pH6 antigen of *Yersinia pestis* interacts with plasma lipoproteins and cell membranes

Elena Makoveichuk,* Peter Cherepanov,† Susanne Lundberg,† Åke Forsberg,†,§ and Gunilla Olivecrona[1,*]

Department of Medical Biosciences,* Physiological Chemistry, Umeå University, SE-901 85 Umeå, Sweden; Department of Medical Countermeasures,† Division of NBC-Defense, Swedish Defense Research Agency, SE-901 82 Umeå, Sweden; and Department of Molecular Biology,§ Umeå University, SE-901 87 Umeå, Sweden

Abstract **The bacterial pathogen *Yersinia pestis* expresses a potential adhesin, the pH6 antigen (pH6-Ag), which appears as fimbria-like structures after exposure of the bacteria to low pH. pH6-Ag was previously shown to agglutinate erythrocytes and to bind to certain galactocerebrosides. We demonstrate that purified pH6-Ag selectively binds to apolipoprotein B (apoB)-containing lipoproteins in human plasma, mainly LDL. Binding was not prevented by antibodies to apoB. pH6-Ag interacted also with liposomes and with a lipid emulsion, indicating that the lipid moiety of the lipoprotein was responsible for the interaction. Both apoB-containing lipoproteins and liposomes prevented binding of pH6-Ag to THP-I monocyte-derived macrophages as well as pH6-Ag-mediated agglutination of erythrocytes. Binding of pH6-Ag to macrophages was not dependent on the presence of LDL receptors. Treatment of the cells with Triton X-100 or with methyl-β-cyclodextrin indicated that the binding of pH6-Ag was partly dependent on lipid rafts. We suggest that interaction of pH6-Ag with apoB-containing lipoproteins could be of importance for the establishment of *Y. pestis* infections. Binding of lipoproteins to the bacterial surface could prevent recognition of the pathogen by the host defence systems. This might be important for the ability of the pathogen to replicate in the susceptible host.**—Makoveichuk, E., P. Cherepanov, S. Lundberg, Å. Forsberg, and G. Olivecrona. **pH6 antigen of *Yersinia pestis* interacts with plasma lipoproteins and cell membranes. *J. Lipid Res.* 2003.** 44: **320–330.**

Supplementary key words THP-I macrophages • erythrocytes • liposomes • low density lipoproteins • apolipoprotein B • lipid rafts

Pathogenic *Yersinia* species, which include the highly pathogenic *Yersinia pestis,* share a number of virulence plasmid encoded determinants [as reviewed in ref. (1)]. These virulence proteins (denoted Yops for *Yersinia* outer proteins) can be regarded as effectors of virulence in that they contribute in different ways to defeat the primary immune defense of the host. Some of the Yop proteins, YopE and YopH, have been shown to mediate resistance to phagocytosis (2–5). Other Yops, like YopJ, prevent the induction of inflammatory cytokines in response to infection (6, 7). The effector Yops of *Yersinia* are delivered into host cells by a type III secretion system. Similar systems are found in many other gram-negative pathogens and the mechanism of delivery of effector proteins is functionally conserved (8). Extracellularly located bacteria deliver effector proteins into the host cell in a contact-dependent process (9, 10). In *Y. pseudotuberculosis* and *Y. enterocolitica*, either YadA or Invasin can serve as adhesins in the delivery of Yop effectors.

Y. pestis is closely related to *Y. pseudotuberculosis*, but still *Y. pestis* expresses neither YadA nor Invasin. The genes encoding these adhesins are present, but are not functional (3, 11). Two potential adhesins expressed by *Y. pestis* are Pla and pH6 antigen (pH6-Ag). Pla localizes to the outer membrane and has a proteolytic activity that can cleave and activate plasminogen, a property that has been suggested to be important for the ability of *Y. pestis* to infect via peripheral routes, i.e., after flea bites (12). Recently however, Cowan et al. (13) reported that Pla also could mediate adherence and invasion of *Y. pestis* into epithelial cells. Therefore, it is likely that Pla can serve as adhesin in the contact-dependent delivery of Yop effectors by *Y. pestis.*

pH6-Ag was first described more than 40 years ago (14) and was initially identified as an antigen expressed only at pH below 6 at 37°C. More recently the operon encoding this antigen was cloned and partially sequenced (15). This analysis revealed that pH6-Ag belongs to a class of adhesins that are secreted/assembled via a so-called chaperone/usher pathway (16). pH6-Ag appears as a surface

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Abbreviations: apo, apolipoprotein; FC, free cholesterol; MBCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; pH6-Ag, pH6 antigen; PL, phospholipid; PNGase, peptide *N*-glycosidase; TC, total cholesterol; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

[1] To whom correspondence should be addressed.
e-mail: gunilla.olivecrona@medbio.umu.se

polymer composed of PsaA. A chaperone postulated to prevent polymerization of PsaA in the periplasm and an outer membrane usher protein are assumed to be involved in secretion and assembly of the surface antigen, *Y. pseudotuberculosis*. *Y. pestis* strains unable to express pH6-Ag are attenuated in mouse infection models, suggesting that the adhesin is of importance during some stages of infection (15). pH6-Ag has been shown to agglutinate erythrocytes from a wide variety of species and to mediate binding to epithelial cells (17). Some insight into the nature of the receptor(s) for pH6-Ag has been obtained from studies in vitro, where it was shown that purified pH6-Ag binds cerebrosides containing β1-linked galactose (18).

Lindler and Tall (15) presented evidence that expression of pH6-Ag is induced inside macrophages. This is logical provided that the pathogen is inside a phagolysosome with a low pH. Recently, by using transcriptional fusions between *psaA* and *gfp* (encoding green fluorescent protein) we have confirmed that expression of PsaA is induced inside macrophages. In addition, PsaA co-localized with the phagosome membrane and interacted with glycolipids extracted from macrophages in an overlay assay (Cherepanov et al., unpublished observations).

It is not clear if pH6-Ag is expressed by extracellular bacteria during infection since expression in vitro is only seen at 37°C at slightly acidic pH. However, bacteria escaping from phagocytes would express pH6-Ag. It is possible that the adhesin that is functional at a pH range of 4 to 10 (18) could also play an important role in extracellular events. Taking into account that bacteria expressing pH6-Ag may interact with a number of factors in circulation, we investigated the possibility that pH6-Ag interacted with components of blood plasma. We found that pH6-Ag bound with an apparent high affinity to lipoproteins containing apolipoprotein B (apoB). The interaction was mainly hydrophobic and involved the lipid moieties of the lipoproteins but not apoB. Bacteria expressing pH6-Ag that had grown in the presence of serum were entirely covered by apoB-containing lipoproteins. Binding of lipoprotein interfered with binding of the bacteria to cells. Thus, some interesting possibilities are that apoB-containing lipoproteins may be of importance in vivo in preventing interaction of the bacteria with host cells and also in preventing recognition of the pathogen by the host immune defense.

MATERIALS AND METHODS

Reagents

Intralipid® (10%) was from Pharmacia-Upjohn (Uppsala, Sweden). Silica G plates for TLC were from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Molecular weight markers for SDS-PAGE were from Bio-Rad Laboratories (Hercules, CA). Heparin (25,000 IU/ml) was obtained from Lövens (Malmö, Sweden). BSA (fraction V), L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin, methyl-β-cyclodextrin (MBCD) and PMA were from Sigma Chemical Co (St. Louis, MO). CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). Cell culture medium (RPMI-1640 with GlutaMAX II) and fetal calf serum were purchased from Gibco BRL/Life Technologies AB (Täby, Sweden). Garamycin® was obtained from Schering Corporation (Kenilworth, NJ). Peptide *N*-glycosidase (PNGase) F was from Boehringer (Mannheim, Germany). Trasylol was from Bayer AG (Leverkusen, Germany). Cholesterol was from BDH Chemicals Ltd. (Poole, England). Carrier-free $Na^{125}I$ was purchased from Nordion Inc., Canada. Polyclonal rabbit anti-human apoB antibody and polyclonal rabbit anti-human apoE antibody were from DAKO (Glostrup, Denmark). Polyclonal anti-pH6-Ag of *Y. pestis* antibody was obtained by immunization of rabbits with purified pH6-Ag. Other reagents, if not specially mentioned, were from Sigma.

Cells, strains, and growth conditions

Y. pestis of the KIM5 and EV76 were used as wild types throughout experiments. *Escherichia coli* DH5α (pJG428) contained the entire operon encoded pH6-Ag cloned from *Y. pestis* EV76 (wild type). Routine cultures of *Y. pestis* were grown at 26°C in blood agar base (Merck). *E.coli* DH5α (pJG428) strains were grown at 26°C in L-agar with 100 μg/ml of ampicillin. When *Yersinia* strains were to be cultured at 37°C, BHI medium (brain heart infusion, Difco) was supplemented with 2.5 mM $CaCl_2$, 0.5% yeast extract, and 0.2% xylose (19). The pH of the medium was adjusted to either 8 or 5.8 with HCl.

THP-I monocytes were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI-1640 with GlutaMAX II (RPMI-1640) supplemented with 10% (v/v) fetal calf serum and 50 μg/ml Garamycin®. The cells were incubated at 37°C in an atmosphere containing 5% CO_2 in air. The growth medium was replaced every 2–3 days. The concentration of cells in the growth medium never exceeded 1×10^6 cells/ml. For induction of macrophage differentiation, cells were suspended in 1.6×10^{-7} M PMA in growth medium (20) and plated at a density of 1×10^6 cells/22-mm well in 12-well Falcon® multidishes (Becton Dickinson and Co., Lincoln Park, NJ). THP-I monocyte-derived macrophages were used in experiments 36–48 h after addition of PMA.

Preparation of lipoproteins

Human chylomicrons ($d < 0.96$ g/ml), VLDL ($d < 1.006$ g/ml), IDL ($1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$ g/ml), a narrow density fraction of LDL ($1.030 < d < 1.045$ g/ml), and HDL ($1.063 < d < 1.21$ g/ml) were isolated by sequential centrifugation in the presence of EDTA-Na_2 (1 mg/ml of blood) from the pool of fresh plasma of healthy volunteers after 16 h of fasting (21). The narrow fraction of LDL was used in some experiments to avoid the presence of apoE. A combination of protease inhibitors (per liter: 100 mg PMSF, 1 mg trasylol, and 0.2 g NaN_3) was added to prevent degradation of the lipoproteins during preparation (22). EDTA-Na_2 and NaN_3 were added to the lipoproteins immediately after isolation to final concentrations of 0.01% (w/v) and 0.02% (w/v), respectively. The lipoproteins were stored at 4°C and used for experiments within 3–4 weeks. Before experiments, the lipoproteins were dialyzed overnight at 4°C against 10 mM Dulbecco's PBS (pH 7.4) with 0.01% (w/v) EDTA-Na_2 and 0.02% (w/v) NaN_3. The protein concentration was determined using BSA as a standard (23).

Purification of pH6-Ag

The pH6-Ag was purified from the *E. coli* strain DH5α transformed with plasmid pJG428, which contains the entire operon encoding pH6-Ag cloned from *Y. pestis* EV76. The recombinant strain was grown overnight in a 5 liter flask containing 1 liter of LB medium, pH 5.8, at 37°C. The bacterial pellet was removed by centrifugation (7,000 *g*, 20 min) at 4°C. The remaining super-

natant was collected and pH6-Ag precipitated at 4°C overnight using $(NH_4)_2SO_4$ (30% of saturation). After centrifugation (17,000 *g* for 20 min at 4°C), the supernatant was carefully discarded. The pH6-Ag-containing pellet was rapidly suspended in 100 ml of PBS and immediately centrifuged at 40,000 *g* for 20 min at 4°C. The resulting pellet was dissolved in about 10 ml of PBS for 10 h at 4°C. The solution of pH6-Ag was further clarified by centrifugation at 40,000 *g* for 20 min at 4°C. Finally, the protein concentration was measured by the Lowry assay (23) or spectrophotometrically at 280 nm. The pH6-Ag-containing sample was diluted in PBS to a final concentration of 1 mg of protein per milliliter of PBS and stored at −20°C. The purity of pH6-Ag was more than 80%, as evaluated by analysis of the data obtained after SDS-PAGE. For preparation of the affinity column, about 10 mg of purified pH6-Ag was coupled to CNBr-activated Sepharose 4B according to the instructions provided by the manufacturer.

Iodination of lipoproteins and pH6-Ag

Lipoproteins and pH6-Ag were iodinated using the iodine monochloride method (24) and dialyzed against several changes of Dulbecco's PBS with 0.01% (w/v) EDTA-Na_2 and 0.02% (w/v) NaN_3.

Analysis of material eluted from the pH6-Ag Sepharose column

Delipidation. Twenty volumes of methanol and diethyl ether, 1:1(v/v), were added to 100 μl of sample. After centrifugation, the lipid extract was removed and evaporated in a stream of nitrogen before analysis by TLC. The protein pellet was washed by diethyl ether and used for analysis by SDS-PAGE. For comparison we used isolated lipoproteins analyzed the same way (25).

Lipid analysis. The evaporated lipid extracts from lipoproteins and from the eluted material were dissolved in 100 μl of chloroform. Fifty microliters of these solutions were applied on Silica G plates. TLC was performed using heptane-diethyl-ether-methanol acidic acid, 85:30:3:2 (v/v/v/v), as the mobile phase. The lipids were visualized by iodine vapors. The concentrations of triglycerides (TGs) and total cholesterol (TC) in the samples were determined enzymatically by kits from Boehringer.

Protein analysis. SDS-PAGE was performed on 7.5% Tris-HCl gel (Bio-Rad) under reducing conditions. The separated proteins were stained by Coomassie® G250 Stain (Bio-Safe™ Coomassie, Bio-Rad) or transferred to a nitrocellulose membrane (BioTrace™ NT, Pall Corporation) and analyzed by Western blot using polyclonal rabbit anti-human apoB antibody.

Interaction of pH6-Ag with lipoproteins

Immunodiffusion. Diffusion of both pH6-Ag (5 μg per well) and polyclonal rabbit anti-human apoB antibody (20 μg per well) was performed at room temperature in 1% agarose (in PBS) against isolated lipoproteins (VLDL, IDL, LDL, HDL; 5 μg protein per well) and material eluted from the pH6-Ag column (26). Precipitation reactions were recorded after 16–24 h.

Agarose electrophoresis. Two microliters of each sample (^{125}I-labeled lipoprotein alone or mixed with different amounts of pH6-Ag) was applied on the commercial agarose gel (Hydragel Lipo+Lp(a), Sebia, France). Electrophoresis was performed at 300V for 3–4 h with fresh human plasma as standard. Lipoprotein bound to pH6-Ag remained on the origin, while unbound lipoprotein migrated into a gel. Radioactivity in the band corresponding to the unbound lipoprotein was counted using the GS-250 Molecular Imager (Bio-Rad). The difference between the radioactivity without addition of pH6-Ag and the radioactivity after addition of pH6-Ag was used for calculation of the amount of lipoprotein bound to pH6-Ag.

Interaction between lipoproteins and bacteria expressing pH6-Ag

Bacterial strains KIM5 (*psaA::gfp*) (wild-type strain expressing pH6-Ag with a *gfp*-fusion to monitor transcription of the *psaABC* operon) and KIM5 (Δ*psaA::gfp*) (Δ*psaA*-deletion strain negative for pH6-Ag with a *gfp*-fusion to monitor transcription of the *psaABC* operon) (Cherepanov et al., unpublished observations) were grown in BHI medium (supplemented with 2.5 mM $CaCl_2$, 0.5% yeast extract, and 0.2% xylose) with pH 5.8 at 37°C to induce expression of pH6-Ag. After 5 h of induction the cell cultures were diluted 2-fold in human serum and incubated at 4°C for 10 min. After washing two times with fresh BHI medium, the bacterial cells were incubated at 4°C for 10 min with polyclonal rabbit anti-human apoB antibody (dilution 1:1,000 in BHI medium) and then washed two times with BHI medium. Incubation with secondary antibody (Alexa Fluor 568 goat anti-rabbit antibody; dilution 1:2,000 in BHI medium) was performed at 4°C for 10 min and followed by two washes with BHI medium. Finally, bacteria were visualized by immunofluorescence microscopy using Leica HC.

Interaction of pH6-Ag with erythrocytes

Studies of the interaction of pH6-Ag with human erythrocytes (hRBCs) were performed by a hemagglutination assay (27). hRBCs were washed and resuspended in PBS to a final concentration of 1.0% (v/v). Purified pH6-Ag (serial dilutions 1:2 in 0.1 ml PBS) was placed in round bottom wells of 96-well micro-titer plate (Sarstedt, Inc., Newton, NC) and then 10 μl of washed 1.0% hRBC was added. Hemagglutination titers were scored after 1–2 h of incubation at room temperature. Less than 0.01 μg of purified pH6-Ag was required for visible hemagglutination. For competition assays, serum or isolated lipoproteins were serially diluted 1:2 in 0.05 ml PBS and then mixed with 0.05 ml of pH6-Ag (0.01 mg/ml in PBS). Finally, 10 μl of washed 1.0% hRBC was added. Competitive hemagglutination was scored after 1–2 h of incubation at room temperature as the highest dilution of serum or lipoprotein at which hemagglutination was visible.

Cell culture experiments

Binding of pH6-Ag to THP-I macrophages at 4°C. Before experiments, the cells were chilled at 4°C for 15 min. Growth medium with nonadherent cells was removed and adherent macrophages were washed twice with ice-cold medium (RPMI-1640) containing 0.2% BSA (RPMI-0.2% BSA). Then lipoproteins (added first) and ^{125}I-pH6-Ag in RPMI-1640 with 2% BSA were added and incubation continued for 2 h at 4°C. At the end of incubation period, the medium was removed and cells were washed three times with ice-cold RPMI-0.2% BSA. Heparin (1,500 IU per ml of RPMI-0.2% BSA) was then added and the cells were incubated for 30 min at 4°C. Aliquots of heparin-containing medium (heparin-releasable fraction) were taken to determine the possible lipoprotein-mediated binding of ^{125}I-pH6-Ag to lipoprotein receptors (28). The macrophages were washed three times with ice-cold Dulbecco's PBS and dissolved in 0.2 M NaOH for determination of ^{125}I-pH6-Ag associated with cells (heparin-resistant pH6-Ag) and for measurement of cellular protein (23). Control wells without macrophages were treated the same way. Radioactivities in the samples were measured using an Automatic γ counter Wallac Wizard 1480 (Wallac Oy, Turku, Finland). The values obtained for the control wells were subtracted from the values obtained for the wells with macrophages.

Binding of pH6-Ag to THP-I macrophages at 37°C. The experiments were carried out as described above for the binding of pH6-Ag to macrophages at 4°C with the following changes: incubation with ^{125}I-pH6-Ag and lipoproteins was performed for 2 h at 37°C and the cells were then chilled for 15 min before the washing procedure.

Studies on the mechanism of interaction of pH6-Ag with cell membranes. THP-I macrophages were washed twice with warm RPMI-0.2% BSA and incubated for 1 h at 37°C in RPMI-1640 containing 2% BSA with or without 10 mM MBCD. After chilling for 15 min at 4°C, the macrophages were washed twice with ice-cold RPMI-0.2% BSA, and incubation continued for 1 h at 4°C in RPMI-1640 containing 2% BSA and 10 μg/ml pH6-Ag with or without 10 mM MBCD. Then the cells were washed with heparin (see above) and incubated for 5 min at 4°C in Dulbecco's PBS alone or with ice-cold Triton X-100 (1%, v/v). Cells in other wells were incubated for 5 min at 37°C with warm Triton X-100 (1%, v/v). Aliquots of the PBS and Triton extracts were taken for measurement of radioactivity to determine the amount of pH6-Ag associated with phospholipid (PL)-rich domains on the cell membrane (fraction soluble in cold Triton) and free cholesterol (FC)-rich domains (fraction insoluble in cold Triton, but soluble in warm Triton).

Studies of the possible interaction between apoB and pH6-Ag

ELISA. In system A, the plates [96-well MaxiSorp™ Surface plates (Nunc A/S, Denmark)] were coated overnight at 4°C with 100 μl LDL (10 μg protein per ml PBS). Unbound LDL was removed and the wells were washed four times with PBS containing 0.05% Tween 20 (the same washing routine was used in all steps) and incubated with PBS containing 1% BSA for 1.5–2 h at room temperature to block nonspecific binding sites. Polyclonal rabbit antibody against human apoB or 21 kinds of monoclonal antibodies against different parts of human apoB (a kind gift from Prof. Ross Milne, Ottawa, Canada) were added in optimal amounts for reaction with the bound LDL (dilutions in PBS with 1% BSA). In some experiments, we used mixtures of monoclonal antibodies: mixture I consisted of antibodies against apoB residues 1–1,500; mixture II: against residues 1,501–3,250; and mixture III: against residues 3,251–4,536. The incubation with antibodies was for 1 h at room temperature. Control wells did not contain antibodies against apoB. After washing of the plate, 100 μl of pH6-Ag was added in each well (dilutions from 5 μg/ml to 1.6 ng/ml in PBS, 1% BSA) and incubation was continued for 1 h at room temperature. For detection of the bound pH6-Ag, the plates were incubated for 1 h at room temperature with polyclonal rabbit antibody against pH6-Ag diluted to 1:1,000 in PBS with 1% BSA, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, dilution 1:25,000). In system B, the plates were coated with monoclonal antibodies against apoB (see above) in PBS. Blocking and washing procedures were the same as above. LDL (dilutions from 5 μg/ml to 1.6 ng/ml in PBS, 1% BSA) was added and incubation was for 1 h at room temperature. After washing, incubation with pH6-Ag was performed as described above. In system C, the plates were coated with pH6-Ag (1 μg/well in PBS) and then LDL or LDL preincubated with different monoclonal antibodies against apoB were added. Detection of LDL bound to the immobilized pH6-Ag was performed using polyclonal rabbit antibody against human apoB (dilution 1:1,000 in PBS, 1% BSA).

Partial deglycosylation of LDL. LDL ($1.030 < d < 1.045$ g/ml, 500 μg protein per ml of 0.2 M sodium-phosphate buffer, pH 8.6) was incubated with different concentrations of PNGase F (up to 75 U/ml) for 29 h at 25°C (29, 30) and then washed twice with the same buffer by centrifugation through Nanosep™ 10K (Pall Filtron). That partial deglycosylation was achieved was confirmed by SDS-PAGE and by the DIG Glycan Detection Kit (data not shown) from Boehringer.

Proteolysis of LDL. LDL ($1.030 < d < 1.045$ g/ml) was incubated with TPCK-treated trypsin (ratio 100:1, w/w) for 2 h at 25°C (31). The reaction was stopped by the addition of trasylol (5 μg/ml of reaction mixture). That partial proteolysis had occurred was confirmed by SDS-PAGE and Western blot (data not shown).

After any of the described treatments, the ability of LDL to interact with pH6-Ag was analyzed by ELISA, system C (see above).

Preparation of liposomes and studies of the interaction of pH6-Ag with lipids

FC/phosphatidylcholine (PC) and PC-only liposomes were prepared by probe sonication. Chloroform solutions of FC and PC (10 mg/ml) were mixed in 1:1 molar ratio and evaporated under a stream of nitrogen. After addition of 5 mM Tris-Cl, 0.15 M NaCl, 0.02% (w/v) NaN_3 (pH 7.4), sonication was for repetitive cycles as previously described (32). For some experiments, a commercial emulsion of soybean TG in egg yolk PC (Intralipid® 10%) was used.

Dilutions of liposomes or Intralipid® in 0.15 M NaCl were mixed with an equal volume of ^{125}I-pH6-Ag (100 μg/ml) and centrifuged at 60,000 *g* for 10 min at 4°C. Radioactivities in the top, middle, and bottom fractions were then analyzed.

In other experiments the effects of liposomes were compared with that of LDL on the ability of pH6-Ag to interact with cells. The experiments were carried out as described above for the interaction of THP-I monocyte-derived macrophages with pH6-Ag in the presence of lipoproteins, but pH6-Ag was used at a concentration of 5 μg of protein per ml of incubation medium. Both LDL and liposomes were added at a concentration of 40 μg PL per ml of incubation medium.

To compare the effect of TG-rich lipid emulsion (Intralipid®) with that of apoB-containing TG-rich lipoprotein (chylomicrons) on the interaction of pH6-Ag with THP-I macrophages, we used Intralipid® at the same concentration (2 mg of TG per ml of incubation medium) as the concentration of TG in the experiments with chylomicrons.

Statistical analysis

Statistical analysis of the results, if it is not specially mentioned in the figure legends, was performed using the two-group paired Student's *t*-test. Results are expressed as mean of triplicate determinations ± SD.

RESULTS

pH6-Ag binds a component of human plasma

The pH6-Ag from bacterial culture supernatants is a soluble polymer of high molecular weight built up of PsaA monomers of about 15 kDa each. Purified pH6-Ag was coupled to CNBr-activated Sepharose 4B and used to identify proteins in human plasma with affinity for the putative adhesin. The dominating band in Coomassie-stained SDS-polyacrylamide gel corresponded to a large protein of around 530 kDa (**Fig. 1**). This protein was excised and peptide fragments were analyzed by N-terminal amino acid sequencing that identified the protein as apoB-100. Comparison with the electrophoretic pattern of LDL, IDL, and VLDL isolated from human plasma (Fig.

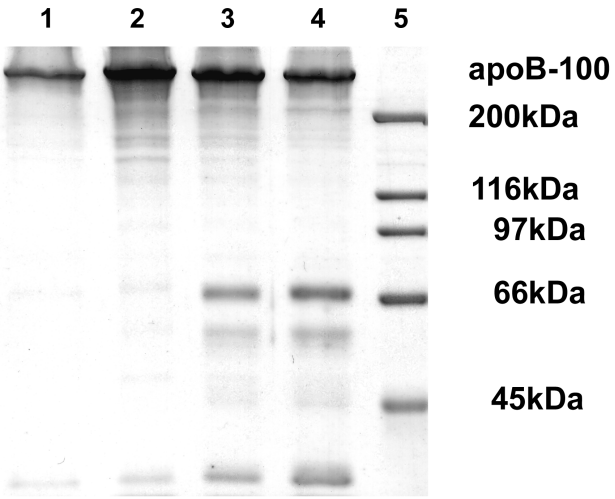

Fig. 1. Affinity purification of components from human plasma on pH6 antigen (pH6-Ag) Sepharose. Human plasma (10 ml) was applied to a PBS-equilibrated Sepharose 4B column with immobilized pH6-Ag (about 1.5 mg protein). After three washings with 20 mM Tris-HCl (pH 7.5, 0.5 M NaCl), the bound proteins were eluted with 3 M potassium thiocyanate (KSCN). After dialysis against PBS, the bound material was analyzed by SDS-PAGE. Lane 1: sample eluted from the affinity column; lane 2: human LDL, $1.019 < d < 1.063$ g/ml; lane 3: human IDL; lane 4: human VLDL; lane 5: protein standard (high-molecular-weight range).

1) and Western blot with anti-human apoB antibody (data not shown) confirmed this finding. Only traces of other apolipoproteins were seen on SDS-PAGE of the material bound to pH6-Ag. Analyses of the lipid content of the bound material showed that the ratio of TG to TC was 1.4 (w/w). This also indicated that other apoB-containing lipoproteins, but more TG-rich than LDL (chylomicrons, VLDL and IDL), had bound to the pH6-Ag on the column.

Interaction of pH6-Ag with lipoproteins

Addition of lipoproteins to a solution of pH6-Ag resulted in a dramatic and immediately observable aggregation. During centrifugation, the precipitate sedimented when VLDL, IDL, or LDL were mixed with pH6-Ag, while with chylomicrons the precipitated material floated, probably due to a higher lipid content (data not shown).

The interaction was further studied by immunodiffusion in agarose. Precipitation lines against the pH6-Ag were obtained both with material eluted from the pH6-Ag column and with the isolated apoB-containing lipoproteins, but no interaction of pH6-Ag was observed with HDL (**Fig. 2**).

We tested the ability of bacteria grown at low pH, which should induce pH6-Ag expression, to interact with lipoproteins. After incubation with human serum, the wild-type strain KIM5 of *Y. pestis* was fluorescence-stained over the whole surface by an anti-human apoB antibody, while the isogenic *psaA* mutant strain showed no surface staining (**Fig. 3**). Thus, only bacteria expressing the pH6-Ag were covered with lipoproteins.

To study the relative affinities in the interaction of different types of lipoproteins with pH6-Ag, we used electrophoresis of ^{125}I-labeled lipoproteins in agarose after mixing with increasing amounts of pH6-Ag. Aggregates of pH6-Ag with the lipoproteins were retained at the origin, while nonaggregated lipoproteins moved in the gel during electrophoresis (**Fig. 4**). Interaction of pH6-Ag with LDL could readily be observed, even with low concentrations of pH6-Ag, while with HDL there was little or no material retained at the origin. The radioactivity in the fraction of nonaggregated lipoproteins was counted, and thus the fraction of lipoproteins that interacted with pH6-Ag could be calculated for each class of lipoproteins. All apoB-containing lipoproteins interacted with pH6-Ag (**Fig. 5**), while essentially no interaction of pH6-Ag with HDL could be detected from these measurements. When the same amounts of lipoprotein protein were added, comparable amounts of protein bound from the VLDL, IDL, and LDL fractions. Binding of LDL appeared to have the highest affinity for pH6-Ag. Addition of more LDL (0.9 mg/ml instead of 0.3 mg/ml) demonstrated that the binding capacity was not limiting in this range (Fig. 5).

Interaction of pH6-Ag with erythrocytes and competition with lipoproteins

Previous work had shown that pH6-Ag agglutinates erythrocytes and that pH6-Ag can mediate binding to epithelial cells (17). We decided to use the agglutinating activity of pH6-Ag to study if the same structures of pH6-Ag are responsible for interaction with erythrocytes as with lipoproteins. Purified pH6-Ag showed high agglutination titers with washed hRBC. Less than 0.01 μg of purified pH6-Ag was required for visible agglutination. Addition of human serum (diluted 1:1,024) prevented agglutination of erythrocytes in the presence of 0.5 μg of pH6-Ag. Isolated human LDL also prevented the agglutination reaction when

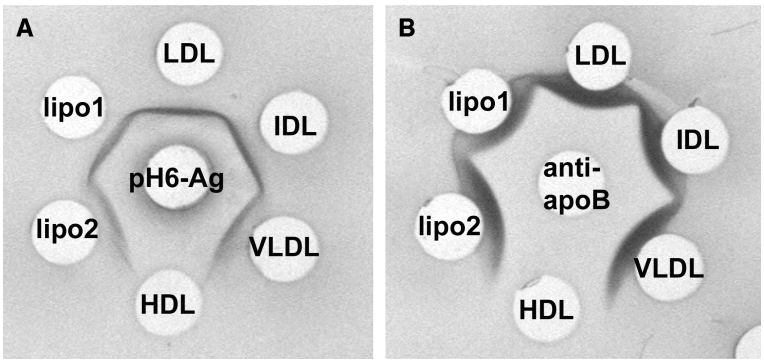

Fig. 2. Interaction of pH6-Ag with apolipoprotein B (apoB)-containing lipoproteins. The interaction was studied by diffusion as described in Materials and Methods. A: The different lipoproteins were tested against purified pH6-Ag. Lipo1 and lipo2 denote two different samples of plasma proteins eluted from the pH6-Ag-Sepharose column. B: The same samples were tested against a polyclonal rabbit antibody to human apoB.

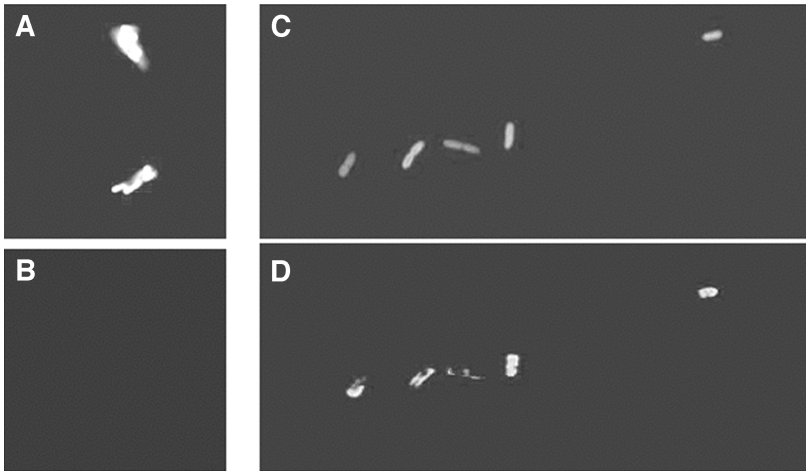

Fig. 3. Interaction of plasma lipoproteins with bacteria expressing pH6-Ag. Bacteria were grown at pH 5.8 to induce expression of pH6-Ag and thereafter incubated with human serum. After washing, the bacteria were fixed and immunostained using anti-human apoB antibody (primary antibody). The bacteria were engineered to express green fluorescent protein (GFP) to allow visualization of bacteria not stained with the anti-apoB antibody. A: KIM5 (*psaA*) GFP expression. B: KIM5 (*psaA*) apoB-staining. C: KIM (wt) GFP expression. D: KIM5 (wt) apoB-staining.

added in dilutions corresponding to the amount of LDL in serum diluted 1:840–1:1,680. HDL showed no significant effect even in much higher concentrations. We did not see any agglutination by the same amounts of pH6-Ag as used above if whole blood was added instead of washed erythrocytes, indicating that the effect of pH6-Ag might be blocked by the lipoproteins.

The interaction of pH6-Ag with RBC and with LDL was very fast and strong. Addition of LDL to pH6-Ag prior to addition of erythrocytes blocked agglutination of the cells, while LDL added to a mixture of pH6-Ag and RBC failed to protect the cells against agglutination.

Interaction of pH6-Ag with macrophages and competition with lipoproteins

We used THP-I monocyte-derived macrophages for studies of binding of ^{125}I-labeled pH6-Ag to cells and of the effects of different lipoproteins on the binding (**Fig. 6**). Binding of pH6-Ag to the cell surface (incubation at 4°C) was dose dependent (more was bound at 10 μg/ml than at 1μg/ml) and it was completed by increasing concentrations of LDL in the incubation medium. After incubation at 37°C for the same time, more pH6-Ag was associated with the cells than at 4°C, indicating that in addition to binding, some pH6-Ag had been internalized. LDL efficiently prevented the association. At higher concentration of pH6-Ag (10 μg/ml), more LDL was required to abolish the interaction of pH6-Ag with the cells.

Competition for interaction of pH6-Ag with THP-I macrophages was marked with all apoB-containing lipoproteins, but was not seen with HDL (**Fig. 7A**). Chylomicrons used at the same protein concentration as the other lipoproteins also blocked the interaction (data not shown). To investigate if LDL receptors were involved, the cells

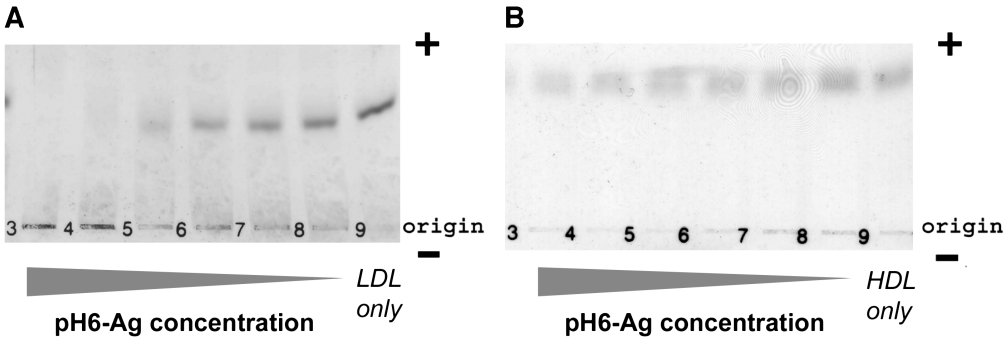

Fig. 4. Quantification of the ability of lipoproteins to interact with pH6-Ag. Serial dilutions of pH6-Ag (1.04 mg/ml) in PBS were mixed with equal volumes of ^{125}I-LDL, 1.05 mg of protein/ml (A) or ^{125}I-HDL, 1.77 mg of protein/ml (B). Two microliters of each mixture containing from 10 ng to 420 ng of pH6-Ag were applied on an agarose gel and electrophoresis was then performed. Lipoproteins bound to the pH6-Ag remained at the origin, while unbound lipoproteins migrated into the gel. The difference between the radioactivity in the original lipoprotein fraction and the radioactivity in the unbound lipoprotein in each sample was used to calculate the radioactivity of lipoprotein bound to pH6-Ag.

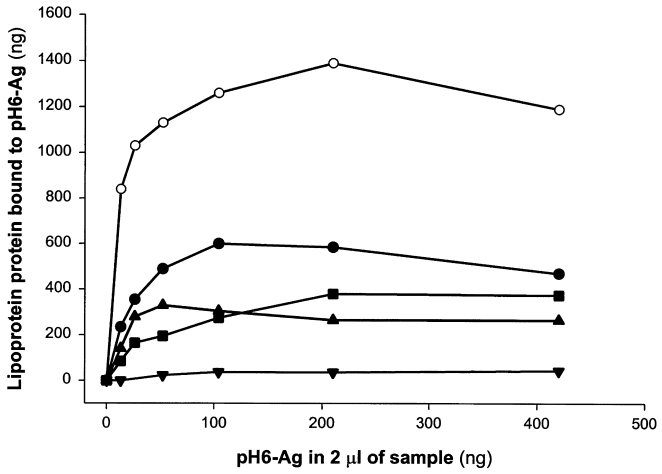

Fig. 5. Interaction of lipoproteins with pH6-Ag as calculated by agarose electrophoresis. The amount of lipoprotein bound to pH6-Ag was determined as described in the legend to Fig.4. The initial concentrations of lipoproteins used for dilution of pH6-Ag are indicated. Inverted triangles, HDL (0.3 mg/ml); triangles, IDL (0.3 mg/ml); squares, VLDL (0.3 mg/ml); solid circles, LDL (0.3 mg/ml); open circles, LDL (0.9 mg/ml). Data from a representative experiment are shown.

were treated with a high concentration of heparin (1,500 IU/ml) that should remove lipoproteins bound to receptors of the LDL receptor family or to proteoglycans (28). As shown in Fig. 7B, heparin did not release much of the pH6-Ag from the cells, indicating that the binding sites for pH6-Ag differed from those for lipoproteins. Moreover, pH6-Ag was able to interact in a similar way with normal and LDL receptor-negative fibroblasts. This interaction was abolished by the presence of LDL in the incubation medium, investigated as described above for the experiments with macrophages (data not shown).

Studies of the interaction of apoB with pH6-Ag

The ability of apoB-containing lipoproteins to interact with the pH6-Ag, and the absence of interaction between HDL and pH6-Ag, indicated that apoB might be responsible for the interaction. LDL was the best candidate for direct studies of the interaction because the protein part of LDL consists of only one molecule of apoB-100. To investigate if apoE participated in the interaction with pH6-Ag, we removed apoE-containing lipoproteins from the LDL fraction by preincubation with a polyclonal antibody against human apoE. In addition, we used a narrow fraction of LDL ($1.030 < d < 1.045$ g/ml) that was free of apoE on SDS-PAGE, and we studied competition between LDL and apoE-containing lipoproteins (VLDL, IDL) for binding to pH6-Ag. We did, however, not see any evidence for participation of apoE in the interaction with pH6-Ag (data not shown).

Several different approaches were used to find evidence for a direct interaction between apoB and the pH6-Ag. Since most attempts were negative, the data are summarized in **Table 1**. Competition was seen with all apoB-containing lipoproteins. Neither partial deglycosylation of LDL nor preincubation of LDL with polyclonal antibodies to apoB or with 19 different monoclonal antibodies to apoB had any effect on the interaction of LDL with pH6-Ag. In contrast, fragmentation of apoB with trypsin led to a slight increase in the interaction (verified by ELISA and in experiments with THP-I macrophages, data not shown). Detailed analysis of the data obtained with the monoclonal antibodies showed that only mixture II (antibodies against amino acid residues 1,501–3,250 of the apoB-100 molecule) and in particular the 2G4 and 3A5 antibodies (against the hydrophobic part of apoB-100) were able to slightly decrease the interaction of pH6-Ag with LDL. Taken together, these data indicated that hydrophobic parts of the lipoprotein, but not apoB itself, were responsible for the interaction of pH6-Ag with the lipoproteins.

Analysis of the interaction of pH6-AG with lipids

To study the interaction of pH6-Ag with lipids, we used liposomes consisting of different lipid components and a commercial lipid emulsion designed for parenteral nutrition (Intralipid®). A strong dose-dependent interaction of

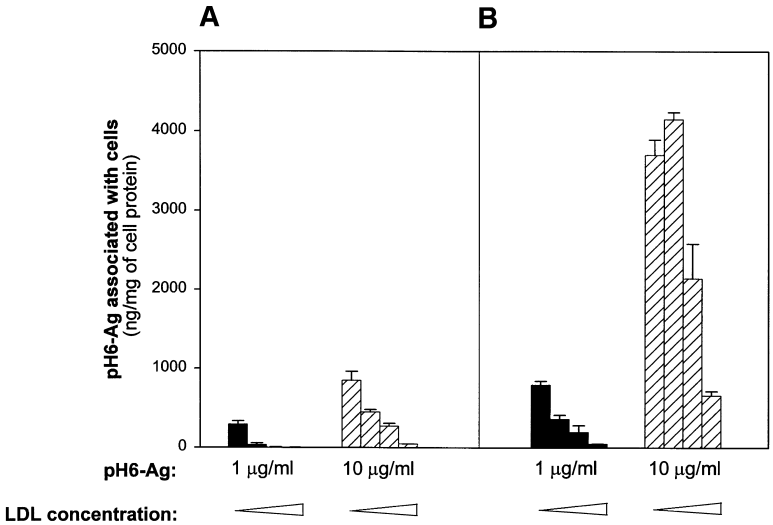

Fig. 6. LDL abolishes the interaction of pH6-Ag with THP-I macrophages. The THP-I monocyte-derived macrophages were incubated for 2 h at 4°C (A) or at 37°C (B) with 1 µg/ml (black bars) or 10 µg/ml (hatched bars) of ^{125}I-pH6-Ag. Incubation was performed without human LDL (the first bar in each group) and with 10 µg (the second bar), 50 µg (the third bar), or 250 µg (the fourth bar) of human LDL per ml incubation medium. The total amount of pH6-Ag associated with the cells was determined as described in Materials and Methods. Data are means of values obtained from three wells ± SD.

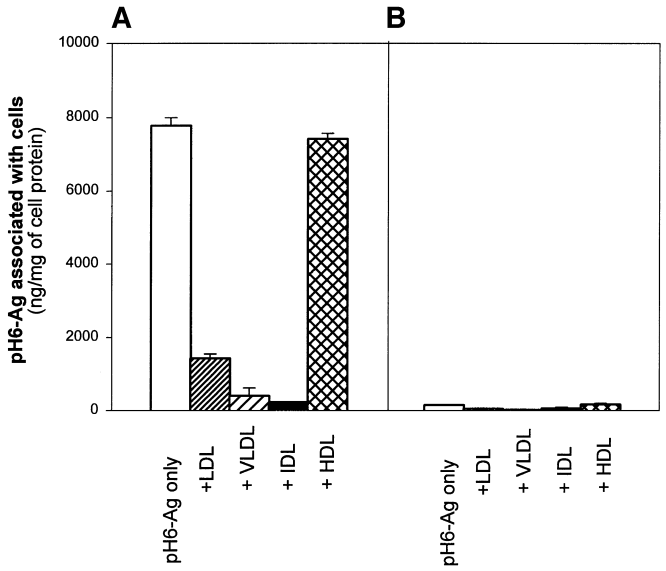

Fig. 7. Lipoproteins have different ability to prevent the interaction of pH6-Ag with THP-I macrophages. THP-I monocyte-derived macrophages were incubated for 2 h at 37°C with ^{125}I-pH6-Ag alone (10 μg per ml incubation medium) or with 250 μg of human lipoproteins per ml incubation medium. After wash, the total amount of pH6-Ag bound to the cells was determined (A). The amounts of pH6-Ag released from the cells with heparin, as described in Materials and Methods, are shown in B. Data are means of the values obtained from three wells ± SD. Open bars, pH6-Ag alone; gray bars, pH6-Ag plus LDL; hatched bars, pH6-Ag plus VLDL; black bars, pH6-Ag plus IDL; cross-hatched bars, pH6-Ag plus HDL.

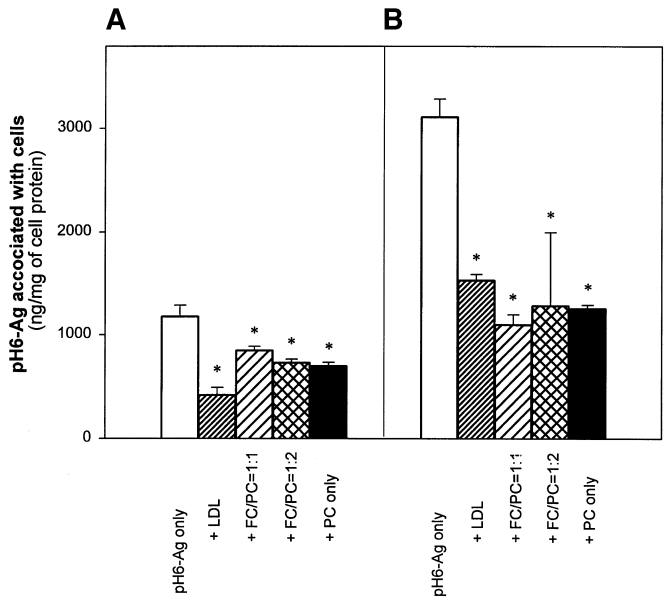

Fig. 8. Liposomes have a similar ability to prevent the interaction of pH6-Ag with THP-I macrophages as human LDL. THP-I monocyte-derived macrophages were incubated for 2 h at 4°C (A) or at 37°C (B) with 5 μg of ^{125}I-pH6-Ag per ml incubation medium alone or in the presence of human LDL or liposomes (40 μg of phosphatidylcholine (PC) per milliliter of incubation medium). Liposomes (LS) were prepared as described in Materials and Methods from PC alone or free cholesterol (FC) and PC in the molar ratio 1:1 and 1:2 and added to the incubation medium to the final concentration of 40 μg of PC per ml. Data are means of the values obtained from three wells for the total radioactivity associated with cells after wash ± SD. Open bars, pH6-Ag alone; gray bars, pH6-Ag plus LDL; hatched bars, pH6-Ag plus LS (FC/PC, 1:1); cross-hatched bars, pH6-Ag plus LS (FC/PC, 1:2); black bars, pH6-Ag plus LS (PC alone). * $P < 0.01$ in comparison with pH6-Ag only.

pH6-Ag occurred with all types of liposomes used in our experiments, as well as with the lipid emulsion. Addition of any of the studied lipid preparations to pH6-Ag immediately resulted in visible aggregation and flotation/sedimentation of the aggregates. From 70% to 95% of the pH6-Ag was found in the lipid-containing aggregates after centrifugation (60,000 *g*, 10 min).

Liposomes blocked the interaction of pH6-Ag with THP-I macrophages at 4°C and 37°C to a similar extent as LDL did at comparable lipid concentrations (**Fig. 8**). Intralipid® blocked the interaction of pH6-Ag with macrophages at 37°C to a similar extent as chylomicrons did when used at the same TG concentration (data not shown). These experiments illustrated the importance of the lipid-dependent interaction of pH6-Ag with the apoB-containing lipoproteins.

To study if the interaction of the pH6-Ag with macrophages was dependent on membrane lipids, we incubated THP-I macrophages with pH6-Ag at 4°C, then washed and incubated the cells in cold or warm Triton X-100 (**Fig. 9**). The pH6-Ag soluble in cold Triton was considered to be associated with PL-rich domains on the cell membrane (33). The cold Triton-insoluble, but warm Triton-soluble, pH6-Ag was considered to be associated with FC-rich domains or rafts (34). These fractions constituted one third each of the total binding of pH6-Ag. Some pH6-Ag could not be removed from the cells. This fraction (warm Triton-insoluble), as well as the cold Triton-insoluble fraction, was sensitive to depletion of cholesterol from the cell membrane by treatment with MBCD. MBCD decreased the total amount of pH6-Ag bound to the cells, but did not influence the binding of pH6-Ag to PL-rich domains (cold Triton-soluble fraction).

TABLE 1. Summary of results from attempts to identify the binding site on LDL responsible for the interaction with pH6-Ag

Experimental Approach	Interaction of pH6-Ag with LDL
Presence of other lipoproteins:	
human VLDL	↓[a]
human IDL	↓↓
human LDL	↓↓
Treatment of LDL with polyclonal anti-apoB antibodies	–[b]
Treatment of LDL with 19 monoclonal anti-apoB antibodies	–
Treatment of LDL with monoclonal antibodies against hydrophobic part of apoB (2G4 and 3A5)	↓
Partial deglycosylation of LDL	–
Treatment of LDL with trypsin	↑[c]

[a] Decrease in the interaction between LDL and pH6-Ag.
[b] Interaction between LDL and pH6-Ag was not changed.
[c] Increase in the interaction between LDL and pH6-Ag.

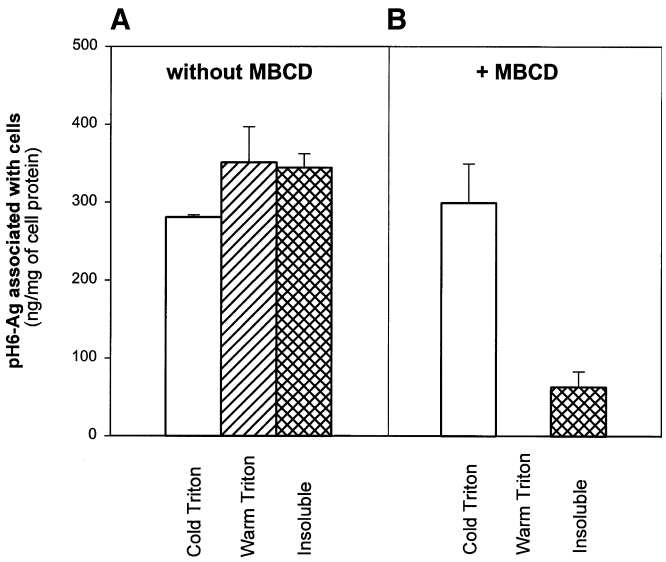

Fig. 9. Role of membrane lipids in the interaction of pH6-Ag with cells. THP-I monocyte-derived macrophages were incubated for 1 h at 4°C with 10 μg of pH6-Ag per ml incubation medium with or without 10 mM methyl-β-cyclodextrin (MBCD). After washing with heparin, the cells were incubated with cold or warm Triton X-100 to determine the amount of pH6-Ag associated with phospholipid (PL)-rich domains (cold Triton-soluble fraction) and FC-rich domains (cold Triton-insoluble, warm Triton-soluble fraction). For details see Materials and Methods. Data are means of the values obtained from three wells ± SD. Open bars, cold Triton-soluble pH6-Ag; hatched bars, cold Triton-insoluble, warm Triton-soluble pH6-Ag; cross-hatched bars, warm Triton-insoluble pH6-Ag.

DISCUSSION

Binding of pH6-Ag to lipoproteins and other lipid-containing structures

The first experiments in this study identified LDL as the dominating component from plasma that bound to the pH6-Ag. LDL bound rapidly and with relatively high affinity and could be released by treatment with a chaotropic agent (KCSN), indicating the interaction had a strong hydrophobic component. The bound material was identified by its content of apoB. We found that other apoB-containing lipoproteins could also bind (chylomicrons, VLDL, IDL), although with lower affinity than that seen for LDL. Their concentrations in fasted human plasma are lower than that of LDL, probably explaining why LDL was the main component bound. We were not able to directly determine the affinity constants for the interaction of pH6-Ag with the different classes of lipoproteins. The interaction is complex since many lipoprotein particles bound per pH6-Ag polymer, polymer aggregation number is not known, and binding led to precipitation.

No binding was seen between pH6-Ag and HDL

A very clear result from our study was that the pH6-Ag did not interact with HDL. This is the smallest lipoprotein class in human plasma (about 10 nm in diameter). Protein and PL represent about 50% and 25% of the dry weight of HDL, respectively (35). The protein consists mainly of apoA-I and apoA-II, but also other exchangeable apolipoproteins can be found, like apoE and members of the apoC family. Compared with the larger lipoproteins, the surface of HDL is densely packed with proteins, leaving limited areas of exposed lipids. However, many factors involved in the metabolism of HDL (enzymes, lipid exchange factors, and receptors) still have access to the lipid part of the HDL particle. The reason for the inability of the pH6-Ag to bind to HDL was not explored here. The most likely explanation, that the pH6-Ag needs apoB for interaction, was excluded by a number of different approaches. It was shown that liposomes and a triglyceride emulsion interacted avidly with the pH6-Ag, indicating that binding occurred directly between hydrophobic parts of the pH6-Ag and lipids.

Binding of pH6-Ag to cells

Previous studies (17), as well as data presented here, have shown that pH6-Ag agglutinates erythrocytes, indicating binding of pH6-Ag to cell membranes. We demonstrated direct binding of purified ^{125}I-labeled pH6-Ag to THP-I macrophages and to human fibroblasts. More pH6-Ag was found associated with the cells after incubation at 37°C than at 4°C, indicating internalization by energy-dependent mechanisms. Depletion of cholesterol from the cell membrane by pretreatment with MBCD affected the interaction, indicating that cholesterol-rich parts of the membrane (lipid rafts) were involved. Components of lipid rafts, for example glycosphingolipids, can act as specific receptors for certain multivalent toxins (36). Interaction with ganglioside GM_1 was described for Cholera toxin (37). A variety of pore-forming toxins are also likely to utilize components of rafts on the membrane of the host cell (cholesterol and sphingomyelin) to promote oligomerization (38). Thus, there is a possible link between the affinity of pH6-Ag to FC-rich areas on the macrophage membrane found in the present study and the ability of pH6-Ag to interact with some glycosphingolipids in vitro (18; Cherepanov et al., unpublished observations).

About one third of the bound pH6-Ag could be released from macrophages with cold Triton X-100, indicating that a large amount was also associated with PL-rich areas. The cold Triton-soluble fraction was not affected by treatment of the cells with MBCD. Our data do not exclude the possibility that the pH6-Ag bound via some receptor protein to these membrane areas. However, the lack of sensitivity of the bound pH6-Ag to high concentrations of heparin, and the studies with LDL receptor-negative human fibroblasts, demonstrated that binding of pH6-Ag to the cells was not dependent on proteoglycans or on receptors from the LDL receptor family.

Lipoproteins and other lipid-composed particles prevented binding of pH6-Ag to cells

The apoB-containing lipoproteins, as well as liposomes and the commercial lipid emulsion, all prevented the interaction of pH6-Ag with cells. This was seen as prevention of agglutination of erythrocytes, or of interaction of radiolabeled pH6-Ag with macrophages and fibroblasts. Addi-

tion of lipoproteins or lipid particles to the incubation medium markedly decreased the association of pH6-Ag with the cells, indicating that similar binding sites were engaged and supporting the hypothesis that the main interactions both with cells and with lipoproteins were with the lipid parts.

Role of pH6-Ag for virulence and for protection against defense mechanisms in the host

We have shown that pH6-Ag specifically interacts with lipoproteins. The role of this interaction during infection is not immediately recognized. The observation that expression of PsaA is induced rapidly after internalization of the bacteria by macrophages suggests that the adhesin is important during the early intracellular phase of the infection. It is possible that pH6-Ag somehow facilitates the escape of *Y. pestis* from the macrophage (Cherepanov et al., unpublished observations). After escaping from the phagosome, the bacteria most likely express the pH6-Ag. Because of the high affinity between apoB-containing lipoproteins (especially LDL) and the pH6-Ag, circulating lipoproteins could be of importance for preventing host cell interaction with *Y. pestis*.

The highest concentration of LDL used in our experiments (250 μg of protein per ml of incubation medium) was close to physiological concentrations of LDL in human blood. At this concentration, LDL almost abolished the interaction of pH6-Ag with cells, probably due to the formation of aggregates in which the pH6-Ag was almost entirely covered by LDL (Fig. 3). As complexes of pH6-Ag with LDL apparently are not internalized via endocytosis, the LDL binding is likely to prevent uptake and promote extracellular replication of *Y. pestis*. In addition, the LDL deposition on the bacterial surface could also prevent recognition of the pathogen by the host defense, and this might be important for the ability of the pathogen to replicate in the susceptible host. This could promote infection, but has as yet not been studied. Increased susceptibility of the liver to toxin-induced injury was recently shown for mice with blocked VLDL secretion from hepatocytes (39).

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