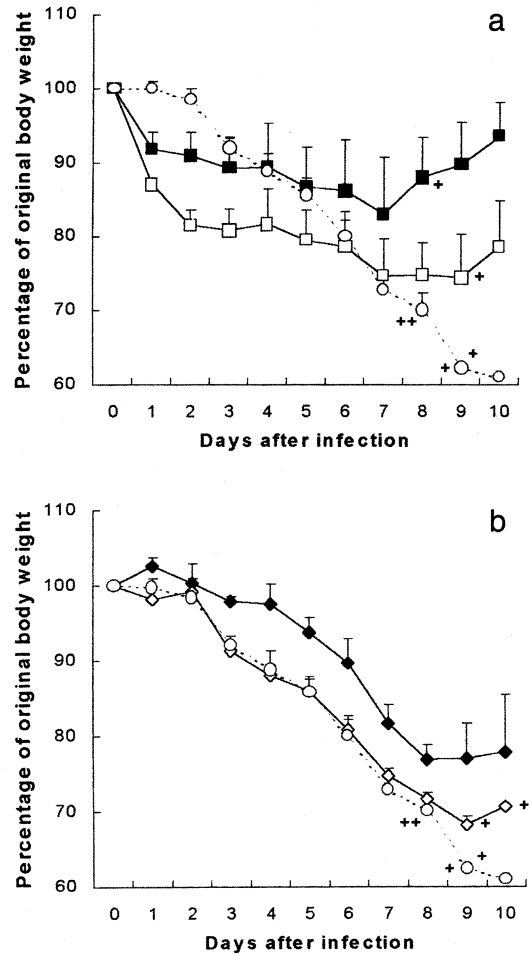

Fig. 5. Body weight and mortality: (a) of mice given SAP (40 μg) and Softigen® (■) or Softigen® only (□); and (b) of mice given SAP and methylcellulose (◆) or methylcellulose only (◇) intranasally 1 h before infection with influenza A Japan/57 virus. Mice receiving no treatment prior to viral exposure (○). Dead mice +.

4. Discussion

Infection with influenza A virus is initiated by the attachment of HA to sialic acid on glycoprotein/glycolipid receptors of the host cells. The receptor binding pocket on the HA molecule includes 5–6 conserved amino acid residues. A further prerequisite for infection with the virus is the enzymatic cleavage of the hemagglutinin molecule by serine proteases of epithelial cells in the respiratory tract giving rise to disulfide-bridge-linked HA1 and HA2 subunits (Webster and Rott, 1987).

Influenza A hemagglutinin molecules are covered by carbohydrates up to 20% (by weight) of the surface. Most of the carbohydrates consist of complex type oligosaccharides but also the oligomannoside type is expressed. The complex type contains mannose, galactose and fucose, the simple type only mannose (Ward, 1981). SAP has the capacity to interact Ca^{2+}-dependently with terminal mannose, galactose and fucose (Hind et al., 1985; Kubak et al., 1988; Loveless et al., 1992), which is compatible with its binding to hemagglutinin of all influenza A strains tested. The contribution of these three monosaccharides to the SAP-reactive site(s) was confirmed by our inhibition studies.

In extension of our earlier studies (Andersen et al., 1997) we have examined SAP's capacity to inhibit the HA reaction of additional influenza virus subtypes and strains of both human, swine and mink origin. We have tested 19 influenza A strains and two B virus isolates and they could all be inhibited by SAP in the presence of Ca^{2+}. These studies have shown strain-related differences in the minimal SAP concentration required to inhibit the HA reaction probably related to differences in glycosylation and expression of the oligosaccharide moieties mannose, galactose and fucose.

Hemagglutinin trimers solubilized from the viral membrane of different influenza A subtypes have a molecular weight of about 220 kDa and the monomers are approximately 75 kDa. Efficient reduction of the monomers yields the HA1 and HA2 subunits with a MW of 50–56 and about 28 kDa, respectively. Both these subunits are glycosylated with 4–6 glycosylation sites on HA1 and 1–2 on HA2. Hemagglutinin subtypes show a varying number and localization of the oligosaccharides, but all express the complex type on the surface of the HA-trimer (Ward, 1981). Our immunoblotting studies of fractionated influenza A split virions have demonstrated a Ca^{2+}-dependent binding of SAP to components of about 220, 75, 56 and 28 kDa, likely representing HA trimers, monomers and HA1 and HA2 subunits. All these components reacted with *anti*-HA antibodies.

In preparation for the in vivo studies the influence of different compounds on SAP's binding to influenza A hemagglutinin was investigated. Purified SAP shows a concentration-dependent tendency to form aggregates, which can be reduced by addition of serum albumin (Hutchinson et al., 2000). Therefore, serum albumin was incorporated in the purified SAP preparations, and it was tested whether serum albumin interfered with the SAP–hemagglutinin interaction. The ELISA results indicated that 1% serum albumin reduced SAP aggregation without inhibiting the binding of SAP to the viral hemagglutinin.

Furthermore, it was desirable to reduce the rate of mucociliary clearance of SAP administered intranasally by the addition of compounds that retard clearance. In vitro binding studies indicated acceptable concentrations of the compounds for in vivo studies. Based on the results of both in vitro binding and in vivo clearance studies, 1% methylcellulose of low viscosity or 4% Softigen® was selected as an additive to the SAP preparations.

Before investigating the effect of intranasal administration of SAP on infection with influenza A virus it was important to establish whether nasal or bronchial secretions would inhibit the binding of SAP to the virus. Preincubating SAP with 1:2 dilutions of the secretions was found to cause only modest inhibition of the binding.

When initiating in vivo experiments in mice, a possible toxic or allergic reaction to intranasal administration of SAP was investigated. We observed no local irritation from the nasal cavity or upper respiratory tract upon repeated exposure to SAP (total dose 24 μg per mouse). The lungs showed a normal appearance.

Although SAP is a relatively large glycoprotein (decameric SAP, MW 255 kDa, Pinteric et al., 1976) which is rather resistant to proteolytic cleavage and would not be expected to penetrate the nasal mucosa, the possible immunogenicity of human SAP given as nose drops (total dose 32 μg per mouse) was investigated. Circulating antibodies against SAP could not be demonstrated in sera collected 2 and 3 weeks after administration of SAP.

SAP inhibits the HA reaction of a wide spectrum of influenza A serotypes and it also inhibited the infection of MDCK cells by both A and B virus (Andersen et al., 1997; Svehag, unpublished). We therefore examined the effect of SAP on infection of mice with influenza A virus. When $2LD_{50}$ of virus was preincubated with a rather high concentration of SAP (800 μg/ml) the infection was completely prevented and no humoral immunity was induced. However, when animals were given a single dose (40 μg) of purified SAP as nasal drops 1 h before virus infection ($2LD_{50}$), a mild infection occurred lasting 4–6 days. Control mice showed progressive loss of weight and died, at the time when most of the SAP-treated mice were approaching normal body weight.

Influenza virus type A is a major cause of morbidity and mortality due to respiratory disease and outbreaks may occur in world-wide epidemics. Existing influenza vaccines become gradually less effective when the viruses undergo antigenic changes. Prophylaxis has been attempted with drugs such as amantadine and its analogue rimantadine. However, their clinical use has been limited by disturbing side effects and the rapid emergence of resistant virus strains. Recently, compounds that inhibit influenza virus sialidase have been reported to limit the spread of virus and to alleviate the symptoms (Hayden et al., 1997, 1999; Mäkelä et al., 2000; Monto et al., 2000; Murphy et al., 2000).

Unlike vaccines, the antiviral effect of SAP is not restricted to a certain variant (serotype) of influenza A virus. It has been effective in inhibiting HA of both influenza A and B serotypes in the laboratory experiments. Unlike influenza sialidase inhibitors, which may limit the spread of the disease, SAP prevents/inhibits the initial infection. Antiviral antibodies are the main effectors against infections with cytopathic viruses like influenza (Kagi and Hengartner, 1996), and locally administered SAP could act similarly to neutralizing antibodies during the initial infection. SAP could prevent the development of serious infection and, concomitantly, promote the generation of efficient antiviral effector and memory immune response.

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References

Andersen, O., Vilsgaard Ravn, K., Juul Sørensen, I., Jonson, G., Holm Nielsen, E., Svehag, S.-E., 1997. Serum amyloid P component binds to influenza A virus haemagglutinin and inhibits the virus infection in vitro. Scand. J. Immunol. 46, 331–337.

Dhawan, S., Fields, R.L., Robey, F.A., 1990. A novel peptide from amyloid P component supports cell attachment. Biochim. Biophys. Acta. 171, 1284–1290.

Emsley, J., White, H.E., O'Hara, B.P., Oliva, G., Srinivasan, N., Tickle, I.J., Blundell, T.L., Pepys, M.B., Wood, S.P., 1994. Structure of pentameric human serum amyloid P component. Nature 367, 338–345.

Feizi, T., 2000. Carbohydrate-mediated recognition systems in innate immunity. Immunol. Rev. 173, 79–88.

Hayden, F.G., Albert, D.M.E., Treanor, J.J., Fleming, D.M., Aoki, F.Y., Nicholson, K.G., Bohnen, A.M., Hirst, H.M., Keene, O., Wightman, K., 1997. Efficacy and safety of the neuraminidase inhibitor Zanamivir in the treatment of influenza virus infections. New Engl. J. Med. 337, 874–880.

Hayden, F.G., Atmar, R.L., Schilling, M., Johnson, C., Poretz, D., Paar, D., Huson, L., Ward, P., Mills, R.G., 1999. Use of the selective oral neuraminidase inhibitor Oseltamivir to prevent influenza. New Engl. J. Med. 341, 1336–1343.

Hind, C.R.K., Collin, P.M., Baltz, M., Pepys, M.B., 1985. Human serum amyloid P component, a circulating lectin with specificity for the cyclic, 4:6-pyruvate acetal of galactose. Biochem. J. 225, 107–111.

Hutchinson, W.L., Hohenester, E., Pepys, M.B., 2000. Human serum amyloid P component is a single uncomplexed pentamer in whole serum. Mol. Med. 6, 482–493.

Juul Sørensen, I., Andersen, O., Holm Nielsen, E., Svehag, S.-E., 1995. Native human serum amyloid P component is a single pentamer. Scand. J. Immunol. 41, 263–267.

Kagi, D., Hengartner, H., 1996. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr. Opin. Immunol. 8, 472–477.

Kubak, B.M., Potempa, L.A., Anderson, B., 1988. Evidence that serum amyloid P component binds to mannose-terminated sequences of polysaccharides and glycoproteins. Mol. Immunol. 25, 851–858.

Loveless, R.W., Floyd-O'Sullivan, G., Raynes, J.G., Yuen, C.-T., Feizi, T., 1992. Human serum amyloid P is a multispecific adhesive protein whose ligands include 6-phosphorylated mannose and the 3-sulfated saccharides galactose, N-acetylgalactosamin and glucuronic acid. EMBO. J. 11, 813–819.

Mäkelä, M.J., Pauksens, K., Rostila, T., Fleming, D.M., Man, C.Y., Keene, O.N., Webster, A., 2000. Clinical efficacy and safety of the orally inhaled neuraminidase inhibitor Zanamivir in the treatment of influenza: a randomized, double-blind, placebo-controlled European study. J. Infect. 40, 42–48.

Monto, A.S., Moult, A.B., Sharp, S.J., 2000. Effect of Zanamivir on duration and resolution of influenza symptoms. Clin. Ther. 22, 1294–1304.

Murphy, K.R., Eivindson, A., Pauksens, K., Stein, W.J., Tellier, G., Watts, R., Leóphonte, P., Sharp, S.J., Loeschel, E., 2000. Efficacy and safety of inhaled Zanamivir for the treatment of influenza in patients with asthma or chronic obstructive pulmonary disease. A double-blind, randomised, placebo-controlled, multicentre study. Clin. Drug. Invest. 20, 337–349.

Nelson, S.R., Tennent, G.A., Sethi, D., Gower, P.E., Ballardie, F.W., Amatayakul-Chantler, S., Pepys, M.B., 1991. Serum amyloid P component in chronic renal failure and dialysis. Clin. Chim. Acta 200, 191–200.

Nybo, M., Holm Nielsen, E., Svehag, S.-E., 1999. Complement activation by the amyloid proteins Aβ peptide and β_2M-microglobulin. Amyloid: Int. J. Exp. Clin. Invest. 6, 265–272.

Pinteric, L., Assimeh, N., Kells, D.I.C., Painter, R.H., 1976. The ultrastructure of C1t, a subcomponent of the first component of complement: an E.M. and ultracentrifuge study. J. Immunol. 117, 79–83.

Ward, W.C., 1981. Structure of influenza virus hemagglutinin. In: Henle,W., et al. (Eds.), Current Topics in Microbiology and Immunology. Springer Verlag, New York, 94/95, pp. 1–74.

Webster, R.G., Rott, R., 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell 50, 665–666.