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Complement-Independent Binding of Microorganisms to Primate Erythrocytes In Vitro by Cross-Linked Monoclonal Antibodies via Complement Receptor 1

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Under certain circumstances, soluble antigens, particulate antigens, and/or microorganisms have been shown to bind to primate erythrocytes via complement receptor 1 (CR1) in the presence of specific antibodies and complement. This immune adherence reaction, specific for CR1, can lead to neutralization of antigens in the circulation and their subsequent clearance from the blood. The present experiments utilized cross-linked monoclonal antibody complexes (heteropolymers) with specificity for both CR1 and either ^{35}S-labeled herpes simplex virus capsid or *Haemophilus influenzae* as prototype viral and bacterial particulate antigens, respectively. In each case, the respective specific heteropolymers facilitated binding of the target antigens (≥70 to 90%) in vitro to erythrocytes in the absence of complement. Several experimental protocols were employed to demonstrate that heteropolymers mediate specific, rapid (≥30 s), and quantitative binding of prototypical particulate pathogens to human and monkey erythrocytes but not to sheep erythrocytes, which lack CR1. These results extend the potential use of the erythrocyte-heteropolymer system to the neutralization and clearance of particulate viral and bacterial pathogens from the blood.

The function of leukocytes in host defense against microbial invasion is well known. Less widely appreciated, however, is the part primate erythrocytes may play in resistance to infection. The potential role of human erythrocytes in the binding and neutralization of pathogens was first described by Nelson (15, 16) more than 40 years ago. He demonstrated in vitro adherence of microorganisms to the surface of erythrocytes in the presence of specific antibodies and complement, terming this phenomenon immune adherence (IA). His work also indicated that the attachment and immobilization of C3b-opsonized pathogens on erythrocytes resulted in enhanced phagocytosis of the adherent bacteria by leukocytes.

In an extension of this work, the effective role of IA in vivo has since been investigated by Cornacoff et al. and Hebert and Cosio (1, 10) and others (3, 21, 12) as a mechanism by which erythrocytes may facilitate the safe and rapid clearance of antigen-antibody immune complexes from the circulation. C3b-opsonized immune complexes attach to the erythrocyte surface via complement receptor 1 (CR1). The erythrocyte-bound immune complex is then transported to the liver and spleen, where the immune complex is removed from the erythrocyte by fixed tissue macrophages (by an as-yet-unknown mechanism) and the erythrocyte is returned to the circulation without hemolysis.

Primate CR1 possesses several properties that allow this receptor to bind and clear the immune complex without erythrocyte clearance. CR1 is composed of long homologous repeating subunits and occurs in clusters on the erythrocyte membrane, properties which allow for multivalent binding of antigens to the erythrocyte surface. Experiments utilizing ^{51}Cr-labeled erythrocytes have shown that the binding and clearance of immune complexes via CR1 does not result in liberation of the radioisotope into the plasma, thus confirming that the cells are returned to the circulation without hemolysis. These unique features of CR1 have led us to attempt to extend the erythrocyte binding phenomenon through the use of monoclonal antibodies (MAb) specific for CR1 cross-linked to MAb specific for a target antigen. These antibody constructs, termed heteropolymers (HP), should facilitate the binding of virtually any antigen to erythrocytes and act independently of complement activation. Work performed previously by Taylor et al. (25) has demonstrated in vitro HP-mediated binding of soluble protein antigens to primate erythrocytes. This binding was found to be saturable and specific for the target antigen. Binding occurred rapidly and was stable over time without factor I cleavage of the complex from the erythrocyte surface as can occur in natural IA, in which complement fixation is a necessary condition for binding. Subsequent in vivo studies (19, 24) have shown that the erythrocyte-HP system facilitates clearance of several soluble proteins from the circulations of squirrels and rhesus monkeys. In the present experiments, we show that use of HP can be extended to bind prototypical viral particles (herpes simplex virus type 1 capsids [HSVc]) and bacteria (*Haemophilus influenzae*) to erythrocytes in vitro.

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MATERIALS AND METHODS

HSVc. ^{35}Sulfur-labeled HSVc was provided by Jay Brown and William Newcomb (University of Virginia, Charlottesville, Va.). All experiments utilized herpes simplex virus type 1 (HSV-1) (17MP) grown on BHK-21 cell monolayers. B capsids were isolated by sonication of infected cells in the presence of Triton X-100, and A, B, and C capsids were separated by centrifugation on linear 20 to 50% sucrose gradients, as previously described (17). The B capsids were labeled with Tran-^{35}S L-methionine (ICN Biochemicals, Irvine, Calif.). Aliquots of B capsids were placed in 850-cm^2 roller bottles in 10% Dulbecco's modified Eagle medium–10% newborn-calf serum–10% tryptose phosphate medium (GIBCO Laboratories, Grand Island, N.Y.) and incubated with 10 μCi of [^{35}S]methionine per ml of medium. The final specific activity was 1,000 dpm/5 × 10^9 capsids.

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TABLE 1. MAb composition of HP

HP specificity	Composition[a]	Individual MAb
Specific anti-HSVc	Anti-CR1 × anti-HSVc	$7G9_{10} \times 5C10_4$
Irrelevant	Anti-CR1 × anti-IgM	$7G9_{10} \times HB57_6$
Specific anti-*H. influenzae*	Anti-CR1 × anti-HI	$7G9_{10} \times 6G3_5$

[a] IgM, immunoglobulin M; HI, *H. influenzae*.

Bacteria. *H. influenzae* 1479, an unencapsulated variant, was the gift of Timothy Murphy (State University of New York, Buffalo). The organisms were cultured at 37°C in 5% CO_2 on chocolate agar (Baxter Scientific Products, Chicago, Ill.) weekly from a skim milk stock broth stored at −70°C. A 0.5-McFarland-unit suspension (≈10^8 CFU/ml) was prepared in phosphate-buffered saline (PBS) from an overnight culture.

Erythrocytes. Whole blood was collected from adult human donors, rhesus monkeys, and sheep and was stored in Alsever's solution (26). In all assays, aliquots of blood were first washed three times in PBS and reconstituted to a 50% hematocrit in 1% bovine serum albumin (BSA)–PBS, pH 7.4.

MAb. Mouse immunoglobulin G MAb against HSVc (5C10), human immunoglobulin M (HB57), dinitrophenol (23D1), and CR1 (7G9) were prepared from ascites fluid by affinity chromatography with immobilized recombinant protein G (25) (Gamma Bind Plus Agarose; Pharmacia, Newark, N.J.), and the anti-CR1 MAb was radiolabeled with ^{125}I by the Iodogen method (6). We used radioimmunoassays (20) to determine the numbers of epitopes for the anti-CR1 MAb 7G9 per human and rhesus monkey erythrocytes; these numbers were approximately 400 and 1,200, respectively. The cell line for the anti-*H. influenzae* MAb (6G3) was a gift from Timothy Murphy (State University of New York, Buffalo); MAb preparation and purification were as described above. This MAb binds an epitope on the P2 outer membrane protein of *H. influenzae* 1479 (9, 22), and approximately 10,000 of these epitopes are present per bacterium, as determined by radioimmunoassays (data not shown). The MAb were biotinylated by established procedures (24, 29), using the biotinyl-*N*-hydroxy succinimide long-arm reagent (Vector Laboratories, Burlingame, Calif.). The molar input ratios of biotinylating agent to MAb varied from 4:1 to 10:1 and are designated by subscripts (e.g., $7G9_{10}$, $6G3_5$, etc.).

HP. HP were constructed by incubation of the biotinylated anti-CR1 MAb ($7G9_{10}$) with a slight excess of streptavidin (Sigma, St. Louis, Mo.) for 30 min at room temperature, followed by addition of the biotinylated MAb for the specific target antigen (19). Previously performed dose-response experiments (data not shown) established the optimal inputs of anti-CR1 MAb, streptavidin, and anti-target MAb to yield soluble HP that maximized specific binding of target antigen to erythrocytes. Both the anti-HSVc and anti-*H. influenzae* HP were constructed at $7G9_{10}$/streptavidin/anti-target MAb ratios of 1.0:1.25:1.5 by weight. Samples of HP were briefly centrifuged to remove any insoluble material (typically <20% of total protein) and used without further purification (see Table 1 for descriptions of each HP).

Binding assays. In binding experiments for *H. influenzae*, human or monkey erythrocytes were mixed with *H. influenzae* at ratios of 500:1. Aliquots of specific anti-*H. influenzae* HP or 1% BSA-PBS were added and incubated for various times at 37°C or on ice. The erythrocytes were washed and centrifuged at a speed which pelleted erythrocytes and erythrocyte-bound bacteria but not free bacteria (258 × *g* for 10 min). Serial dilutions of the pellets and supernatants were cultured on chocolate agar at 37°C, at 5% CO_2, overnight.

For the HSVc experiments, human or monkey erythrocytes and ^{35}S-labeled HSVc were combined at a 3:1 ratio of antigen to erythrocytes in order to obtain an adequate number of input counts for the measurement. Aliquots of either a specific anti-HSVc HP, an irrelevant HP, or 1% BSA-PBS were added to this solution and incubated for various times at 37°C or on ice. The erythrocytes were

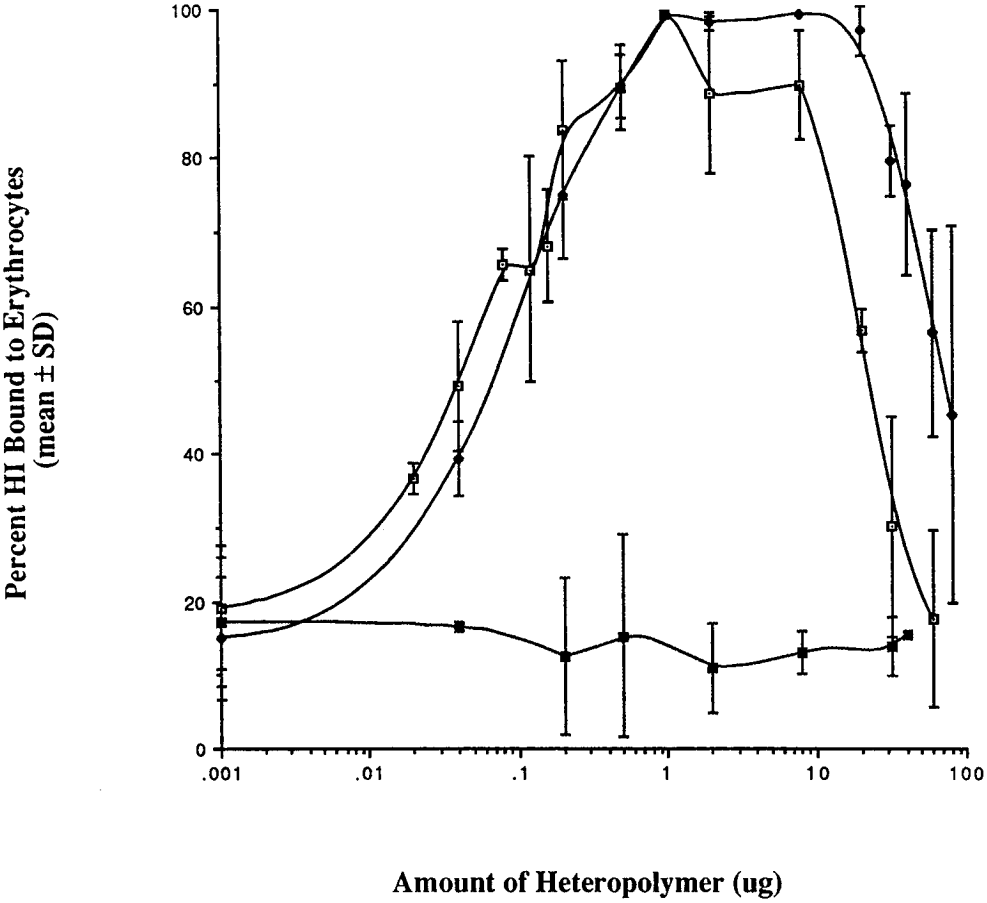

FIG. 1. Percent binding of *H. influenzae* to primate and sheep erythrocytes in the presence of specific HP. Erythrocytes were mixed with bacteria at a ratio of 500:1. Aliquots of anti-*H. influenzae* HP were added and incubated at 37°C. The erythrocytes were washed and centrifuged at a speed which pelleted erythrocytes and erythrocyte-bound bacteria but not free bacteria. Serial dilutions of the pellets and supernatants were cultured overnight. Bacteria exhibited maximal binding to human erythrocytes (⊡) and rhesus monkey erythrocytes (◆) over a broad range of HP concentrations. Only low-level background binding was observed with HP binding assays with sheep erythrocytes (■) (which lack CR1). SD, standard deviation.

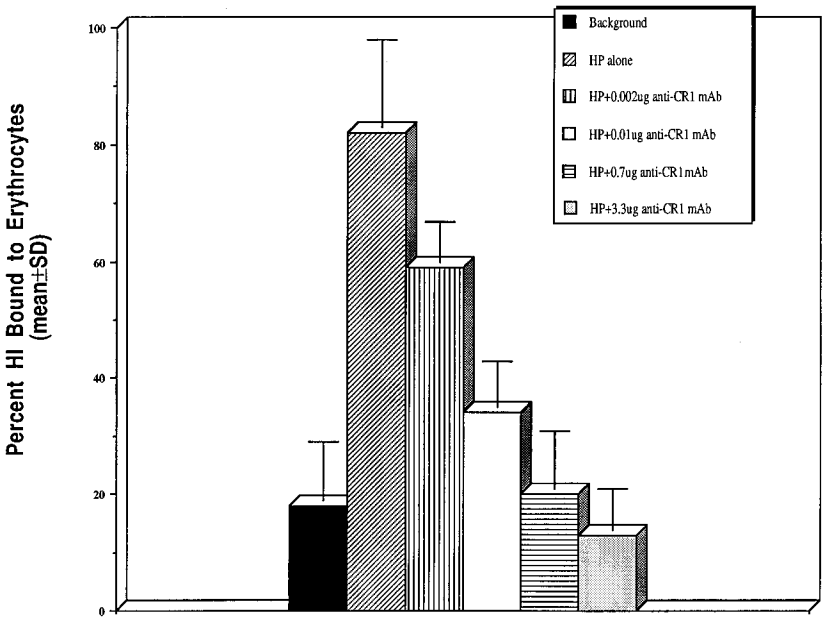

FIG. 2. Inhibition of HP-mediated binding of *H. influenzae* to human erythrocytes by preincubation with various concentrations of homologous anti-CR1 MAb. Erythrocytes and bacteria at a ratio of 500:1 were mixed with increasing amounts of nonbiotinylated anti-CR1 MAb. Specific anti-*H. influenzae* HP was then added, and the samples were incubated at 37°C. The erythrocytes were washed and centrifuged at a speed which pelleted erythrocytes and erythrocyte-bound bacteria but not free bacteria. Serial dilutions of the pellets and supernatants were cultured overnight. A high degree of binding was seen with HP alone. Preincubation with increasing amounts of anti-CR1 MAb resulted in significant inhibition of binding ($P < 0.0001$ by analysis of variance). SD, standard deviation.

washed and pelleted (1,000 × *g* for 5 min), and the supernatants were counted in a scintillation counter to determine the amount of unbound HSVc.

Competition assays. In order to demonstrate the specificity of binding, several assays were performed in the presence of a large excess of competing nonbiotinylated MAb. For HSVc, human erythrocytes and HSVc were first mixed with either excess nonbiotinylated anti-CR1 MAb (7G9), anti-HSVc MAb (5C10), or an irrelevant MAb (23D1). Aliquots of the specific anti-HSVc HP were then added and incubated for 5 min at 37°C. The cells were washed and processed as described above.

Similarly, in the *H. influenzae* assays, erythrocytes and *H. influenzae* were mixed with increasing amounts of nonbiotinylated anti-*H. influenzae* MAb (6G3) or anti-CR1 MAb (7G9). Specific anti-*H. influenzae* HP was then added, and binding was assayed as described above.

Electron microscopy. Pelleted erythrocyte samples containing bound particles were fixed in 5% glutaraldehyde-PBS for several days and then postfixed in 2% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol concentrations (40 to 100%), and embedded in Epon 812. Tissue blocks were trimmed and sectioned for electron microscopy. Thin sections (80 nm) were stained with 10%

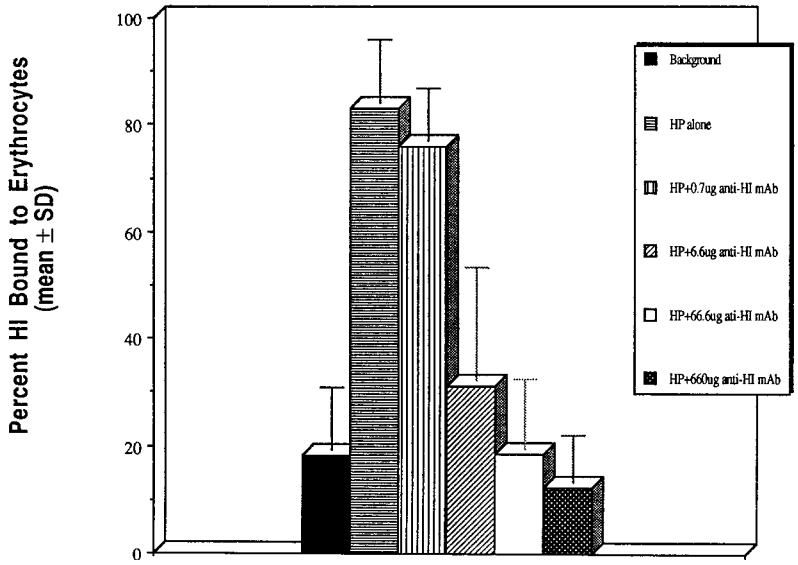

FIG. 3. Inhibition of HP-mediated binding of *H. influenzae* to human erythrocytes by preincubation with various concentrations of anti-*H. influenzae* MAb. Erythrocytes and *H. influenzae* at a ratio of 500:1 were mixed with increasing amounts of nonbiotinylated anti-*H. influenzae* HP MAb. Specific anti-*H. influenzae* HP was then added, and the samples were incubated at 37°C. The erythrocytes were washed and centrifuged at a speed which pelleted erythrocytes and erythrocyte-bound bacteria but not free bacteria. Serial dilutions of the pellets and supernatants were cultured overnight. HP-mediated binding was not significantly inhibited by low concentrations of anti-*H. influenzae* MAb, but at higher concentrations (>6.6 μg) the anti-*H. influenzae* MAb significantly inhibited binding ($P < 0.0001$ by analysis of variance). SD, standard deviation.

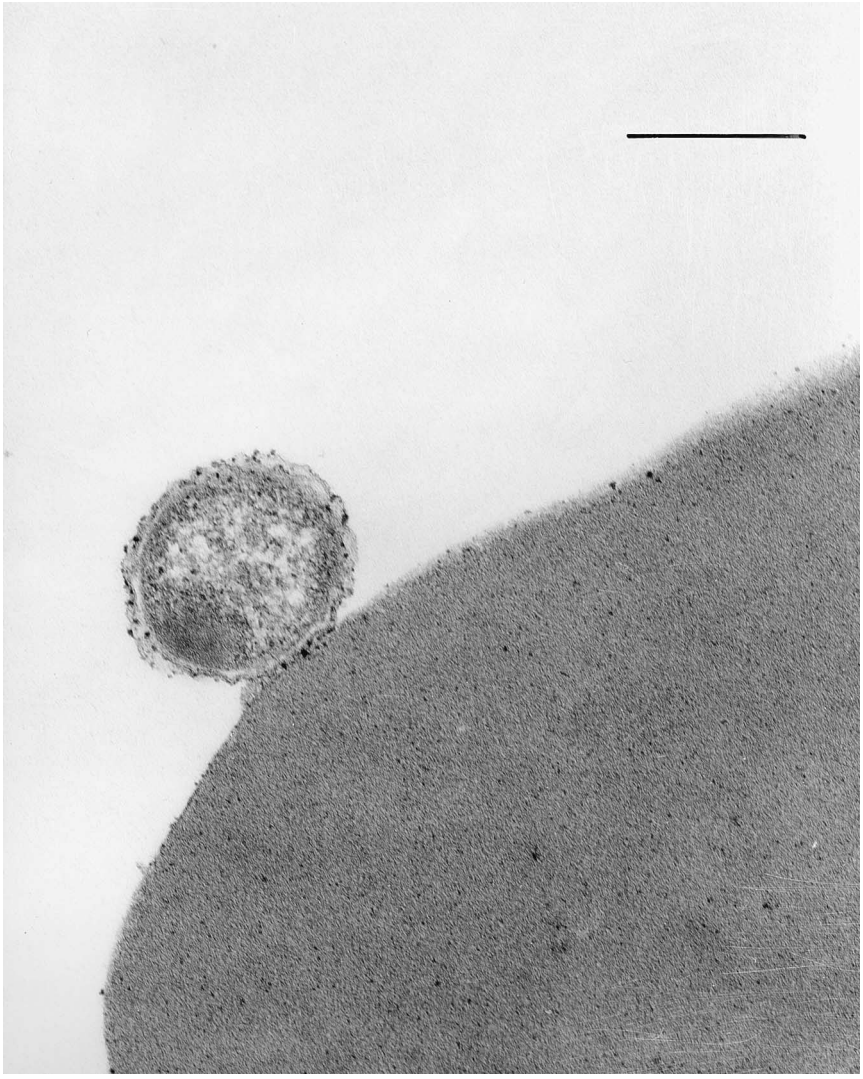

FIG. 4. Transmission electron micrograph showing *H. influenzae* (in cross section) bound to the surface of a human erythrocyte via HP. Bar = 0.5 μm.

uranyl acetate and 0.25% lead citrate and observed and photographed with a 100CX (JEOL, Tokyo, Japan) electron microscope.

Statistical analysis. Data were analyzed for significant differences by the unpaired Student's *t* test or by analysis of variance (32) in the case of linear dose-response data; probability values which were <0.05 were considered significant.

RESULTS

H. influenzae. The effect of increasing concentrations of specific anti-*H. influenzae* HP on the binding of *H. influenzae* to human, monkey, and sheep erythrocytes is shown in Fig. 1. Binding of *H. influenzae* to human and monkey erythrocytes demonstrated an approximate bell-shaped curve, which we believe represents titration of the CR1 epitopes on the erythrocytes (25), but optimal binding of *H. influenzae* occurred over a broad range of HP concentrations. The curve representing binding of *H. influenzae* to monkey erythrocytes was comparable to that for human erythrocytes but was shifted to the right. This effect may be due to the greater number of CR1 epitopes on rhesus monkey erythrocytes (approximately 3 to 4 times as many) relative to that on human erythrocytes. There was no significant binding of *H. influenzae* to sheep erythrocytes at any concentration of specific anti-*H. influenzae* HP.

The specificity of HP-mediated binding of *H. influenzae* to human erythrocytes was demonstrated by incubating the reactants with increasing concentrations of nonbiotinylated anti-CR1 or anti-HSVc MAb. As anticipated, binding of the bacteria to human erythrocytes was significantly inhibited ($P < 0.0001$) by preincubation with the anti-CR1 MAb at all concentrations tested (Fig. 2). Relatively low concentrations of the anti-*H. influenzae* MAb (Fig. 3) did not inhibit binding, but at higher concentrations statistically significant inhibition was noted ($P < 0.0001$). The requirement for a greater concentration of the anti-*H. influenzae* MAb (6G3) than of the anti-CR1 MAb (7G9) to effect the same degree of inhibition may be due to the greater number of P2 outer membrane protein epitopes on the *H. influenzae* cells or a lower relative avidity, compared with the CR1 epitopes on the erythrocytes. Similar inhibition experiments demonstrated comparable results with monkey erythrocytes (data not shown).

The kinetics of HP-associated binding at optimal HP inputs was quite rapid. Maximal binding of the bacteria to human

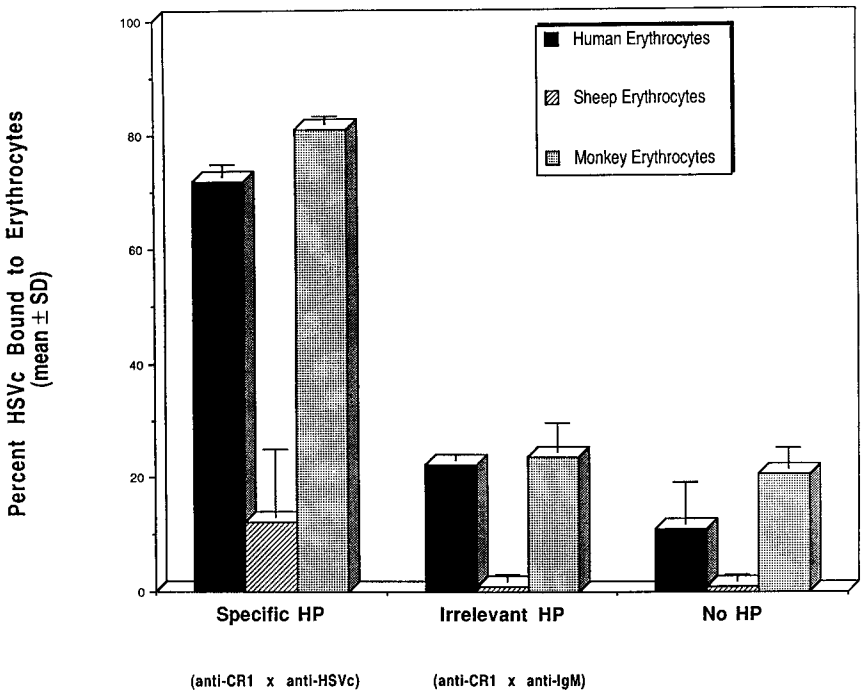

FIG. 5. Percent binding of ^{35}S-labeled HSVc to primate and sheep erythrocytes in the presence of specific HP or irrelevant HP and in the absence of HP. Human or monkey erythrocytes and ^{35}S-labeled HSVc were combined at a ratio of 3:1 (virus/erythrocytes). Aliquots of either a specific anti-HSVc HP, an irrelevant HP, or 1% BSA-PBS were added to this solution and incubated at 37°C. The erythrocytes were washed and pelleted at a speed which pelleted erythrocytes and erythrocyte-bound viral capsids but not free viral particles. The supernatants were counted in a scintillation counter to determine the amount of unbound HSVc. High levels of binding to human and rhesus monkey erythrocytes were seen in the presence of specific HP. Low-level background binding was observed with binding assays utilizing an irrelevant HP or with incubations with sheep erythrocytes (which lack CR1). Anti-IgM, anti-immunoglobulin M. SD, standard deviation.

erythrocytes occurred within 30 s (data not shown). Incubation for longer periods of time showed no significant change in binding.

Binding of *H. influenzae* to human erythrocytes was visually confirmed by transmission electron microscopy (Fig. 4).

HSVc. In the presence of the specific anti-HSVc HP, HSVc demonstrated significant binding to human and monkey erythrocytes ($P = 0.006$) but was not bound in the absence of HP (Fig. 5). When an HP of irrelevant specificity was used, binding was slightly greater than but not statistically different from background ($P = 0.13$) but there was a small increase in nonspecific binding. Binding to sheep erythrocytes, which do not possess CR1, also was not statistically different from background in the presence of either the specific or irrelevant HP ($P = 0.93$). HP-mediated binding of HSVc to human erythrocytes (73.0% ± 14%) was suppressed to background levels (12.0% ± 7.9%; $P = 0.03$) by preincubation of erythrocytes and HSVc with 1 μg of anti-CR1 MAb. There was a trend, although not statistically significant (54.5% ± 0.7%; $P = 0.21$), toward decreased binding when preincubation of viral capsids and erythrocytes was performed with 1 μg of the anti-HSVc MAb.

Binding of HSVc to human erythrocytes is rapid under a variety of conditions. Maximal binding occurred within 1 min at 37°C, and incubation for longer periods did not significantly increase binding (data not shown). Incubation on ice for 5 min or longer resulted in lower (but not statistically different) binding (59% ± 4%).

As with *H. influenzae*, the binding of HSVc to human erythrocytes was confirmed by transmission electron microscopy (Fig. 6).

DISCUSSION

Previous work from one of our laboratories (19, 23–25) has demonstrated binding to erythrocytes and clearance from the primate circulation of a number of soluble proteins utilizing HP to bind these protein antigens via CR1. A first step in extending this work to the clearance of particulate pathogens is to prove that specific HP can bind prototypical bacterial or viral particles to primate erythrocytes in vitro. Results of the present experiments showed specific, rapid, and quantitative binding of *H. influenzae* and HSVc, utilizing the erythrocyte-HP system. These pathogens were chosen as representative prototypes; one of the goals of this research is to make use of the erythrocyte-HP system to clear any given particulate pathogen from the blood. *H. influenzae* and HSVc were chosen for their ready availability and because of our laboratories' familiarity with these prototypes from prior experimentation.

Several experimental protocols confirmed the specificity of binding for both the target antigens and the CR1 receptor: (i) 70 to 90% binding was obtained in the presence of specific anti-target HP; (ii) experiments performed with an irrelevant HP or in the absence of HP showed considerably less or negligible binding; (iii) sheep erythrocytes (which do not possess CR1), when utilized in place of primate erythrocytes, failed to show significant binding, as expected (Fig. 1 and 5). Binding assays performed in the presence of a large excess of competing nonbiotinylated anti-CR1 or anti-target MAb abrogated specific HP-mediated binding (Fig. 2 and 3). Transmission electron microscopy provided a visual confirmation of HP-mediated binding of pathogens to the erythrocyte surface (Fig. 4 and 6).

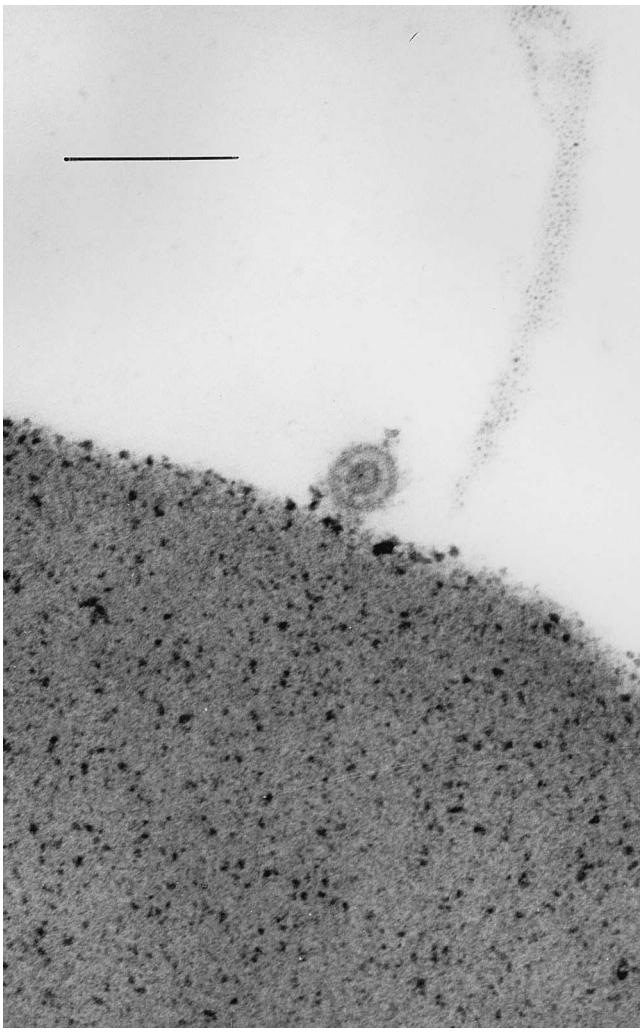

FIG. 6. Transmission electron micrograph showing HSVc bound to the surface of a human erythrocyte via HP. Bar = 0.25 μm.

Evaluation of the kinetics of HP-mediated substrate binding to primate erythrocytes demonstrated maximal binding in less than 1 min. In vivo experiments (19, 24) have shown the average half-life of HP bound to erythrocytes in the circulation to be approximately 5 min. Thus, binding of particulate antigens to erythrocytes via HP can occur rapidly and ultimately allow clearance of the antigens at the same rates that the HP themselves are removed from the erythrocytes and cleared from the circulation.

There is a large body of literature (1, 3, 10, 12, 21) which describes the binding of complement-opsonized multivalent antigen-antibody immune complex to primate erythrocyte CR1 and the subsequent clearance reaction. In view of this, one might expect a single MAb to affect binding and clearance of any target antigen via this complement-mediated mechanism. However, unless it is effective in neutralizing the pathogen directly or is coupled to an effector molecule, a single MAb is often not effective at destroying and/or clearing an antigen from the circulation, because it does not efficiently activate complement and opsonize the antigen at antigen-antibody concentrations likely to be encountered in the circulation (4, 14, 18, 31). However, cross-linking an anti-target MAb with an anti-CR1 MAb in an HP complex achieves significant binding of substrates to erythrocytes at rather low MAb concentrations, which, when normalized per milliliter of blood, correspond to total human body doses of between ≈1 and 10 mg. This is in contrast to several other MAb-based therapies, some of which require a dose in the range of several hundred milligrams (8, 33).

The large numbers of erythrocytes in the circulation should allow the erythrocyte-HP system to facilitate pathogen binding and clearance even at high circulating-pathogen concentrations. In the present experiments, erythrocytes and antigen were incubated at ratios of 1:3 and 500:1. Thus, the erythrocyte-HP system should be able to clear particulate pathogens present in the circulation at concentrations as high as 10^7 or more organisms per ml of blood. Even in clearly septic patients, most pathogens are present at much lower concentrations in the bloodstream (11, 27, 28).

The erythrocyte-HP system, unlike natural IA, does not require the presence and activation of complement. HP-mediated binding to erythrocytes can, therefore, still occur in complement-deficient states, including sepsis (5, 7). As the binding of the target antigen to erythrocytes is mediated by the anti-CR1 component of the HP rather than C3b, the target antigen cannot be released by the action of factor I, as happens in complement-mediated IA (13). Target antigens bound to erythrocytes via HP have been shown to remain stably bound in serum for 2 h in vitro (25), but removal from the erythrocyte in vivo is quite rapid. The erythrocyte CR1 must be present, however, for binding to occur in the erythrocyte-HP system, and in disease states in which there is a decrease in CR1 or a complement deficiency (30), there can be compromise of the erythrocyte-bound immune complex clearance mechanism (2). This potential confounding effect may be overcome by the transfusion of fresh erythrocytes, which first can be primed with HP.

The next step in extending the present experiments is to investigate clearance of a prototypical particulate pathogen in vivo. Unfortunately, the unencapsulated serum-sensitive strain of *H. influenzae* used in these experiments was rapidly killed when injected into the circulation of rhesus monkeys, precluding the measurement of any HP-mediated effects with this strain. The lack of availability of sufficiently large quantities of ^{35}S-labeled HSVc also prevented performance of possible in vivo experiments with the viral capsid. A number of MAb specific to a variety of pathogens, however, are available commercially, and these may prove more suitable for future in vivo experimentation.

The present experiments demonstrate the utility of HP in the binding of particulate pathogens to primate erythrocytes without the requirement for complement. This represents an important step toward the possible therapeutic use of HP in the clearance of bacterial and viral pathogens from the bloodstream. Implicit in our approach is the assumption that clearance of the HP-antigen complexes bound to primate erythrocyte CR1 from the circulation will occur in an analogous fashion to C3b-mediated erythrocyte-bound immune complex clearance in primates. Previous in vivo studies with primates of HP-mediated clearance of injected circulating protein target antigens (dinitrophenyl-bovine gamma globulin [24] and immunoglobulin M [23]) indicate that clearance of HP-bound bacteria and viruses indeed may be possible. Thus, it is feasible to extend this work to the development of a novel adjunctive therapy for bloodborne infectious diseases.

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