日本財団補助金による
２０００年度日中医学学術交流促進事業

⑤．在留中国人研究者研究助成

(5) 水性バクテリアより新規なシアル酸を含まない抗インフルエンザウイルス活性物質の分離及びその化学構造の同定

水性バクテリアより新規なシアル酸を含まない抗インフルエンザウイルス活性物質の分離およびその化学構造の同定

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要旨

バクテリアは、日本の各地から取った2000種の水サンプル(samples)の中から、分離された257株のうち、増殖能のよい27株を選び、大量培養を行いました。それから、クロロホルム・メタノール（1:1)で総脂質を抽出して、TLC/virus-binding assayを用いて、抗インフルエンザウイルス物質の探索を行いました。そこで、これら一つ株水性バクテリア*Rhodococcus equi* S_{420}株より活性物質を分離精製して、NMRやMASSよりその化学構造を明らかにしました。構造はC-14にメチル化された分枝した脂肪酸(14-methylocatadecanic acid) (palmitic acid)を含むユニークなホスファチジルイノシトールでした。このホスファチジルイノシートルが、ヒト、トリやブタから分離されたの１８種インフルエンザウイルスとの結合性をTLC/virus-binding assayで示唆されました。さらに、この物質の抗インフルエンザウイルスの活性を赤血球凝集、溶血及び細胞感染の阻害アッセイで確認しました。この物質の赤血球凝集、溶血および細胞感染に対する阻害活性の結果より、この物の赤血球凝集阻害活性は、シアル酸誘導体 (Neu5Ac-PE)に比べて低いけれども、ウイルス感染や溶血阻害活性は、いずれのウイルス株でも、３倍から８倍の強いことが認められました。

一方、この物質がウイルスのどの部位に結合するかを調べるため、ブロメライン (bromelain)で分解したヘマグルチニンを用いて、TLC/binding assayを行いました。その結果より、このホスファチジルイノシートルはNeu5AcnLc4Cerと同じように結合することが確認できました。したがって、この物質はヘマグルチニンに結合することがわかりました。

また、生物化学的にＴＬＣで検出する結果より、このホスファチジルイノシートルは菌体の総脂質の中に多く存在していました（普通のバクテリア中より４倍高いでした）。したがって、この物質は抗インフルエンザ剤として、利用することが可能と思われます。さらに、シアリダーゼの水解に対しても抵抗性を有することが分かりました。この物質はインフルエンザの化学療法剤として有用であると考えられます。

KEY WORDS: influenza virus; phosphatidylinositol; branched-chain fatty acid; Rhodococcus equi

INTRODUCTION

Influenza virus initiates infection through a receptor-ligand interaction and subsequent receptor-mediated endocytosis. After entry[1,2], the components of the viral particle are disassembled in the endosome where a low pH induces the irreversible conformational change of hemagglutinin (HA), resulting in fusion of the viral and endosomal membranes [3-6]. The viral genome is then released from the endosome and transported into the nucleus where replication occurs [7]. After replication, viral genomes leave the nucleus and progeny virus particles assemble and bud from the cellular membrane of the infected cells [6,8].

Viral proteins, which provide essential functions during the life cycle of influenza virus, are potential targets for the development of antiviral agents. Several agents involved in the infectious cycle have already been documented [9-14]. It is known that the sialic acid-containing glycoproteins or glycolipids on cell surfaces are receptors for influenza viruses [15-20]. Two major virus glycoproteins, HA and sialidase, mediate the interaction between influenza viruses and cellular receptors. They are responsible for the attachment to target cells and for release of progeny viruses from the surface of infected cells, respectively [1,8]. Therefore, many inhibitors of influenza viruses have been developed against virus HA or/and sialidase [14,21-24]. Several studies reported that neoglycoprotein or a synthetic co-polymer containing sialic acid residue strongly inhibited the HA and sialidase activities, resulting in prevention of influenza virus infection [24-30]. However, the effects of most inhibitors are usually restricted to HA subtypes of influenza virus isolates, and their stability has been influenced by the hydrolysis of sialic acid-linkage by viral sialidase. Influenza virus vaccines are also dependent on viral specific antigen [31].

Recent studies have shown that a desialylated glycolipid sulfatide also has receptor-like activity [47] and that two desialylated glycoglycerolipids from *Coryneform* bacteria have binding and neutralizing activities against influenza virus [32]. As desialylaled glycolipids are not substrates for sialidase [64,65], which is a receptor-destroying enzyme and is integrated into the influenza virus envelope, they might be useful as anti-influenza drugs.

EXPERIMENTAL PROCEDURES

Preparation of lipids from *R. equi* strain S420: Two thousand aquatic soil samples were selected in Japan and 257 bacteria were isolated, producing more than detectable levels of glycolipids, of which 27 strains were selected because of their high productivity. In *R. equi* strain S420 (Table I), we found virus-binding substance, which was screened by a thin layer chromatography (TLC)/virus-binding immunostaining method.

R. equi strain S420 was cultivated in a 250-ml Erlenmeyer flask containing 30 ml of YMPG medium. The incubated-aliquot (0.7 ml) was added to a 500-ml Erlenmeyer flask containing 70 ml of YSGG mediumand cultivated for 96 hr at 28° C. The culture was extracted with an equal volume of a chloroform/methanol (1:1, v/v) solvent. The extract was used for this experiment.

Total lipids from strain S420 were fractionated by Q-Sepharose column chromatography [36]. The developing solvent was a mixture of chloroform/methanol/water (60:35:8, v/v/v). Lipids were separated on a TLC plate. The virus-binding substance was purified with LH-20 column chromatography [63].

Structure of the virus-binding substance: The virus-binding substance: colorless oil. [α]D +8° (c 0.37, $CHCl_3$). Figure 2A shows ^1H NMR ($CD_3OD/CDCl_3$, 10:1, v/v): δH 0.84 (3H, d, *J* = 6.4 Hz, CH_3), 0.88 (6H, t, *J* = 6.9 Hz, CH_3 x 2), 1.10 (2H, m, CH_2), 1.27 (41H, m, CH and CH_2 x 20), 1.30 (2H, m, CH_2), 1.35 (4H, m, CH_2 x 2), 1.59 (4H, m, CH_2 x 2), 2.30 (2H, t, *J* = 7.3 Hz, CH_2-C=O), 2.33 (2H, t, *J* = 7.3 Hz, CH2-C=O), 3.20 (1H, t, *J* = 9.4 Hz, 5'-H), 3.37 (1H, dd, *J* = 9.4 and 3.0 Hz, 3'-H), 3.62 (1H, t, *J* = 9.4 Hz, 4'-H), 3.76 (1H, t, *J* = 9.4 Hz, 6'-H), 3.89 (1H, br t, *J* = 9.4 Hz, 1'-H), 4.06 (2H, m, 3-H2), 4.17 (1H, t, *J* = 3.0 Hz, 2'-H), 4.20 (1H, dd, *J* = 12.2 and 7.0 Hz, 1-H), 4.45 (1H, dd, *J* = 12.2 and 3.1 Hz, 1-H), 5.24 (1H, m, 2-H). Figure 2B shows 13C NMR ($CD_3OD/CDCl_3$, 10:1, v/v): δC 15.3 (2C), 21.0, 24.4 (2C), 26.7 (2C), 30.9-31.8 (19C), 33.8 (2C), 34.6, 35.9 (2C), 38.9 (2C), 64.5 (C-1), 65.6 ($J_{C\text{-}P}$ = 5.7 Hz, C-3), 72.6 ($J_{C\text{-}P}$ = 7.6 Hz, C-2), 73.5 (C-3'), 73.7 (br, C-2'), 73.9 ($J_{C\text{-}P}$ = 5.7 Hz, C-6'), 74.7 (C-4'), 76.9 (C-5'), 79.0 (br, C-1'), 175.4, 175.7. (+)FABMS m/z: 875 $[M+Na]^+$, 593. (-)FABMS m/z: 851 $[M-H]^-$, 689. (-)HRFABMS m/z 851.5666 $[M-H]^-$ (Calcd. For $C_{44}H_{84}O_{13}P$: 851.5650). Fatty acid analysis: 10% sodium methylate (0.6 m) was added to the sample (0.1 mg) in 0.2 ml benzene and the reaction mixture was held at 60° C for 20 min. After the addition of a few drops of 1 N AcOH to stop the reaction followed by 2 ml of distilled water, the reaction mixture was extracted with 2 ml of n-hexane. The n-hexane extract was evaporated to dryness and was then followed by fatty acid analysis by gas chromatographic mass (GC-MS). The conditions for GC were: column, J & W Scientific DB-1 (0.25 mm-by-15 m); column temperature, 100-270° C, raised at 20° C/min; injection temperature, 280° C; carrier gas, He, 1.2 kg/cm. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX500 (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR). FABMS and HRFABMS were obtained on a JEOL SX102A spectrometer (glycerol as matrix). Optical rotation was measured on a JASCO DIP-370 digital polarimeter.

Virus-binding immunostaining assay: Lipids were spotted on the Silica gel plastic plates (Polygram Sil G; Macherey-Nagel, Germany). Immunochemical detection of virion or BHA on the TLC plates was performed. In the inhibition assay for virus binding, viruses were preincubated with inhibitors for 1 hr at 4° C and were then used on the binding assay.

Anti-influenza virus assay: The inhibition activity of the PtdIns to influenza virus was detected with hemagglutination, hemolysis inhibition assays and neutralization assay.

RESULTS

Presence of a substance that binds to influenza viruses in *R. equi* strain S420: Two thousand aquatic soil samples were selected in Japan and 257 bacteria were isolated, producing more than detectable levels of glycolipids, of which 27 strains were selected because of their high productivity. The TLC-separated glycolipids were assayed for binding activity to human influenza viruses. *R. equi* strain S420 showed one active spot on all H1 (A/PR/8/34 strain), H2 (A/Singapore/1/57 strain) and H3 (A/Aichi/2/68 strain) subtypes of human influenza A viruses (Fig. 1).

Structure of the virus-binding substance: The virus-binding substance gave a positive sign for Dittmer's test [51], indicating the presence of a phosphate group. The compound showed a pseudomolecular ion peak at *m/z* 851, matching $C_{44}H_{85}O_{13}P$ (HRFABMS: *m/z* 851.5666 $[M-H]^-$, calcd. for $C_{44}H_{85}O_{13}P$: 851.5650). Both ^1H and ^{13}C NMR spectra (Fig. 2A and Fig. 2B), together with a DEPT spectrum, revealed that the substance contains two long-chain acyl groups, two oxygenated methylenes, and seven oxygenated methines. The ^1H-^1H COSY and 1D homonuclear Hartman-Hahn (HOHAHA) spectra indicated the presence of a cyclitol moiety and a glycerol unit. The cyclitol was shown to be myo-inositol on the basis of the 1H vicinal coupling constants of H-1' to H-6' [*J*(1'/2') = 3.0 Hz, *J*(2'/3') = 3.0 Hz, *J*(3'/4') = 9.4 Hz, *J*(4'/5') = 9.4 Hz, *J*(5'/6') = 9.4 Hz, *J*(6'/1') = 9.4 Hz]. The phosphate was determined to be attached at C-3 and C-1' on the basis of the 13C-P coupling constants of C-2 (7.6 Hz), C-3 (5.7 Hz), C-1' (< 2 Hz), C-2' (< 2 Hz), C-6' (5.7 Hz). The ^1H chemical shifts of H_2-1 (δ 4.45 and 4.20) and H-2 (δ 5.24) signals indicate that both the C-1 and C-2 positions of the glycerol moiety are acylated.

Treatment of the compound with MeOH/MeONa afforded 16:0 and 19:0 fatty acid methyl esters which showed molecular ion peaks at m/z 270 and 312, respectively, in the gas chromatography-electron impact mass spectrum (GC-EIMS) (Fig. 2C). The 16:0 fatty acid methyl ester showed GLC retention time and mass spectrum identical to those of authentic palmitic acid methyl esters. On the other hand, the EIMS of 19:0 fatty acid methyl ester showed relatively intense peaks at *m/z* 255 $[M-C_4H_9]^+$, originating from a-cleavages with respect to the methine carbon atom carrying the methyl branch, and the absence of a peak at *m/z* 241, corresponding to the ion $[M-C_5H_{11}]^+$. These findings indicated that the 19:0 fatty acid is 14-methyloctadecanoic acid. Thus, the structure of the virus-binding substance was determined to be a PtdIns bearing a branched-chain fatty acid (Fig. 3).

By chemical staining analyses, it was shown that the purified PtdIns was the main lipid (16.5% of total lipids) and further is present as a major phospholipid (about 55% of phospholipids) in *R. equi* strain S420 (Fig. 2D, 2E). Purification rate of PtdIns was approximate 60%.

The binding specificity of the purified PtdIns to influenza viruses: The binding specificity of the purified PtdIns to influenza viruses was determined by a TLC/virus-binding immunostaining assay. As shown in Table II, all the isolates tested from human (A and B types) and animal (avian and swine) species bound to the purified PtdIns. The binding specificity of various influenza viruses to the purified PtdIns was different from their viral sialic acid-linkage specificity. These findings indicate that the binding of purified PtdIns to virus were not dependent on the different isolates or HA types of influenza viruses.

Inhibition activity of the purified PtdIns on the virus-mediated hemolysis and the cell infection by influenza viruses: To determine the anti-viral activity of the purified PtdIns, we carried out hemagglutination inhibition, hemolysis inhibition and neutralization assays. As shown in Table III, the purified PtdIns potently reduced the releasing LDH activity of virus-infected MDCK cells in a dose dependent manner. The IC_{50}s of the purified PtdIns (a concentration of purified PtdIns for 50% infection being inhibited) to A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2) and A/Aichi/2/68 (H3N2) strains were 20.1±2.3, 14.7±3.2 and 10.4±4.1 μM

(n = 3 experiments), respectively. The inhibition activity of purified PtdIns was about 6-fold stronger than that of Neu5Ac-PE which was indicated previously a potent inhibitor against human influenza virus. Similarly, the inhibition activity of the purified PtdIns to virus-mediated hemolysis of erythrocytes was also stronger than that of Neu5Ac-PE. However, the purified PtdIns exhibited weaker inhibition of viral hemagglutination relative to Neu5Ac-PE. These findings show that the purified PtdIns significantly prevented both virus-mediated hemolysis and infection by human influenza A virus in vitro, and was effective against influenza A viruses regardless of subtypes.

Inhibition activities of PtdInses containing different fatty acid residues: To examine the influence of species of fatty acid residues to viral infection, we tested PtdInses containing three types of fatty acid residues as inhibitors of influenza viruses. As shown in Table IV, the inhibition activities of PtdInses each other were significantly different. The effect of the PtdIns from R. equi strain S420 on virus-mediated hemolysis and cell infection by influenza virus in vitro were 15-fold and 40-fold stronger than those of PtdInses from bovine liver or soybean, respectively. We also used a fatty acid-free PtdIns (GPI) in these experiments. Although the GPI inhibited viral hemagglutination at a concentration of 1 mM, it did not inhibit either the virus-mediated hemolysis or viral infection of MDCK cells even at a concentration of 2 mM (data not shown). These findings indicate that the fatty acid residue on PtdIns might be an indispensable part of their inhibition of viral hemolysis and viral replication.

Binding of the purified PtdIns to bromelain-cleaved hemagglutinin: The above findings indicate that the purified PtdIns prevents influenza virus infection. In order to clarify the mechanism of its anti-influenza activity, we used the bromelain-cleaved hemagglutinin (BHA) of A/Aichi/2/68 (H3N2) virus instead of virus particles for TLC/virus-binding assay. The binding specificity of PtdIns to BHA on plates was examined using mouse monoclonal anti-HA (H3) antibody and HRP-conjugated goat anti-mouse IgG+IgM (H+L) (Jackson Immunoresearch Laboratories). As shown in Fig. 4, the BHA clearly bound to the purified PtdIns as well as IV6Neu5AcnLc4Cer. This experimental finding indicates that HA of influenza virus is a component in the binding to purified PtdIns.

DISCUSSION

In this study, we isolated and purified a unique type of phosphatidylinositol (PtdIns) from *R. equi*, bearing a branched-chain fatty acid (14-methyloctadecanoic acid). Furthermore, we first found that it strongly inhibited the virus-induced hemolysis and the infection of influenza virus in vitro. The discovery evidently indicates existence of new type anti-influenza compounds in microorganisms. Otherwise, it is shown PtdIns in R. equi strain S420 is the main phospholipid and accounts for about 55% of total phospholipids, because the ratio of PtdIns in Corynebacteria is usually lower than 10% of total phospholipids. This creates favorable condition for opening up new agents against influenza.

Phosphatidylinositol is usually present in the inner layer of the cellular membrane lipid bilayer and is a precursor for several prominent intracellular signaling molecules [35,57-59]. In this study we have first studied phosphatidylinositol regard as an inhibitor against influenza virus.

The binding of influenza virus to sialic acid has been shown to depend on the molecular species of sialic acid, the sialic acid-Gal linkage and the carbohydrate core structure of ganglioside [18]. Therefore, the anti-influenza effects of the sialic acid-containing inhibitors are usually restricted by these factors. It is a novel finding that the PtdIns from *R. equi* strain S420 binds to influenza viruses and that it significantly inhibits the influenza virus infection in vitro. The inhibitory activity of the purified PtdIns on influenza viruses is not restricted to animal species or types (or subtypes) of viral HA. The purified PtdIns from strain S420 may be considered a new type of anti-influenza virus agent, widely effective against human and animal influenza A viruses.

The finding show that purified PtdIns from strain S420 strongly inhibited the virus-mediated hemolysis in comparison with sialic acid-containing inhibitors, or other PtdInses from different materials. We also confirmed that the purified PtdIns attached to viral HA and that the binding position of PtdIns differs from the sialic acid-binding pocket and may be close to the pocket on the head of the HA trimer. The finding on HAI also indicated that the effect of purified PtdIns on the sialic acid-binding pocket of viral HA is weaker. Regarding the anti-influenza virus mechanism of the purified PtdIns, one possibility is that PtdIns adheres to the viral HA and then enters the endosome together with the virion, resulting in interference with the fusion of viral and endosomal membranes.

We also used other phospholipids in the same experiment to compare their effects against influenza viruses (data not shown). Bovine liver PtdIns, phosphatidylserine (PS, from bovine brain, Sigma P-7769) and phosphatidylenthanolamine (PE, from bovine brain, Sigma P-7693), all of which carry a negative charge, bound to influenza viruses, but neutral glycophospholipids such as phosphatidylcholine (PC, from bovine liver, Sigma P-6638) and sphingomyelin (SM, from bovine brain, Sigma S-7004) did not. None of PC, PE and SM showed inhibition activities of virus-induced hemolysis at a concentration of 2 mM. Both bovine liver PtdIns and PS showed inhibition activities, but their effects were much lower than that of the purified PtdIns. Adhesion of phospholipids to viral HA may be an indispensable step in preventing influenza virus infection. It is noteworthy that the virus binding activity of bovine liver PtdIns to influenza viruses was similar to that of purified PtdIns, however the inhibitory activity of bovine liver PtdIns on the influenza virus-mediated fusion was lower than that of the purified bacterial PtdIns. The structural difference between the purified PtdIns and bovine liver PtdIns is that it bears a branched-chain fatty acid. L-α-glycerophospho-D-myo-inositol from soybean, which has no fatty acid residue, showed no inhibitory activity of virus-mediated hemagglutination and hemolysis. These findings indicate that the phosphatidylinositol and branched fatty acid residue may be important for the binding of viral HA and for interfering with membrane fusion, respectively, leading to inhibition of infection by influenza viruses.

Some sialic acid-containing derivatives usually inhibit the attachment of viral HA to target cells and/or the enzymatic activity of viral sialidase, thus preventing infection by influenza virus. In contrast, the purified PtdIns preferentially targets virus-mediated cellular membrane fusion. Our hypothesis is that PtdIns binds near the fusion loop of HA, resulting in interference with the fusogenic function of viral HA, thus preventing the growth of human influenza A virus.

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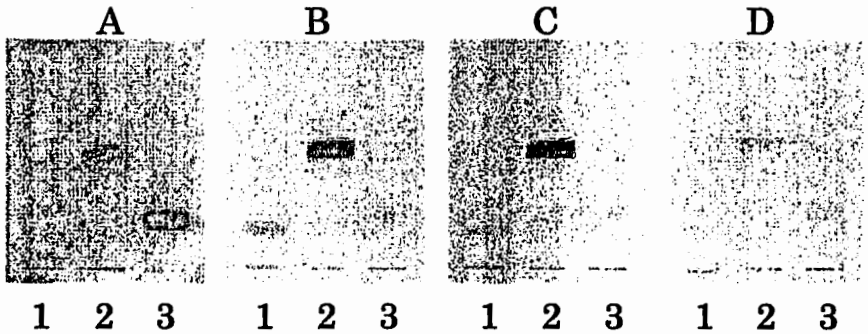

Fig. 1. Binding specificity of the purified PtdIns to human influenza A viruses. The binding specificity of the PtdIns isolated from aquatic bacteria R. equi strain S420 (2 nmol) (lane 2) to human influenza A viruses [A/PR/8/34 (H1N1) (a), A/Singapore/1/57 (H2N2) (b), and A/Aichi/2/68 (H3N2) (c)] was determined by TLC/virus-binding immunostaining assay as described under "Experimental Procedures". TLC Plate was visualized with orcinol-H2SO4 reagent (d). IV6Neu5AcnLc4Cer (lane 1) and IV3Neu5AcnLc4Cer (lane 3) (1 nmol) were used as positive controls.

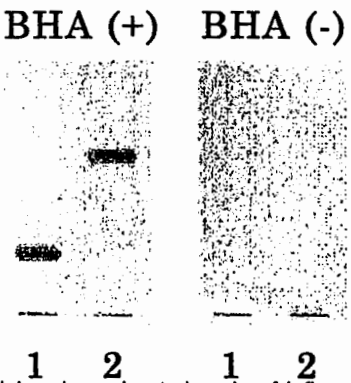

Fig. 4. Binding of the purified PtdIns to bromelain released ectodomain of influenza hemagglutinin from A/Aichi/2/68 (H3N2) strain. The binding of purified PtdIns (2 nmol) (lane 2) to bromelain-released ectodomain of influenza HA (BHA) from A/Aichi/2/68 (H3N2) strain was determined using a TLC/HA-binding immunostaining assay as described under "Experimental Procedures". IV6Neu5AcnLc4Cer (1 nmol) (lane 1) was used as a positive control.

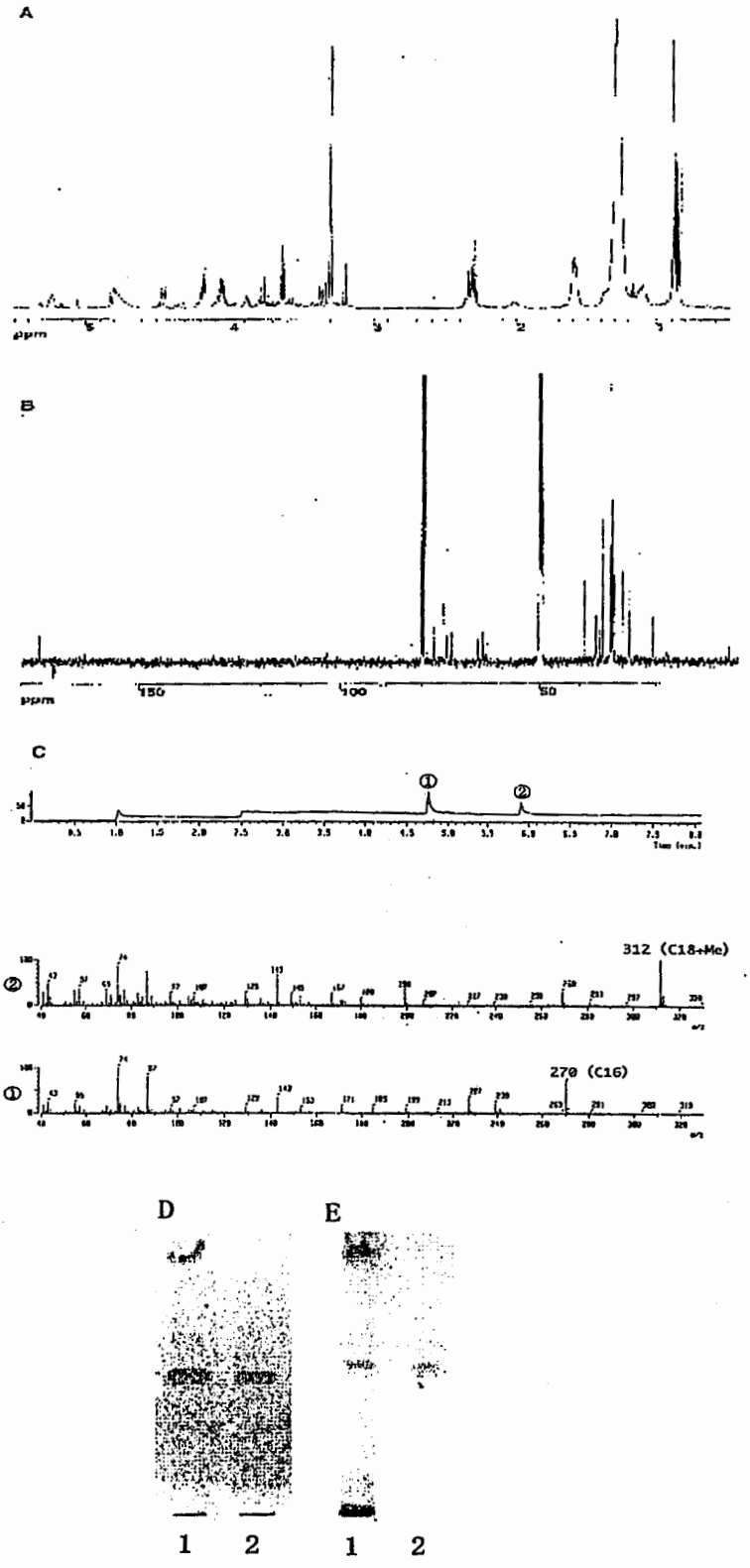

Fig. 2. A: An ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from R. equi strain S420.

B: A ^{13}C-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from R. equi strain S420.

C: A gas chromatography-electron impact mass spectrum of the PtdIns from *R. equi* strain S420.

D and E: Profile of phospholipids and total glycolipids from *R. equi* strain S420. Profiles of phospholipids (plate D) and glycolipids (plate E) were visualized with Dittmer's reagent and orcinol-H_2SO_4 reagent, respectively. Lane 1 is total lipids and lane 2 is the purified PtdIns (2 nmol).

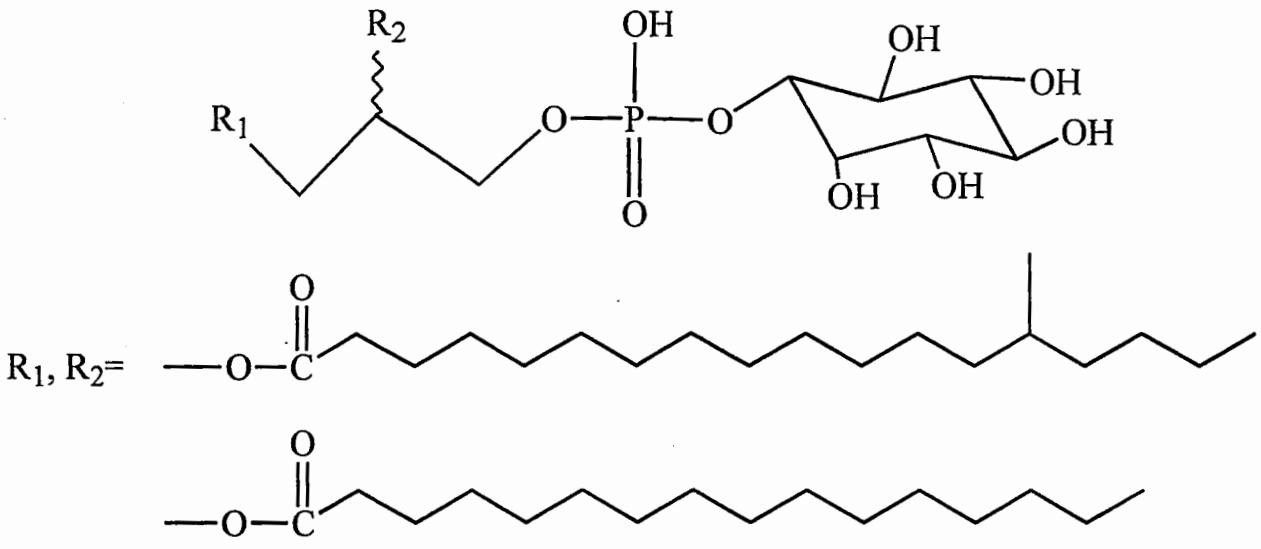

Fig. 3. Structure of the PtdIns from *R. equi* strain S420.

TABLE I. **Microbiological property of *Rhodococcus equi* strain S_{420}.**

	Strain S_{420}
Colony	
Shape	Flat and round
Tone	Buttery, opaque, and beige
Cell shape	
Shape	Long-rod
Width	0.8 μm
Moving	-
Gram-staining	+
Spore formation	-
Enzymatic properties	
Pyrazinamidase	+
Pyrrolidonearylamidase	-
Alkaline phosphatase	+
Catalase	+
β-Glucuronidase	-
β-Galactosidase	-
β-Glucosidase	+
N-Acetyl-β-glucosaminidase	-
Urease	+
Esculin hydrolyzation	+
Gelatin hydrolyzation	-
Nitrate reduction	-
Fermentation ability	
Glucose	-
Ribose	-
Xylose	-
Mannitol	-
Maltose	-
Lactose	-
Sucrose	-
Glycogen	-

TABLE II. **Binding reactivities of human, avian and swine isolates of influenza virus to the purified PtdIns from *R. equi* strain S_{420}.**

Influenza virus	Relative binding reactivity (%)		
	IV^3Neu5AcnLc4Cer	IV^6Neu5AcnLc4Cer	PtdIns
Human isolates			
A/PR/8/34 (H1N1)	100	12±6	26±6
A/Singapore/1/57 (H2N2)	21±4	100	43±5
A/Aichi/2/68 (H3N2)	26±5	100	48±7
B/Lee/40	16±2	100	28±5
B/Bangkok/163/90	18±8	100	31±6
Avian isolates			
A/duck/HK/36/76 (H1N1)	100	42±8	51±8
A/duck/HK/273/78 (H2N2)	100	13±5	19±6
A/duck/HK/24/76 (H3N2)	100	10±3	38±6
A/duck/HK/849/76 (H4N1)	100	16±8	34±7
A/duck/HK/313/76 (H5N3)	100	56±6	31±6
A/duck/HK/13/76 (H6N1)	100	15±6	36±5
A/duck/HK/47/76 (H7N2)	100	22±9	33±7
A/duck/HK/86/76 (H9N2)	100	32±6	40±5
A/duck/HK/33/76 (H10N1)	100	28±5	33±5
A/duck/HK/44/76 (H11N3)	100	21±4	30±8
A/duck/HK/862/80 (H12N5)	100	14±2	37±7
Swine isolates			
A/swine/Hokkaido/2/81 (H1N1)	13±5	100	33±5
A/swine/Italy/309/83 (H3N2)	15±3	100	31±7

The binding activities of human, avian and swine isolates of influenza virus to purified PtdIns were determined with a TLC/virus-binding immunostaining assay as described under "Experimental Procedures". IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer were used as positive controls. The data are expressed as mean±SD of three independent experiments and as a percentage of that of IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer.

TABLE IV. **Influence of fatty acid residues of PtdIns to viral hemagglutination, virus-induced hemolysis and influenza virus infection.**

Phosphatidylinositols	Fatty acid residue	Binding activities	Inhibition activities		
			HA	Hemolysis (IC_{50},μM)	Cell infection (IC_{50},μM)
PtdIns from strain S420	14-Methylocatadecanoic acid and palmitic acid	43±5	500	38.9±4.9	10.4±4.1
PtdIns from bovine liver	Stearic acid and arachidonic acid	41±6	500	578.4±5.6	360.7±5.2
PtdIns from soybean	Palmitic acid and linoleic acid	39±7	500	616.4±9.7	396.1±8.3
Glycerophosphatidylinositol from soybean	No fatty acid residue	ND	>1000	>1000	>1000

Inhibition activities of PtdIns containing different fatty acid residues on viral hemagglutination, virus-induced hemolysis and infection of MDCK cells by human influenza A virus (A/Aichi/2/68 strain) to MDCK cells were detected as described under "Experimental Procedures". The maximum dilutions of samples (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) are defined as the hemagglutination inhibition titer. The inhibition activities of samples against viral hemolysis or infection of MDCK cells are expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean±SD of three independent experiments. Each experiment was carried out in duplicate.

TABLE III. **Inhibition of the purified PtdIns from *R. equi* strain S_{420} on viral hemagglutination, virus-induced hemolysis and infectivity by human influenza A viruses.**

Viruses	Inhibition activity of purified PtdIns			Inhibition activity of Neu5Ac-PE		
	HA (μM)	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)	HA (μM)	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)
A/PR/8/34	500	50.3±7.2	20.1±2.3	>1000	>1000	>1000
A/Singapore/1/57	500	42.4±5.6	14.7±3.2	32	158.5±7.4	94.7±5.2
A/Aichi/2/68	250	38.9±4.9	10.4±4.1	16	142.3±8.2	64.3±7.8

Inhibition activity of the purified PtdIns on viral hemagglutination, virus-induced hemolysis and infectivity of human influenza A viruses was detected as described under "Experimental Procedures". The maximum dilution of the PtdIns (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) is defined as the hemagglutination inhibition titer. The inhibition activity of the PtdIns against viral hemolysis or infection of MDCK cells by viruses is expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean±SD of three independent experiments. Each experiment was carried out in duplicate. Neu5Ac-PE, which is a sialylphosphatidylenthanolamine derivative (*24*), was used as a control.

A Phosphatidylinositol Bearing a Novel Branched-chain Fatty Acid from *Rhodococcus equi* Binds to Viral Hemagglutinin and Inhibits the Infection of Cells*

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Running title: PtdIns inhibits infection by human influenza virus

SUMMARY

From aquatic bacteria *Rhodococcus equi* strain S_{420}, we isolated a substance that strongly binds to influenza virus. Structural analyses revealed that it is a unique type of phosphatidylinositol (PtdIns) bearing a branched-chain fatty acid (14-methyloctadecanoic acid). Using a TLC/virus-binding immunostaining assay, this PtdIns bound to all subtypes of hemagglutinin (HA) of influenza A viruses tested, isolated from humans, ducks and swine, and also to human influenza B viruses. Furthermore, the PtdIns significantly prevented the infection of MDCK cells by influenza viruses, and also inhibited the virus-mediated hemagglutination and low pH-induced hemolysis of human erythrocytes. We also used purified hemagglutinin instead of virions to examine the interaction between viral HA and PtdIns, showing that the PtdIns binds to hemagglutinin. These findings indicate that the inhibitory mechanism of PtdIns on the influenza virus infection may be through its binding to HA and then enter the host cells membrane together with virus, following inhibition the fusion between cell and virus membrane.

INTRODUCTION

Influenza virus initiates infection through a receptor-ligand interaction and subsequent receptor-mediated endocytosis (*1*, *2*). After entry, the components of the viral particle are disassembled in the endosome where a low pH induces the irreversible conformational change of hemagglutinin (HA)[1], resulting in fusion of the viral and endosomal membranes (*3-6*). The viral genome is then released from the endosome and transported into the nucleus where replication occurs (*7*). After replication, viral genomes leave the nucleus and progeny virus particles assemble and bud from the cellular membrane of the infected cells (*6*, *8*).

Viral proteins, which provide essential functions during the life cycle of influenza virus, are potential targets for the development of antiviral agents. Several agents involved in the infectious cycle have already been documented (*9-14*). It is known that the sialic acid-containing glycoproteins or glycolipids on cell surfaces are receptors for influenza viruses (*15-20*). Two major virus glycoproteins, HA and sialidase, mediate the interaction between influenza viruses and cellular receptors. They are responsible for the attachment to target cells and for release of progeny viruses from the surface of infected cells, respectively (*1*, *6*). Therefore, many inhibitors of influenza viruses have been developed against virus HA or/and sialidase (*14*, *21-24*). Several studies reported that neoglycoprotein or a synthetic co-polymer containing sialic acid residue strongly inhibited the HA and sialidase activities, resulting in prevention of influenza virus infection (*24-30*). However, the effects of most inhibitors are usually restricted to HA subtypes of influenza virus isolates, and their stability has been influenced by the hydrolysis of sialic acid-linkage by viral sialidase. Influenza virus vaccines are also dependent on viral specific antigen (*31*).

Recent studies have shown that a desialylated glycolipid sulfatide also has receptor-like activity (*49*) and that two desialylated glycoglycerolipids from *Coryneform bacteria* have binding and neutralizing activities against influenza virus (*32*). As desialylaled glycolipids are not substrates for sialidase (*58*, *59*), which is a receptor-destroying enzyme and is integrated into the influenza virus envelope, they might be useful as anti-influenza drugs.

Coryneform bacteria are gram-positive microorganisms, which are widely distributed in the environment. Aquatic bacteria *Rhodococcus equi* (*R. equi*), a strictly aerobic gram-positive co-bacillus belonging to the *Corynebacterium* family, is a facultative intracellular bacterium that can cause pneumonia in both young horses and immunocompromised patients (*33*, *34*). We purified a unique phosphatidylinositol (PtdIns) bearing a novel branched-chain fatty acid from *R. equi* strain S_{420}, which binds to all subtypes of HA of influenza virus tested. Furthermore, it was demonstrated that significantly inhibits virus-mediated hemolysis and viral growth in tissue culture.

EXPERIMENTAL PROCEDURES

Preparation of lipids from R. equi strain S_{420}—Two thousand aquatic soil samples were selected in Japan and 257 bacteria were isolated, producing more than detectable levels of glycolipids, of which 27 strains were selected because of their high productivity as described previously (*32*). In *R. equi* strain S_{420} (Tab. I), we found virus-binding substance, which was screened by a thin layer chromatography (TLC)/virus-binding immunostaining method as described previously (*24*).

R. equi strain S_{420} was cultivated in a 250-ml Erlenmeyer flask containing 30 ml of YMPG medium [0.3% yeast extract (Difco), 0.3% malt extract (Difco), 0.5% polypepton (Nihon Pharmaceutical Co.), 1% glucose, pH 7.0] for 20 hr at 28°C in a rotary shaker (220 rpm with a 50-mm stroke). The incubated-aliquot (0.7 ml) was added to a 500-ml Erlenmeyer flask containing 70 ml of YSGG medium [0.4% yeast extract, 0.1% soy flour, 1% glucose, 2% glycerol, 0.1% K_2HPO_4, 0.1% $MgSO_4$-$7H_2O$, 0.2% NaCl, 0.2% $CaCO_3$] and cultivated for 96 hr at 28°C. The culture was extracted with an equal volume of a chloroform/methanol (1:1, v/v) solvent. The extract was used for this experiment.

Total lipids from strain S_{420} were fractionated by Q-Sepharose column chromatography (*36*). The developing solvent was a mixture of chloroform/methanol/water (60:35:8, v/v/v). Lipids were separated on a TLC plate. The virus-binding substance was purified with LH-20 column chromatography (*57*).

Structure of the virus-binding substance—The virus-binding substance: colorless oil. $[\alpha]_D$ +8° (c 0.37, $CHCl_3$). Figure 2A shows ^1H NMR ($CD_3OD/CDCl_3$, 10:1, v/v): δ_H 0.84 (3H, d, J = 6.4 Hz, CH_3), 0.88 (6H, t, J = 6.9 Hz, CH_3 x 2), 1.10 (2H, m, CH_2), 1.27 (41H, m, CH and CH_2 x 20), 1.30 (2H, m, CH_2), 1.35 (4H, m, CH_2 x 2), 1.59 (4H, m, CH_2 x 2), 2.30 (2H, t, J = 7.3 Hz, CH_2-C=O), 2.33 (2H, t, J = 7.3 Hz, CH_2-C=O), 3.20 (1H, t, J = 9.4 Hz, 5'-H), 3.37 (1H, dd, J = 9.4 and 3.0 Hz, 3'-H), 3.62 (1H, t, J = 9.4 Hz, 4'-H), 3.76 (1H, t, J = 9.4 Hz, 6'-H), 3.89 (1H, br t, J = 9.4 Hz, 1'-H), 4.06 (2H, m, 3-H_2), 4.17 (1H, t, J = 3.0 Hz, 2'-H), 4.20 (1H, dd, J = 12.2 and 7.0 Hz, 1-H), 4.45 (1H, dd, J = 12.2 and 3.1 Hz, 1-H), 5.24 (1H, m, 2-H). Figure 2B shows ^{13}C NMR ($CD_3OD/CDCl_3$, 10:1, v/v): δ_C 15.3 (2C), 21.0, 24.4 (2C), 26.7 (2C), 30.9-31.8 (19C), 33.8 (2C), 34.6, 35.9 (2C), 38.9 (2C), 64.5 (C-1), 65.6 ($J_{C\text{-}P}$ = 5.7 Hz, C-3), 72.6 ($J_{C\text{-}P}$ = 7.6 Hz, C-2), 73.5 (C-3'), 73.7 (br, C-2'), 73.9 ($J_{C\text{-}P}$ = 5.7 Hz, C-6'), 74.7 (C-4'), 76.9 (C-5'), 79.0 (br, C-1'), 175.4, 175.7. (+)FABMS *m/z*: 875 $[M+Na]^+$, 593. (-)FABMS m/z: 851 $[M-H]^-$, 689. (-)HRFABMS m/z 851.5666 $[M-H]^-$ (Calcd. For $C_{44}H_{84}O_{13}P$: 851.5650).

Fatty acid analysis: 10% sodium methylate (0.6 m) was added to the sample (0.1 mg) in 0.2 ml benzene and the reaction mixture was held at 60°C for 20 min. After the addition of a few drops of 1 N AcOH to stop the reaction followed by 2 ml of distilled water, the reaction mixture was extracted with 2 ml of *n*-hexane. The *n*-hexane extract was evaporated to dryness and was then followed by fatty acid analysis by gas chromatographic mass (GC-MS). The conditions for GC were: column, J & W Scientific

DB-1 (0.25 mm-by-15 m); column temperature, 100-270°C, raised at 20°C/min; injection temperature, 280°C; carrier gas, He, 1.2 kg/cm.

^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR). FABMS and HRFABMS were obtained on a JEOL SX102A spectrometer (glycerol as matrix). Optical rotation was measured on a JASCO DIP-370 digital polarimeter.

Viruses and antibodies—Influenza viruses from human isolates [A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2), A/Aichi/2/68 (H3N2), B/Lee/40 and B/Bangkok/163/90 strains], swine isolates [A/swine /Hokkaido/2/81 (H1N1) and A/swine/Italy/309/83 (H3N2) strains] and avian isolates [A/duck/HK/36/76 (H1N1), A/duck/HK/273/78 (H2N2), A/duck/HK/24/76 (H3N2), A/duck/HK/849/80 (H4N1) A/duck/HK/313/78 (H5N3) A/duck/HK/13/76 (H6N1) A/duck/HK/47/76 (H7N2), A/duck/HK /86/76 (H9N2), A/duck/HK/33/76 (H10N1), A/duck/HK/44/76 (H11N3) and A/duck/HK/862/80 (H12N5) strains] were used for this study. Virus strains were propagated in the allantoic cavity of 11-day-old chicken eggs for 48 h at 35°C and purified by sucrose density gradient centrifugation (*24*, *37*, *38*). To obtain bromelain-cleaved HA (BHA), purified virus (A/Aichi/2/68 (H3N2) strain) was digested with bromelain and purified as described previously (*39*, *40*). Rabbit anti-influenza virus antibodies were raised by immunization with various strains grown in eggs as described previously (*18*, *41*). Monocolonal anti-HA (H3 subtype) antibody (MAb 2E10) was propagated as described previously (*42*).

Phospholipids and glycolipids—L-α-PtdIns [1,2-diacyl-sn-glycero-3-phospho-(1-D-myo-inositol)] from bovine liver (Sigma P-8443) or from soybean (Sigma P-5954), L-α-glycerophospho-D-myo-inositol from soybean (Sigma G-1891) and sialylphosphatidylethanolamine derivative (Neu5Ac-PE) (*24*) were used this study as controls. IV^{3}Neu5AcnLc4Cer (Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal-β1-4Glc-β1-ceramide) and IV^{6}Neu-5AcnLc4Cer (Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide) were prepared from human erythrocytes (*43*) and from human meconium (*44*), respectively.

Virus-binding immunostaining assay—Lipids were spotted on the Silica gel plastic plates (Polygram Sil G; Macherey-Nagel, Germany). Immunochemical detection of virion or BHA on the TLC plates was performed as described previously (*18*, *24*). In the inhibition assay for virus binding, viruses were preincubated with inhibitors for 1 hr at 4°C and were then used on the binding assay.

Hemagglutination and hemolysis inhibition assays—Hemagglutination and hemolysis inhibition assays were performed as described previously (*24*, *38*, *45*, *46*).

Neutralization assay—The neutralization of the PtdIns on human influenza A virus infection to Madin Darby canine kidney (MDCK) cells was detected as described previously (*47*, *48*).

RESULTS

Presence of a substance that binds to influenza viruses in R. equi strain S_{420}—Two thousand aquatic soil samples were selected in Japan and 257 bacteria were isolated, producing more than detectable levels of glycolipids, of which 27 strains were selected because of their high productivity. The TLC-separated glycolipids were assayed for binding activity to human influenza viruses. *R. equi* strain S_{420} showed one active spot on all H1 (A/PR/8/34 strain), H2 (A/Singapore/1/57 strain) and H3 (A/Aichi/2/68 strain) subtypes of human influenza A viruses (Fig. 1).

Structure of the virus-binding substance—The virus-binding substance gave a positive sign for Dittmer's test (*50*), indicating the presence of a phosphate group. The compound showed a pseudomolecular ion peak at *m/z* 851, matching $C_{44}H_{85}O_{13}P$ (HRFABMS: *m/z* 851.5666 $[M-H]^-$, calcd. for $C_{44}H_{85}O_{13}P$: 851.5650). Both ^1H and ^{13}C NMR spectra (Fig. 2A and Fig. 2B), together with a DEPT spectrum, revealed that the substance contains two long-chain acyl groups, two oxygenated methylenes, and seven oxygenated methines. The ^1H-^1H COSY and 1D homonuclear Hartman-Hahn (HOHAHA) spectra indicated the presence of a cyclitol moiety and a glycerol unit. The cyclitol was shown to be *myo*-inositol on the basis of the ^1H vicinal coupling constants of H-1' to H-6' [J(1'/2') = 3.0 Hz, J(2'/3') = 3.0 Hz, J(3'/4') = 9.4 Hz, J(4'/5') = 9.4 Hz, J(5'/6') = 9.4 Hz, J(6'/1') = 9.4 Hz]. The phosphate was determined to be attached at C-3 and C-1' on the basis of the ^{13}C-P coupling constants of C-2 (7.6 Hz), C-3 (5.7 Hz), C-1' (< 2 Hz), C-2' (< 2 Hz), C-6' (5.7 Hz). The ^1H chemical shifts of H_2-1 (δ 4.45 and 4.20) and H-2 (δ 5.24) signals indicate that both the C-1 and C-2 positions of the glycerol moiety are acylated.

Treatment of the compound with MeOH/MeONa afforded 16:0 and 19:0 fatty acid methyl esters which showed molecular ion peaks at *m/z* 270 and 312, respectively, in the gas chromatography-electron impact mass spectrum (GC-EIMS) (Fig. 2C). The 16:0 fatty acid methyl ester showed GLC retention time and mass spectrum identical to those of authentic palmitic acid methyl esters. On the other hand, the EIMS of 19:0 fatty acid methyl ester showed relatively intense peaks at *m/z* 255 $[M-C_4H_9]^+$, originating from α-cleavages with respect to the methine carbon atom carrying the methyl branch, and the absence of a peak at *m/z* 241, corresponding to the ion $[M-C_5H_{11}]^+$. These findings indicated that the 19:0 fatty acid is 14-methyloctadecanoic acid. Thus, the structure of the virus-binding substance was determined to be a PtdIns bearing a branched-chain fatty acid (Fig. 3).

By chemical staining analyses (*50*, *51*), it was shown that the purified PtdIns was the main lipid (16.5% of total lipids) and further is present as a major phospholipid (about 55% of phospholipids) in *R. equi* strain S_{420} (Fig. 2D, 2E). Purification rate of PtdIns was approximate 60%.

The binding specificity of the purified PtdIns to influenza viruses—The binding specificity of the purified PtdIns to influenza viruses was determined by a TLC/virus-binding immunostaining assay. As

shown in Table II, all the isolates tested from human (A and B types) and animal (avian and swine) species bound to the purified PtdIns. The binding specificity of various influenza viruses to the purified PtdIns was different from their viral sialic acid-linkage specificity. These findings indicate that the binding of purified PtdIns to virus were not dependent on the different isolates or HA types of influenza viruses.

Inhibition activity of the purified PtdIns on the virus-mediated hemolysis and the cell infection by influenza viruses—To determine the anti-viral activity of the purified PtdIns, we carried out hemagglutination inhibition, hemolysis inhibition and neutralization assays. As shown in Table III, the purified PtdIns potently reduced the releasing LDH activity of virus-infected MDCK cells in a dose dependent manner. The IC_{50}s of the purified PtdIns (a concentration of purified PtdIns for 50% infection being inhibited) to A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2) and A/Aichi/2/68 (H3N2) strains were 20.1±2.3, 14.7±3.2 and 10.4±4.1 μM ($n = 3$ experiments), respectively. The inhibition activity of purified PtdIns was about 6-fold stronger than that of Neu5Ac-PE which was indicated previously a potent inhibitor against human influenza virus (*24*). Similarly, the inhibition activity of the purified PtdIns to virus-mediated hemolysis of erythrocytes was also stronger than that of Neu5Ac-PE. However, the purified PtdIns exhibited weaker inhibition of viral hemagglutination relative to Neu5Ac-PE. These findings show that the purified PtdIns significantly prevented both virus-mediated hemolysis and infection by human influenza A virus *in vitro*, and was effective against influenza A viruses regardless of subtypes.

Inhibition activities of PtdInses containing different fatty acid residues—To examine the influence of species of fatty acid residues to viral infection, we tested PtdInses containing three types of fatty acid residues as inhibitors of influenza viruses. As shown in Table IV, the inhibition activities of PtdInses each other were significantly different. The effect of the PtdIns from *R. equi* strain S_{420} on virus-mediated hemolysis and cell infection by influenza virus *in vitro* were 15-fold and 40-fold stronger than those of PtdInses from bovine liver or soybean, respectively. We also used a fatty acid-free PtdIns [*sn*-glycero-3-phosphatidylinositol (GPI)] in these experiments. Although the GPI inhibited viral hemagglutination at a concentration of 1 mM, it did not inhibit either the virus-mediated hemolysis or viral infection of MDCK cells even at a concentration of 2 mM (data not shown). These findings indicate that the fatty acid residue on PtdIns might be an indispensable part of their inhibition of viral hemolysis and viral replication.

Binding of the purified PtdIns to bromelain-cleaved hemagglutinin---The above findings indicate that the purified PtdIns prevents influenza virus infection. In order to clarify the mechanism of its anti-influenza activity, we used the bromelain-cleaved hemagglutinin (BHA) of A/Aichi/2/68 (H3N2) virus instead of virus particles for TLC/virus-binding assay. The binding specificity of PtdIns to BHA on plates

was examined using mouse monoclonal anti-HA (H3) antibody and HRP-conjugated goat anti-mouse IgG+IgM (H+L) (Jackson Immunoresearch Laboratories). As shown in Fig. 4, the BHA clearly bound to the purified PtdIns as well as IV^6Neu5AcnLc4Cer. This experimental finding indicates that HA of influenza virus is a component in the binding to purified PtdIns.

DISCUSSION

In this study, we isolated and purified a unique type of phosphatidylinositol (PtdIns) from *R. equi*, bearing a branched-chain fatty acid (14-methyloctadecanoic acid). Furthermore, we first found that it strongly inhibited the virus-induced hemolysis and the infection of influenza virus *in vitro*. The discovery evidently indicates existence of new type anti-influenza compounds in microorganisms. Otherwise, it is shown PtdIns in *R. equi* strain S_{420} is the main phospholipid and accounts for about 55% of total phospholipids, because the ratio of PtdIns in *Corynebacteria* is usually lower than 10% of total phospholipids (*52*, *56*). This creates favorable condition for opening up new agents against influenza.

Phosphatidylinositol is usually present in the inner layer of the cellular membrane lipid bilayer and is a precursor for several prominent intracellular signaling molecules (*35*, *53-55*). In this study we have first studied phosphatidylinositol regard as an inhibitor against influenza virus.

The binding of influenza virus to sialic acid has been shown to depend on the molecular species of sialic acid, the sialic acid-Gal linkage and the carbohydrate core structure of ganglioside (*19*). Therefore, the anti-influenza effects of the sialic acid-containing inhibitors are usually restricted by these factors. It is a novel finding that the PtdIns from *R. equi* strain S_{420} binds to influenza viruses and that it significantly inhibits the influenza virus infection *in vitro*. The inhibitory activity of the purified PtdIns on influenza viruses is not restricted to animal species or types (or subtypes) of viral HA. The purified PtdIns from strain S_{420} may be considered a new type of anti-influenza virus agent, widely effective against human and animal influenza A viruses.

The finding show that purified PtdIns from strain S_{420} strongly inhibited the virus-mediated hemolysis in comparison with sialic acid-containing inhibitors, or other PtdInses from different materials. We also confirmed that the purified PtdIns attached to viral HA and that the binding position of PtdIns differs from the sialic acid-binding pocket and may be close to the pocket on the head of the HA trimer. The finding on HAI also indicated that the effect of purified PtdIns on the sialic acid-binding pocket of viral HA is weaker. Regarding the anti-influenza virus mechanism of the purified PtdIns, one possibility is that PtdIns adheres to the viral HA and then enters the endosome together with the virion, resulting in interference with the fusion of viral and endosomal membranes.

We also used other phospholipids in the same experiment to compare their effects against influenza

viruses (data not shown). Bovine liver PtdIns, phosphatidylserine (PS, from bovine brain, Sigma P-7769) and phosphatidylenthanolamine (PE, from bovine brain, Sigma P-7693), all of which carry a negative charge, bound to influenza viruses, but neutral glycophospholipids such as phosphatidylcholine (PC, from bovine liver, Sigma P-6638) and sphingomyelin (SM, from bovine brain, Sigma S-7004) did not. None of PC, PE and SM showed inhibition activities of virus-induced hemolysis at a concentration of 2 mM. Both bovine liver PtdIns and PS showed inhibition activities, but their effects were much lower than that of the purified PtdIns. Adhesion of phospholipids to viral HA may be an indispensable step in preventing influenza virus infection. It is noteworthy that the virus binding activity of bovine liver PtdIns to influenza viruses was similar to that of purified PtdIns, however the inhibitory activity of bovine liver PtdIns on the influenza virus-mediated fusion was lower than that of the purified bacterial PtdIns. The structural difference between the purified PtdIns and bovine liver PtdIns is that it bears a branched-chain fatty acid. L-α-glycerophospho-D-myo-inositol from soybean, which has no fatty acid residue, showed no inhibitory activity of virus-mediated hemagglutination and hemolysis. These findings indicate that the phosphatidylinositol and branched fatty acid residue may be important for the binding of viral HA and for interfering with membrane fusion, respectively, leading to inhibition of infection by influenza viruses.

Some sialic acid-containing derivatives usually inhibit the attachment of viral HA to target cells and/or the enzymatic activity of viral sialidase, thus preventing infection by influenza virus. In contrast, the purified PtdIns preferentially targets virus-mediated cellular membrane fusion. Our hypothesis is that PtdIns binds near the fusion loop of HA, resulting in interference with the fusogenic function of viral HA, thus preventing the growth of human influenza A virus.

FOOTNOTES

* This work was supported in part by Grants-in-aid 06454211, 05274101, 08457098, 07044286, 07557166 (Y. S.) for Scientific Research from the Ministry of Education, Science and Culture of Japan, and for Japan-Chain Medical Association.

[1] The abbreviations used are: HA, HA; MDCK, Madin Darby canine kidney; Neu5AcnLc4Cer, Neu5Ac-Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide; PtdIns, phosphatidylinositol; TLC, thin layer chromatography; YMPG medium, 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% polypepton, pH 7.0; YSGG medium, 1% glucose, 2% glycerol, 0.4% yeast extract, 0.1% soy flour, 0.1% K_2HPO_4, 0.1% $MgSO_4$-$7H_2O$, 0.2% NaCl, 0.2% $CaCO_3$.

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Table and Figures

TABLE I. **Microbiological property of *Rhodococcus equi* strain S_{420}.**

TABLE II. **Binding reactivities of human, avian and swine isolates of influenza virus to the PtdIns from *R. equi* strain S_{420}.** The binding activities of human, avian and swine isolates of influenza virus to purified PtdIns were determined with a TLC/virus-binding immunostaining assay as described under "Experimental Procedures". IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer were used as positive controls. The data are expressed as mean±SD of three independent experiments and as a percentage of that of IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer.

TABLE III. **Inhibition of the purified PtdIns from *R. equi* strain S_{420} on viral hemagglutination,**

virus-mediated hemolysis and cell infection by human influenza A viruses. Inhibition activity of the purified PtdIns on viral hemagglutination, virus-mediated hemolysis and cell infection by human influenza A viruses was detected as described under "Experimental Procedures". The maximum dilution of the PtdIns (starting concentration, 1 mM) showing complete inhibition of the hemagglutination (HAI) is defined as the hemagglutination inhibition titer. The inhibition activity of the PtdIns against viral hemolysis or viral infection of MDCK cells is expressed as IC_{50} (the concentration at which hemolysis was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean±SD of three independent experiments. Each experiment was carried out in duplicate. Neu5Ac-PE, which is a sialylphosphatidyl-enthanolamine derivative (*24*), was used as a control.

TABLE IV. **Influence of fatty acid residues of PtdIns on viral hemagglutination, virus-mediated hemolysis and cell infection by influenza virus.** Inhibition activities of PtdInses containing different fatty acid residues on viral hemagglutination, virus-mediated hemolysis and infection of MDCK cells by A/Aichi/2/68 (H3N2) strain were detected as described under "Experimental Procedures". The maximum dilutions of samples (starting concentration, 1 mM) showing complete inhibition of the hemagglutination (HAI) are defined as the hemagglutination inhibition titer. The inhibition activities of samples against viral hemolysis or viral infection of MDCK cells are expressed as IC_{50} (the concentration at which hemolysis was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean ± SD of three independent experiments. Each experiment was carried out in duplicate.

Fig. 1. **Binding specificity of the purified PtdIns to human influenza A viruses.** The binding specificity of the PtdIns isolated from aquatic bacteria *R. equi* strain S_{420} (2 nmol) (lane 2) to human influenza A viruses [A/PR/8/34 (H1N1) (a), A/Singapore/1/57 (H2N2) (b), and A/Aichi/2/68 (H3N2) (c)] was determined by TLC/virus-binding immunostaining assay as described under "Experimental Procedures". TLC Plate was visualized with orcinol-H_2SO_4 reagent (d). IV^6Neu5AcnLc4Cer (lane 1) and IV^3Neu5AcnLc4Cer (lane 3) (1 nmol) were used as positive controls.

Fig. 2. **A: An ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from *R. equi* strain S_{420}.**

B: A ^{13}C-detected multiple-bond heteronuclear multiple quantum coherence spectrum of the PtdIns from *R. equi* strain S_{420}.

C: A gas chromatography-electron impact mass spectrum of the PtdIns from *R. equi* strain S_{420}.

D and E: Profile of phospholipids and total glycolipids from *R. equi* strain S_{420}. Profiles of

phospholipids (plate D) and glycolipids (plate E) were visualized with Dittmer's reagent and orcinol-H_2SO_4 reagent, respectively. Lane 1 is total lipids and lane 2 is the purified PtdIns (2 nmol).

Fig. 3. **Structure of the PtdIns from *R. equi* strain S_{420}.**

Fig. 4. **Binding of the purified PtdIns to bromelain released ectodomain of influenza hemagglutinin from A/Aichi/2/68 (H3N2) strain.** The binding of purified PtdIns (2 nmol) (lane 2) to bromelain-released ectodomain of influenza HA (BHA) from A/Aichi/2/68 (H3N2) strain was determined using a TLC/HA-binding immunostaining assay as described under "Experimental Procedures". IV^{6}Neu5AcnLc4Cer (1 nmol) (lane 1) was used as a positive control.

Fig. 1

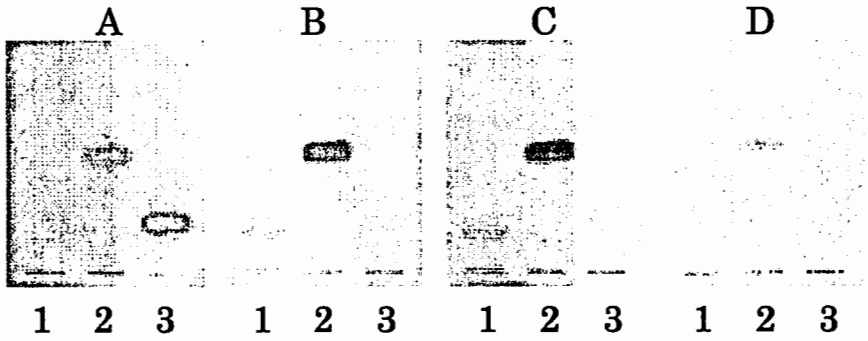

Fig. 3

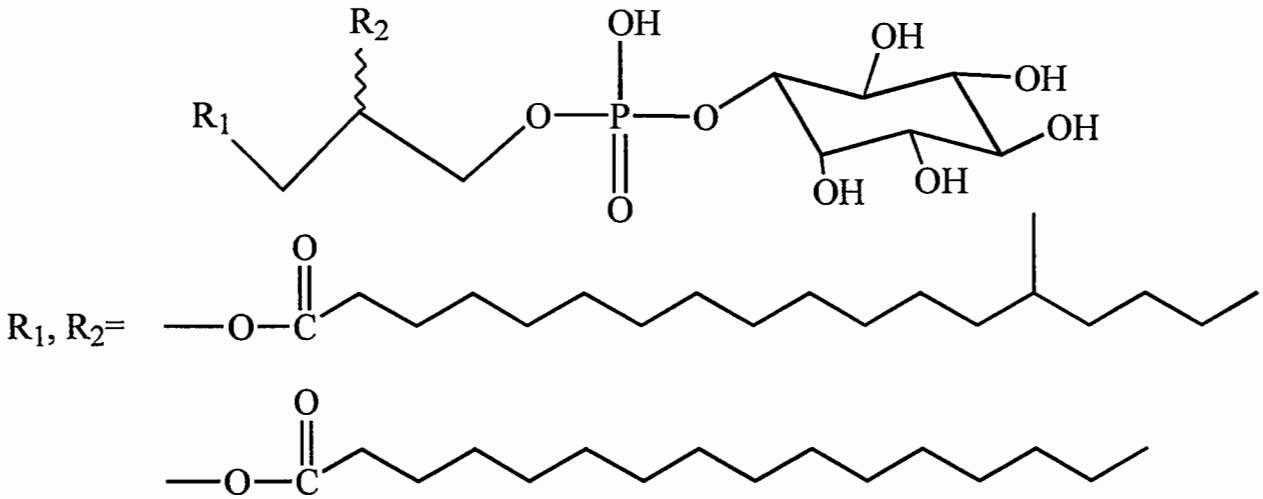

Fig. 4

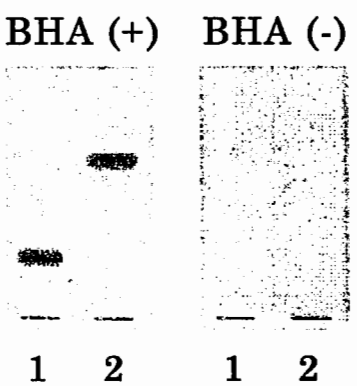

Fig. 2

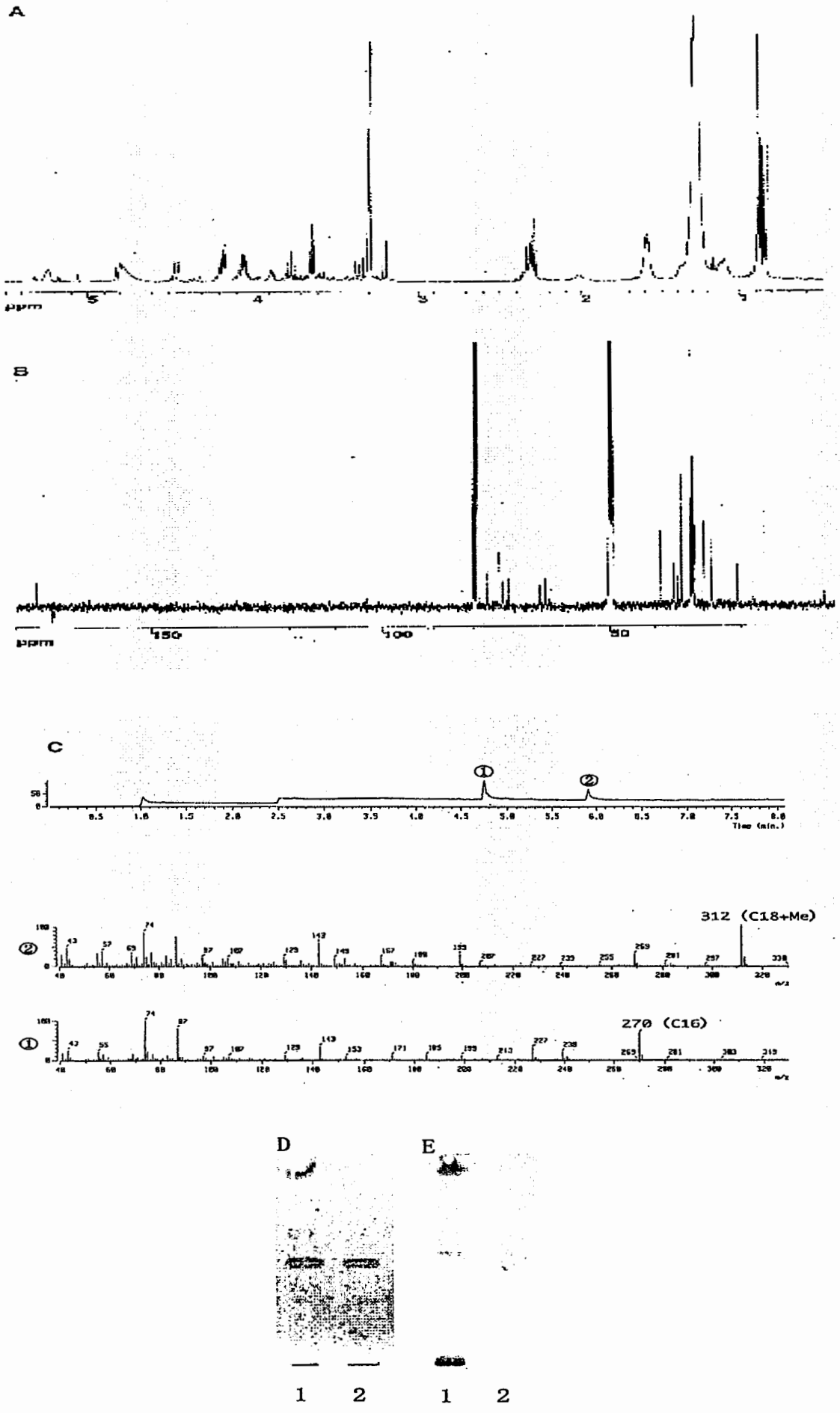
A
ppm
5
4
3
2
1
B
ppm
150
100
50
C
①
②
Time (min.)
312 (C18+Me)
270 (C16)
D
E
1
2
1
2

TABLE I. **Microbiological property of *Rhodococcus equi* strain S_{420}.**

	Strain S_{420}
Colony	
Shape	Flat and round
Tone	Buttery, opaque, and beige
Cell shape	
Shape	Long-rod
Width	0.8 μm
Moving	-
Gram-staining	+
Spore formation	-
Enzymatic properties	
Pyrazinamidase	+
Pyrrolidonearylamidase	-
Alkaline phosphatase	+
Catalase	+
β-Glucuronidase	-
β-Galactosidase	-
β-Glucosidase	+
N-Acetyl-β-glucosaminidase	-
Urease	+
Esculin hydrolyzation	+
Gelatin hydrolyzation	-
Nitrate reduction	-
Fermentation ability	
Glucose	-
Ribose	-
Xylose	-
Mannitol	-
Maltose	-
Lactose	-
Sucrose	-
Glycogen	-

TABLE III. **Inhibition of the purified PtdIns from *R. equi* strain S_{420} on viral hemagglutination, virus-induced hemolysis and infectivity by human influenza A viruses.**

Viruses	Inhibition activity of purified PtdIns			Inhibition activity of Neu5Ac-PE		
	HA (μM)	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)	HA (μM)	Hemolysis (IC_{50}, μM)	Cell infection (IC_{50}, μM)
A/PR/8/34	500	50.3±7.2	20.1±2.3	>1000	>1000	>1000
A/Singapore/1/57	500	42.4±5.6	14.7±3.2	32	158.5±7.4	94.7±5.2
A/Aichi/2/68	250	38.9±4.9	10.4±4.1	16	142.3±8.2	64.3±7.8

Inhibition activity of the purified PtdIns on viral hemagglutination, virus-induced hemolysis and infectivity of human influenza A viruses was detected as described under "Experimental Procedures". The maximum dilution of the PtdIns (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) is defined as the hemagglutination inhibition titer. The inhibition activity of the PtdIns against viral hemolysis or infection of MDCK cells by viruses is expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean±SD of three independent experiments. Each experiment was carried out in duplicate. Neu5Ac-PE, which is a sialylphosphatidylenthanolamine derivative (*24*), was used as a control.

TABLE II. **Binding reactivities of human, avian and swine isolates of influenza virus to the purified PtdIns from *R. equi* strain S_{420}.**

Influenza virus	Relative binding reactivity (%)		
	IV^3Neu5AcnLc4Cer	IV^6Neu5AcnLc4Cer	PtdIns
Human isolates			
A/PR/8/34 (H1N1)	100	12±6	26±6
A/Singapore/1/57 (H2N2)	21±4	100	43±5
A/Aichi/2/68 (H3N2)	26±5	100	48±7
B/Lee/40	16±2	100	28±5
B/Bangkok/163/90	18±8	100	31±6
Avian isolates			
A/duck/HK/36/76 (H1N1)	100	42±8	51±8
A/duck/HK/273/78 (H2N2)	100	13±5	19±6
A/duck/HK/24/76 (H3N2)	100	10±3	38±6
A/duck/HK/849/76 (H4N1)	100	16±8	34±7
A/duck/HK/313/76 (H5N3)	100	56±6	31±6
A/duck/HK/13/76 (H6N1)	100	15±6	36±5
A/duck/HK/47/76 (H7N2)	100	22±9	33±7
A/duck/HK/86/76 (H9N2)	100	32±6	40±5
A/duck/HK/33/76 (H10N1)	100	28±5	33±5
A/duck/HK/44/76 (H11N3)	100	21±4	30±8
A/duck/HK/862/80 (H12N5)	100	14±2	37±7
Swine isolates			
A/swine/Hokkaido/2/81 (H1N1)	13±5	100	33±5
A/swine/Italy/309/83 (H3N2)	15±3	100	31±7

The binding activities of human, avian and swine isolates of influenza virus to purified PtdIns were determined with a TLC/virus-binding immunostaining assay as described under "Experimental Procedures". IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer were used as positive controls. The data are expressed as mean±SD of three independent experiments and as a percentage of that of IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer.

TABLE IV. **Influence of fatty acid residues of PtdIns to viral hemagglutination, virus-induced hemolysis and influenza virus infection.**

Phosphatidylinositols	Fatty acid residue	Binding activities	Inhibition activities		
			HA	Hemolysis (IC_{50},μM)	Cell infection (IC_{50},μM)
PtdIns from strain S_{420}	14-Methylocatadecanoic acid and palmitic acid	43 ± 5	500	38.9 ± 4.9	10.4 ± 4.1
PtdIns from bovine liver	Stearic acid and arachidonic acid	41 ± 6	500	578.4 ± 5.6	360.7 ± 5.2
PtdIns from soybean	Palmitic acid and linoleic acid	39 ± 7	500	616.4 ± 9.7	396.1 ± 8.3
Glycerophosphatidylinositol from soybean	No fatty acid residue	ND	>1000	>1000	>1000

Inhibition activities of PtdIns containing different fatty acid residues on viral hemagglutination, virus-induced hemolysis and infection of MDCK cells by human influenza A virus (A/Aichi/2/68 strain) to MDCK cells were detected as described under "Experimental Procedures". The maximum dilutions of samples (starting concentration, 1 mM) showing complete inhibition of hemagglutination (HAI) are defined as the hemagglutination inhibition titer. The inhibition activities of samples against viral hemolysis or infection of MDCK cells are expressed as IC_{50} (the concentration at which the hemolysis or the infection was inhibited by 50% or 50% infection cells being inhibited). The data are expressed as mean ± SD of three independent experiments. Each experiment was carried out in duplicate.