Escherichia coli Bound to the Primate Erythrocyte Complement Receptor via Bispecific Monoclonal Antibodies Are Transferred to and Phagocytosed by Human Monocytes in an In Vitro Model

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Escherichia coli Bound to the Primate Erythrocyte Complement Receptor via Bispecific Monoclonal Antibodies Are Transferred to and Phagocytosed by Human Monocytes in an In Vitro Model

Susan E. Kuhn,* Alessandra Nardin,* Philip E. Klebba, † and Ronald P. Taylor 2 *

We have prepared cross-linked, bispecific mAb complexes (heteropolymers) that facilitate rapid and quantitative binding of a prototype pathogen, Escherichia coli, to the complement receptor (CR1) on primate erythrocytes. Incubation of the erythrocyte-heteropolymer-E. coli complexes with freshly isolated human mononuclear cells leads to rapid removal of the E. coli from the erythrocytes, and phagocytosis and killing of the bacteria. The erythrocytes are not lysed or phagocytosed during this transfer reaction, but both heteropolymer and CR1 are removed from the erythrocytes along with the E. coli. These findings parallel observations made in previous in vivo experiments in which heteropolymers were used to facilitate clearance of innocuous prototype pathogens in a monkey model. It should now be possible to extend the heteropolymer paradigm to a live pathogen in a primate model. The Journal of Immunology, 1998, 160: 5088–5097.

In 1953, Nelson proposed an immunologic role for primate erythrocytes (E)3 based on his investigations of the immune adherence reaction (1). He observed that bacteria opsonized with both Abs and complement adhere to E and that this binding leads to enhanced phagocytosis and killing of the micro-organisms. It is now recognized that E binding in this reaction is mediated by complement receptor 1 (CR1), a type I membrane-associated glycoprotein receptor found on primate E and other cells that is specific for C3b/C4b (covalently associated with the opsonized substrates) (2, 3). This enhanced killing of the E-bound and complement-opsonized micro-organisms led Nelson to suggest that E presentation of such bound particulate pathogens could play a role in controlling bacterial infections in the bloodstream. Inherent in this idea is the presumption that once bound to E, particulate pathogens would be more easily taken up and destroyed by appropriate phagocytic cells, which could include neutrophils, monocytes, or fixed tissue macrophages. Studies of the E-immune complex (IC) clearance reaction, initiated 30 yr after Nelson, have extended this reaction to soluble substrates. This work has revealed that a fraction of the soluble Ab-protein Ag IC (nonparticulate) that forms in the circulation can fix complement, bind to E, and then be cleared from the circulation and destroyed in the liver and spleen (3–5).

We have developed an alternative procedure to bind target pathogens (both micro-organisms and protein Ags) to primate E via CR1 with a very high level of efficiency in the complete absence of complement (6–9). The method is based on using bispecific mAb complexes that are constructed by cross-linking a mAb specific for CR1 (which serves as a surrogate for C3b) with a mAb specific for the target pathogen. Based on Nelson’s original work and the more widely studied E-based IC clearance phenomenon, we have proposed that these bispecific complexes (heteropolymers (HP); anti-CR1 mAb × anti-pathogen mAb) have the potential to bind both soluble and particulate pathogens to E in the bloodstream and then to present the pathogens to acceptor cells for phagocytosis and destruction. In fact, our in vivo experiments in monkey models have verified that once bound to E CR1 via specific HP, both soluble proteins and a prototype virus are cleared from the circulation and destroyed in the liver by a mechanism quite similar, in many respects, to the E-IC clearance reaction (8, 10, 11).

We have now extended our examination of the HP paradigm to an in vitro model, similar to that examined by Nelson, which in this case focuses on E. coli as a model particulate pathogen. We have used specific HP to bind E. coli to primate E, and we have studied the transfer of this E-bound substrate to human monocytes. The results of these studies, performed in the absence of complement, indicate that Escherichia coli bound to E CR1 via HP are indeed phagocytosed and destroyed by human monocytes. We also demonstrate that this transfer reaction, which includes the concomitant loss of E CR1, shows a striking similarity to the in vivo reaction by which substrates bound to E CR1 are cleared from the circulation in primates.

Materials and Methods

Mouse mAbs and HP

mAb 7G9 (IgG2a) and mAb 44 (IgG2b), respectively specific for CR1 and E. coli, have been reported (8, 10, 12–14). Other mAbs used and their specificities are as follows: HB8592 (IgG1, anti-CR1) (15), 7B7 (IgG2a, 2

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3 Abbreviations used in this paper: E, erythrocytes; CR1, the primate E complement receptor; IC, immune complex; HP, heteropolymers; FcR, Fc receptor; WBC, white blood cells (mononuclear cells); MESF, molecules of equivalent soluble fluorochrome; GPI, glycoporphaphatidylinositol.

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irrelevant specificity) (11), IV.3 (IgG2b, anti-FcRII, Medarex, Annandale, NJ). HP were formed by covalently cross-linking mAb 7G9 or mAb HB8592 with mAb 44 via a thioether bond as previously described (11, 16) and purified by fast protein liquid chromatography. The subfractions corresponding to dimers through tetramers were used in all experiments.

E. coli

The BN1071 strain of E. coli, previously shown to bind mAb 44 due to a high level of expression of the iron-binding protein Fep A (12, 13), was used in all experiments. In certain studies the bacteria were covalently labeled with either FITC (17) (isomer I, Sigma, St. Louis, MO; final concentration of 0.01 mg/ml) or biotin N-hydroxysuccinimide (long arm, Vector Laboratories, Burlingame, CA; final concentration, 5–30 μg/ml). After three washes, the labeled bacteria were stored at 4°C in PBS containing 1% glucose.

Cells

E were obtained from rhesus and stump-tailed macaque monkeys or from normal human donors. Except where explicitly noted (e.g., Fig. 4 and Table IV), monkey E were used in all the experiments. The blood was stored as a 25% dispersion in Alsever’s solution and washed three times in PBS with 1% BSA (BSA/PBS) before use. Mononuclear cells (designated white blood cells (WBC)) were isolated from fresh human blood drawn into EDTA using Ficoll-Hypaque Plus (Pharmacia Biotech, Uppsala, Sweden) (19). They were washed twice in HBSS, diluted to 1 x 10^6 cells/ml in RPMI 1640, stored on ice, and used within 4 h. The WBC preparations generally contained 15 to 20% monocytes as determined by light scattering. Both aliquots were then incubated for 2 to 20 min at 37°C with end-over-end shaking.

The E and WBC were separated using a Percoll gradient. The subfractions corresponding to a ratio of E:monocyte of 1:1 to 10:1 were collected, eluted from Percoll, concentrated to 10^8 E in BSA/PBS (thus, E were in excess), and then an aliquot of HP (mAb 7G9 x mAb 44) was added. Following a 1- to 30-min incubation at 37°C, the controls were centrifuged at 4500 rpm for 20 min to pellet all E. coli. The supernatants were substituted for nonspecific loss of E. coli from E due to the processing steps. E-HP-E. coli complexes were simply mixed with RPMI (no WBC) but otherwise processed in the same fashion.

To test for nonspecific loss of E. coli from E due to the processing steps, E-HP-E. coli complexes were mixed with RPMI (no WBC) but otherwise processed in the same fashion.

The fate of the HP and monkey E CR1 were also examined during the course of the transfer reaction. Biotinylated E. coli were substituted for FITC-labeled E. coli, and following transfer and cell separation, the E were independently probed with FITC-labeled anti-CR1 mAb HB8592. FITC-labeled goat anti-mouse IgG (to probe for HP), or FITC-avidin (to probe for biotinylated E. coli). mAb HB8592 was selected because binding of this mAb to monkey E is the same for both naive and HP (mAb 7G9 x mAb 44)-opsonized E (21). A similar approach was used to analyze human E for loss of CR1 after the transfer reaction. However, due to inhibition of binding of mAb HB8592 to human E-HP-E. coli complexes if the mAb 7G9 x mAb 44 HP was used (not observed with the monkey E), in this case the HP were prepared with anti-CR1 mAb HB8592 and mAb 44, and CR1 was measured by probing with FITC-labeled anti-CR1 mAb 7G9. No inhibition of binding of mAb 7G9 was evident for this combination of reagents.

FACS analysis was conducted on a Becton Dickinson FACScan using CellQuest software. Monocytes were selected from the WBC population using light scattering. During the course of each experiment, FITC standards (Quantum 26 Beads, Flow Cytometry Standards, San Juan, Puerto Rico) were run with the samples, allowing the mean fluorescence data to be normalized to molecules of equivalent soluble fluorochrome (MESF).

Internalization of E. coli by WBC

Transfer was conducted with E-HP-biotinylated-E. coli complexes incubated with WBC. After the separation of E and WBC, the WBC samples were divided into two aliquots; one aliquot was held on ice, while the other was permeabilized using Becton Dickinson FACS Permeabilizing Solution. Both aliquots were then probed simultaneously with Texas Red-avidin (Biomeda, Foster City, CA) and FITC-anti-CD14 (Becton Dickinson), washed multiple times in PBS, and fixed with paraformaldehyde. Slides were prepared for fluorescence microscopy using an antifading agent (Fluoroguard, Bio-Rad, Hercules, CA), and the cells were counted and photographed on an Olympus BX60 microscope at x1000 magnification. The percent positive cells is defined as the number of Texas Red-positive cells (with bound biotinylated E. coli) per 100 FITC-positive (CD14-positive) cells. The phagocytic index refers to the number of biotinylated E. coli bound per 100 CD14-positive cells. Typically, 200 CD14-positive cells were counted per experimental determination.

Inhibition of transfer

The WBC were treated with a variety of reagents in an attempt to block transfer of E. coli from E to monocytes. For example, WBC were preincubated with 40 μg/ml mAb IV.3 (anti-FcRII), 1 mg/ml mAb 7G9 (anti-CR1), or 1 mg/ml mAb 7B7 (isotype-matched control) for 4 h on ice to block monocyte receptors. Protease activity was inhibited by incubating WBC in buffer containing either a protease inhibitor mixture (Sigma General Use Protease Inhibitor Mixture; broad specificity against serine, cysteine, aspartic, and metalloproteases, augmented with pepstatin A) or 10 mM EDTA for 30 min on ice. The importance of Ca^{2+} was tested by including 10 mM Ca^{2+} in one of the EDTA samples (22). Following these incubations, opsonized E were added directly to the incubation mixture, and a transfer experiment was performed as described above.

The potential role of various monocyte cellular processes in transfer was examined by incubating the WBC in 5 μg/ml cytochalasin D (23), 10 μM colchicine (24), 10 mM EDTA, 200 μM genistein (25), 50 μM spinogosine (23), or 0.1 μM staurosporine (23) (all purchased from Sigma) for 30 min at 37°C. Following these incubations, opsonized E were added directly to the incubation mixture, and an internalization experiment was performed as described above. The concentration of inhibitor was maintained throughout all washes.

Monocyte-mediated killing of E. coli bound to E

E. coli were bound to E via HP (the E were washed twice after binding) or were simply mixed with E in solution (and not washed) in E. coli:E ratios varying between 1:1 and 1:100. These samples were then added to WBC, with final E. coli:monocyte ratios ranging from 1:1 to 1:50, and incubated for 1 h at 37°C. Following the incubation, the samples were centrifuged for 15 min at 4500 rpm to pellet all E. coli and cells. The supernatants were removed, and the pelleted cells were lysed with distilled water. CFU assays were performed on multiple dilutions of the lysates.
**Results**

**E binding of E. coli via HP**

HP specific for E CR1 and E. coli were prepared and tested for their ability to facilitate binding of E. coli to E. After a slow centrifugation step to pellet the E, binding was examined by measuring CFU in the pellets and supernatants. The results in Table I demonstrate that the HP (anti-CR1 mAb 7G9 × anti-E. coli mAb 44) was able to promote rapid and efficient binding of bacteria to E; typically 2 log units or more of E. coli were removed from solution and bound to the surface of the E in 15 min, with substantial binding in as little as 2 min. Virtually all specific binding was eliminated in the absence of HP or in the presence of excess mAb 7G9 or mAb 44, proving that binding was HP mediated and specific for both CR1 and E. coli (Fig. 1). In the absence of E, there was a small amount of E. coli precipitation, possibly due to complexes formed by cross-linking. FACS analysis confirmed that the HP do mediate binding of FITC-labeled E. coli to both monkey and human E (Figs. 2 and 4).

**Transfer of E-bound E. coli to monocytes**

E with HP-bound FITC-labeled E. coli were incubated at 37°C with freshly isolated WBC at an E. coli:E:monocyte ratio (assuming that 20% of the WBC are monocytes) of 0.75:1:1. Following incubation and separation of cells, FACS analysis demonstrated that the E had been partially depleted of bound E. coli, while a subset of the monocytes had taken up the fluorescent bacteria (Fig. 2). The rate of the reaction was quite rapid at 37°C, and the majority of transfer occurred within 5 min (Fig. 3 and Table II). When the transfer reaction was conducted on ice instead of at 37°C, loss of E. coli from E was only partially inhibited, with loss

### Table I. HP mediated binding of E. coli to E

<table>
<thead>
<tr>
<th>Expt</th>
<th>E:E. coli Ratio</th>
<th>Incubation Conditions</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>166:1</td>
<td>2 min, 37°C</td>
<td>88.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>166:1</td>
<td>5 min, 37°C</td>
<td>98.1 ± 0.1</td>
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<tr>
<td></td>
<td>166:1</td>
<td>10 min, 37°C</td>
<td>98.6 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>50:1</td>
<td>15 min, 37°C</td>
<td>99.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>500:1</td>
<td>15 min, 37°C</td>
<td>99.5 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td>20:1</td>
<td>15 min, 37°C</td>
<td>99.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>200:1</td>
<td>15 min, 37°C</td>
<td>91.2 ± 2.9</td>
</tr>
</tbody>
</table>

*E were incubated with HP and E. coli at a variety of E:E. coli ratios. Following the incubation, the samples were centrifuged to pellet the E, but not free E. coli, and the CFU remaining in the supernatant were measured. The percent E. coli bound (and SD) mediated by HP was calculated by comparing samples with HP to controls in which no HP was added. Nonspecific binding in the absence of HP averaged 10 to 30%.*

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#### FIGURE 1. Specificity of HP-mediated binding of E. coli to monkey E.

E were incubated with E. coli in the absence of HP or in the presence of both HP and an excess of one of its component parts (mAb 7G9 or mAb 44). E were omitted to test for precipitation of the bacteria. Following a 15-min incubation at 37°C, the E were pelleted through a slow centrifugation step, CFU in the pellets and supernatants were measured, and the percentage of E. coli bound was calculated.

#### FIGURE 2. Transfer of E-bound E. coli to human monocytes.

E. coli were bound to E via HP and then the opsonized E were incubated for 5 min at 37°C with WBC. Following the incubation, the E and WBC were separated by a Percoll density gradient and analyzed by FACS. Control samples (bold) were prepared as follows. E-HP-E. coli complexes or WBC were respectively incubated with buffer alone, but otherwise underwent all processing steps. The percentage of cells that are FACS positive (mean fluorescence >15 units) is displayed. A and B represent two independent experiments. Calculations for E of Expt. A: MESF values are 321 for naive E, 1,950 for the control sample (mock transfer), and 730 for the posttransfer sample. The percent loss in E. coli-associated MESF = (1,950 - 730)/(1,950 - 321) = 75%. The original (unprocessed) E-HP-E. coli sample was also 37% FACS positive and had a MESF of 1830. The respective values for E in Expt. B are 426 (naive), 41,889 (control), and 5,753 (posttransfer), thus giving a net loss in E. coli-associated MESF of 87%. MESF values for the monocytes in parts A and B are, respectively, 4,300 and 4,573 for naive cells and 7,537 and 22,350 for the posttransfer sample.
of bacteria from the E after 5 min on ice approximately half the loss observed at 37°C (MESF values: 6049 down to 3722 compared with 7341 down to 2575; Table II). However, uptake of *E. coli* by monocytes on ice was blocked considerably.

Control experiments were performed in which free FITC-labeled *E. coli*, E, and WBC were mixed in the above ratio (no HP added), and negligible binding of *E. coli* to monocytes was observed (results not shown). To ensure that monocyte uptake of FITC-labeled *E. coli* was specifically facilitated by HP-mediated E binding, further control experiments were performed by substituting either mAb 44 or an irrelevant HP (mAb 7G9 × mAb 7B7) for the *E. coli*-specific HP. Monocyte uptake remained at background levels after an incubation of 5 min at 37°C (results not shown).

We also conducted a number of experiments in which *E. coli* were first bound to human E via HP (mAb 7G9 × mAb 44, as with monkey E), and then transfer to autologous monocytes was studied. The results (Fig. 4) demonstrate robust transfer in this system as well. We also determined that direct opsonization of *E. coli* with HP in the absence of E led to a very high level of binding of the bacteria to monocytes (Fig. 4) after a 20-min incubation at 37°C. This high level of direct binding is quite reasonable. The input ratio of HP-opsonized *E. coli* to WBC for this particular system was approximately 3 times higher than the input ratio when E-HP-*E. coli* complexes were incubated with WBC because only 30 to 40% of the *E. coli* bound to E (see Materials and Methods).

Spontaneous release of *E. coli* bound to E via HP was quite low. Monkey and human E samples containing HP-bound *E. coli* were processed in the control experiments in an identical protocol, except WBC were omitted from the incubation mixtures and retained >90% of bound *E. coli*, as defined by both FACS-positive cells and mean fluorescence intensities.

A number of in vivo and in vitro studies have demonstrated that E opsonized with IgG Abs bound exclusively to CR1 are not lysed or phagocytosed, even when IC bound to the E are transferred to phagocytes (3, 4, 19, 21, 26). To ensure that there was no E loss in the present system, following incubation of the E-HP-*E. coli* complexes with WBC, samples were washed and then subjected to conditions that lysed the E while leaving the monocytes intact (19). After a centrifugation step, the OD540 of the resulting supernatants were measured to determine the original E concentrations. Samples incubated with WBC yielded absorbances equal to those incubated in medium alone (WBC omitted), confirming that no loss of E occurred during the HP-mediated transfer (results not shown).

When CR1 and HP levels on the E were measured before and after transfer, FACS analyses indicated that, along with the transfer of the majority of HP-bound *E. coli* from the E, the majority of both HP and E CR1 were also removed (Fig. 5). E with HP alone also sustained losses of the majority of their HP and CR1, although the decreases were not as great as those seen for E with HP-*E. coli* complexes. These results are consistent with other studies that suggest that a key step in the transfer reaction includes proteolysis of E CR1 by a protease presumably associated with the acceptor cell, a step that would lead to release of the HP-*E. coli*-CR1 complex to the phagocyte (8, 21, 26, 27). Interestingly, even naive E lost approximately one-third of their CR1 upon incubation with WBC, indicating that for the monkey E/human monocyte system, the putative protease-like activity of the monocytes may be constitutive, although this activity is enhanced in the presence of immune complexes bound to E CR1. Birmingham et al. reported that E CR1 of baboons and cynomolgus monkeys is membrane bound in a glycoprophosphatidylinositol (GPI) linkage (28). We found that E CR1 of both the rhesus and stump-tailed macaques (but not human)
used in the present study has the same anchor (results not shown). Therefore, we attempted to block the transfer reaction in the monkey E system with phospholipase C inhibitors such as neomycin sulfate or p-chloromercuriphenylsulfonic acid. Neomycin sulfate (10 mM) had no effect on the transfer reaction or on the CR1 loss, and p-chloromercuriphenylsulfonic acid caused E lysis (results not shown).

We also examined human E-HP-E. coli complexes (mixed with autologous WBC) for loss of E. coli, HP, and CR1 after the transfer reaction. HP were prepared with anti-CR1 mAb HB8592, and CR1 was assayed with FITC-labeled anti-CR1 mAb 7G9 (see Materials and Methods). The results of two independent studies (two different blood donors; in all cases results are compared with the mock transfer; no WBC condition) are as follows: loss of E. coli, 68 ± 4%; loss of HP, 48 ± 2%; and loss of CR1, 13 ± 1%. On the average, less than one E. coli was bound per E (see Materials and Methods).
and Methods), and thus a large fraction of total E CR1 could not have been directly involved in binding the bacteria. In addition, only 68% of the bacteria were transferred, and therefore, the relatively small decrease in E CR1 is quite reasonable. Finally, control experiments indicated that there was no constitutive loss of E CR1 when naive human E were incubated with autologous WBC.

Internalization and killing of E. coli

For HP-mediated clearance to be useful therapeutically, HP-pathogen complexes must be removed from E by phagocytes and then internalized and degraded. To assay for internalization, HP-mediated transfer was conducted with biotinylated E. coli, and both permeabilized and nonpermeabilized aliquots of the WBC were probed with Texas Red-avidin. As demonstrated in the fluorescence micrographs in Figure 6, monocytes with intact membranes have very few bound E. coli. However, in comparable permeabilized samples it is evident that the majority of the monocytes have internalized substantial amounts of biotinylated E. coli, with some monocytes containing as many as 10 bacteria. Virtually all monocyte-associated bacteria were found to be internalized after both 5 and 15 min of transfer (Table III).

The question of bacterial killing was addressed by measuring CFU in samples incubated with and without WBC for 1 h at 37°C. Both WBC and E were lysed following the incubation, allowing all live E. coli, internal or external, to be counted (Table IV). In experiments without HP, WBC did show a limited ability to kill free E. coli; however, in all cases bacteria bound to E via HP were killed to a greater extent. Specific killing of approximately 1 log unit or more is evident over a wide range of E. coli to monocyte ratios. The killing capabilities of monocytes varied from donor to donor, but HP consistently augmented killing. To test whether the increased killing was the result of immune complex formation alone, killing was assayed for E. coli opsonized with mAb 44 and mixed with E and WBC (Table IV, Expt. 2). Killing in this system occurred at an intermediate level, between that of free E. coli and that of HP-opsonized E-bound E. coli, indicating that although specific (mAb 44) IgG binding facilitates bacterial killing, the E-HP system enhances it further.

Blockade of transfer and internalization

To determine which monocyte receptors might be instrumental in transfer, monocytes were preincubated with saturating amounts of HP before transfer and then incubated with E. coli. The results presented in Figure 7A indicate that mAbs respectively specific for

| Table III. Internalization of E. coli by human monocytes during the transfer reaction* |
|---|---|---|---|
| Expt. | Incubation Conditions | Permeabilized | Nonpermeabilized |
| | | Phagocytic index | % positive | Phagocytic index | % positive |
| 1 | 15 min, 37°C | 68 ± 7 | 158 ± 15 | 15 ± 6 | 26 ± 12 |
| 2 | 15 min, 37°C | 73 ± 7 | 257 ± 6 | 24 ± 15 | 30 ± 19 |
| 3 | 5 min, 37°C | 64 ± 0 | 132 ± 11 | 11 ± 4 | 13 ± 6 |
| 4 | 15 min, 37°C | 83 ± 4 | 190 ± 1 | 11 ± 2 | 15 ± 5 |

*Quantification for experiments such as those illustrated in Figure 6. Means and SD are reported for both percent positive and phagocytic index.

| Table IV. Monocyte killing of E. coli* |
|---|---|---|---|
| Expt. | E. coli E: Monocyte Ratio | E Condition | % Killing |
| 1 | 1:1:2 | E. coli | 28 ± 0.5 |
| 2 | 1:2:0.8 | E. coli, HP | 95 ± 0.5 |
| 3 | 1:100:50 | E. coli | 0 ± 3 |
| 4 | 1:100:50 | E. coli, mAb 44 | 30 ± 1 |
| 5 (human E) | 1:100:50 | E. coli, HP | 53 ± 1 |
| 6 | 1:100:50 | E. coli, HP | 25.2 ± 0.2 |
| 7 | 1:100:50 | E. coli, HP | 98 ± 0.5 |
| 8 | 1:100:50 | E. coli, HP | 33 ± 2 |
| 9 | 1:100:50 | E. coli, HP | 89 ± 3 |
| 10 | 1:100:50 | E. coli, HP | 27 ± 3 |
| 11 | 1:100:50 | E. coli, HP | 76 ± 5 |

*E. coli were bound to E via HP or simply mixed with E and then incubated with WBC for 1 h at 37°C. After a pelleting step the cells were lysed with distilled water, and CFU were measured. Control samples not incubated with WBC determined the baseline for killing.
partially blocked release of common protease inhibitors available. Incubation with EDTA only involved in the transfer reaction, it is clearly not inhibited by the uptake (Fig. 7). The results indicate that if indeed a protease is blocked in this case. For both removal from E and uptake by E. coli (7B7) only weakly blocked monocyte-mediated removal of FcRII (IV.3), CR1 (7G9), or an isotype-matched control for 7G9, and EDTA, and n = 2 for IV.3/7B7/7G9, protease inhibitor, and EDTA plus Ca^{2+}.

FcRII (IV.3), CR1 (7G9), or an isotype-matched control for 7G9 (7B7) only weakly blocked monocyte-mediated removal of E. coli from E. However, these reagents were far more effective in blocking uptake by the monocytes of putatively released E. coli (Fig. 7B). This effect was most evident when a mixture of all three mAbs for 4 h on ice or with a protease inhibitor mixture, EDTA, or EDTA plus Ca^{2+} for 30 min on ice. Control samples were preincubated in RPMI. E-HP-FITC-E. coli complexes were added, and transfer was allowed to occur for 5 min at 37°C. The cell populations were then separated, and E (A) and monocytes (B) were analyzed by FACS. Values represent the mean ± SD, with n = 4 for IV.3, 7B7, 7G9, and EDTA, and n = 2 for IV.3/7B7/7G9, protease inhibitor, and EDTA plus Ca^{2+}.

We also sought to demonstrate directly the importance of a protease by using a mixture of protease inhibitors to block transfer and uptake (Fig. 7). The results indicate that if indeed a protease is involved in the transfer reaction, it is clearly not inhibited by the common protease inhibitors available. Incubation with EDTA only partially blocked release of E. coli from E, but it totally abrogated uptake of the bacteria by monocytes (Fig. 7). However, when Ca^{2+} was restored to the system, transfer resumed near control levels, suggesting that EDTA may inhibit Ca^{2+} signaling, rather than inhibiting a metalloprotease. To test for the secretion of a protease-like factor resistant to the inhibitors used, supernatants were collected from the monocyte/E mixture after 5 min of transfer. These supernatants were then added to suspensions of fresh E-HP-E. coli complexes and allowed to incubate for 5 min at 37°C. No release of E. coli occurred (results not shown).

To ascertain which monocyte cellular processes might be important in the removal of E. coli from E or the subsequent uptake and internalization of the bacteria by the phagocyte, the monocytes were incubated with a variety of metabolic inhibitors and then used in the transfer reaction (Table V). Only EDTA and cytochalasin D (an inhibitor of actin polymerization) (29) had significant inhibitory effects on the removal of bacteria from the E. However, use of genistein (a tyrosine kinase inhibitor) (30) and staurosporine (a protein kinase C inhibitor) (31) along with EDTA and cytochalasin D consistently led to lower numbers of E. coli being taken up and internalized by monocytes, as indicated by the decreases in both the percentage of positive cells and phagocytic indexes in the permeabilized samples (Table V). With EDTA and staurosporine, the reduced number of E. coli internalized appears to be simply a natural consequence of having fewer E. coli bound to the monocytes and available for internalization. The results for the use of cytochalasin D, and to a lesser extent genistein, are more complex. The relatively high level of positive cells in the nonpermeabilized samples suggest that these reagents may block internalization as well as uptake. Compared with the control samples in which approximately 95% of the E. coli associated with the monocytes were internalized, only 61% were internalized when incubated in the presence of genistein (95-37)/95 (based on phagocytic indices (Table V)), and cytochalasin completely blocked internalization. In the studies with cytochalasin D (Table V), the slightly higher number of E. coli (56) bound to the nonpermeabilized cells compared with the permeabilized cells (42) is probably due to a small error in counting. Colchecine (an inhibitor of microtubule assembly) (32) and sphingosine (another inhibitor of protein kinase C) (33) have no effect on transfer or internalization.

**Discussion**

HP have shown promise as potential therapeutic agents for the clearance of blood-borne pathogens from the circulation. In in vivo experiments, HP were able to clear from the circulation up to 6 log units of a circulating prototype innocuous target pathogen (11). Current work is focused on extending the HP approach to biologically relevant bacterial and viral pathogens. In the in vitro model presented herein, we demonstrate that specific HP-mediated E binding of E. coli occurs quite efficiently and rapidly (Table I and Fig. 1), and such binding can then promote the transfer (Figs. 2–4 and Table II), internalization (Fig. 6 and Table III), and killing (Table IV) of this bacterium by human monocytes. In this respect our findings reinforce and extend the original description of the biologic role of immune adherence in host defense first described by Nelson (1). However, as opposed to classic immune adherence, the reactions mediated by HP are accomplished in the absence of complement. The HP complex, rather than C3b, insures binding of the IC to E via CR1; once bound, the HP-Ag IC is recognized and processed by human monocytes. Our recent in vivo studies of HP-mediated clearance of target substrates also suggest that IC bound to E CR1 via HP are removed from the circulation by a mechanism quite similar to the process by which complement-opsonized immune complex substrates bound to E are cleared to the liver (8, 10, 11, 21).

Uptake of HP-opsonized E. coli by monocytes was greater than that observed when the bacteria were first bound to E via HP,
findings are therefore consistent with a proteolytic step during E. coli in all cases loss of CR1 from monkey E after transfer of HP-bound be due to a mismatch between the monkey E and human mono-
coli human monocytes. Due to this background constitutive activity, significant, loss in CR1 (Fig. 5) when naive E were incubated with
in vitro model for monkey E. CR1 is lost during transfer of either
during the course of transfer, releasing the CR1-HP-Ag complex
in very large excess over bacteria in the bloodstream.

With respect to both the in vitro and in vivo transfer reaction,
there are several, presumably interrelated and possibly sequential,
steps that must take place for the monocytes to destroy pathogens
bound to E via HP. The HP-Ag complex must be released from the
E, and then it must be bound, internalized, and degraded by the
monocyte. These steps would in aggregate constitute the complete
transfer reaction. Since no E sequestration or phagocytosis is seen
in either the in vivo clearance studies (3, 4, 11, 21, 26) or in the
present in vitro model, a critical step in transfer most likely in-
volves monocyte-mediated release of the HP-Ag complex from E
either before or shortly after it binds to the phagocyte. In previous
in vivo clearance studies in monkeys using similar model com-
plexes, we demonstrated that as IgG-containing IC were removed
from E, there was a concerted loss in E CR1 (8, 21). In the present
in vitro model system, a small (13%) but significant loss of CR1
from human E was demonstrable after transfer of E. coli (initially
bound to the E via HP) to monocytes. Loss of CR1 has also been
observed in conjunction with in vivo clearance of complement-
opsonized IC in both human and primate models, and low CR1
levels are a characteristic of several diseases involving chronic
opsonized IC in both human and primate models, and low CR1
observed in conjunction with in vivo clearance of complement-
mediated clearance of bacteria under conditions in which the E are
in very large excess over bacteria in the bloodstream.

We anticipate that a similar pathway would be followed for HP-
mediated clearance of bacteria under conditions in which the E are
mediated by a protease that is not susceptible to common
protease inhibitors. Certain metalloproteases that mediate shedding
of several surface proteins (including TNF-α and IL-6R) are sim-
ilarly difficult to inhibit, responding only to specifically engineered
protease inhibitors (41, 42). There is evidence for a similar pro-
etlytic cleavage, in cells other than E, of specific membrane gly-
coproteins that share structural characteristics (the short consensus
repeat motif) with CR1 (43–45). For example, Kazatchkine’s
monocyte-as-
associated protease will be a focal point of future research. No
inhibition of transfer was seen in the presence of a protease inhibitor
mixture (Fig. 7), and supernatants collected from transfer mixtures
did not release HP-bound E. coli from fresh E-HP- E. coli samples.
These results suggest that proteolysis may occur in a solution-
inaccessible pocket created by tight cell-cell interactions or that it
may be mediated by a protease that is not susceptible to common
protease inhibitors. Certain metalloproteases that mediate shedding
of several surface proteins (including TNF-α and IL-6R) are sim-
larly difficult to inhibit, responding only to specifically engineered
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The isolation and characterization of the putative monocyte-as-
associated protease will be a focal point of future research. No
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The blocking experiments (Fig. 7) indicate that the role of
monocyte receptors in distinct steps in the transfer reaction may be
complex. Several different approaches led to the blockade of the
majority of the net (complete) transfer to monocytes. However,
there were marked quantitative differences in the effects of inhibi-
tion protocols on the loss of bacteria by the E compared with the
uptake of bacteria by the monocytes. Whether inhibition was ac-
complished by performing the incubation on ice, blocking the
monocyte receptors, or chelating divalent cations, removal of
HP-Ag complexes from the E was inhibited to a lesser extent than
monocyte uptake (Figs. 3 and 7). That is, incubation with WBC led
to robust release of substrate from E, and it was difficult to sub-
stantially inhibit this step. However, the same inhibition protocols
proved to be far more effective in blocking uptake by the mon-
ocytes of the released complexes, which as a consequence of block-
ade were presumably released into the supernatant. These differ-
ences are consistent with the earlier CR1 loss data (Fig. 5) in
implying that removal of substrate from E and subsequent mono-
ocyte uptake are not necessarily linked. Monocytes may have the

### Table V. Inhibition of transfer and internalization by metabolic inhibitors

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment</th>
<th>% positive</th>
<th>Phagocytic index</th>
<th>% positive</th>
<th>Phagocytic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer</td>
<td>87 ± 3</td>
<td>368 ± 30</td>
<td>10 ± 4</td>
<td>12 ± 6</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin D</td>
<td>32 ± 14</td>
<td>42 ± 20</td>
<td>39 ± 3</td>
<td>56 ± 2</td>
</tr>
<tr>
<td></td>
<td>Colchicine</td>
<td>89 ± 7</td>
<td>348 ± 14</td>
<td>8 ± 0</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>33 ± 1</td>
<td>49 ± 7</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>Buffer</td>
<td>76 ± 4</td>
<td>273 ± 37</td>
<td>13 ± 1</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>44 ± 4</td>
<td>95 ± 1</td>
<td>30 ± 12</td>
<td>37 ± 17</td>
</tr>
<tr>
<td></td>
<td>Sphingosine</td>
<td>74 ± 6</td>
<td>256 ± 16</td>
<td>18 ± 2</td>
<td>36 ± 12</td>
</tr>
<tr>
<td></td>
<td>Staurosporine</td>
<td>51 ± 5</td>
<td>167 ± 11</td>
<td>12 ± 0</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

* WBC were preincubated with a variety of metabolic inhibitors for 30 min at 37°C and were then mixed with E-HP-E. coli complexes. Transfer was allowed to occur for 15 min at 37°C. After separation from the E, the WBC were divided into two aliquots, one of which was permeabilized and both of which were double stained with Texas Red-Avidin and FITC-anti-CD14. Data are representative of multiple experiments. By FACS analysis, all treatments led to >90% of control removal of E. coli from E with the exception of the use of cytochalasin D and EDTA, which gave 60% and 75% of control, respectively.
ability to release HP-Ag complexes in a first step that may be independent of the second phase of the transfer reaction.

Binding and internalization of substrate by monocytes appear to take place in a concerted fashion; aside from the studies with cytochalasin D and genistein, virtually all monocyte-associated E. coli were found inside the cell, even after short incubations (Fig. 6 and Table III). The receptors that facilitate these processes must be delineated. The findings in the inhibition studies (Fig. 7B and Table V) suggest that FcR participate in the binding and uptake of HP-Ag IC. On the other hand, in Emlen’s earlier in vitro IC transfer studies, he found monocyte CR1 to be the most important receptor in facilitating transfer of IC from E (46). This difference is probably attributable to differences in the nature of the substrates studied. The IC examined by Emlen were highly opsonized with complement (C3b), whereas the IC used in the present studies were formed in the absence of complement, but obviously may be able to bind to monocyte associated CR1 via the anti-CR1 mAbs in the HP (if they are not specifically engaged on E CR1). It is, therefore, likely that both FcR and CR1 play a role in the transfer reaction, with the influence of the individual receptors being to some extent proportional to the distribution of ligands on the transferred complex. Several groups have studied the differential requirements for CR- and FcR-mediated internalization and have found that the two have distinct mechanisms (23, 47). In inhibition studies, Newman demonstrated that while both CR1 and FcR require intact actin filaments and active protein kinase C for phagocytosis, only CR1-mediated internalization requires microtubule polymerization (23). Internalization in our in vitro model is consistent with FcR-mediated phagocytosis; it is sensitive to inhibitors of microfilament formation and tyrosine phosphorylation, the latter being a required signaling step in FcR-mediated internalization (48), but does not appear to require intact microtubules (Table V). The data using staurosporine and sphingosine to inhibit protein kinase C were inconclusive.

CR3 on monocytes is known to bind unopsonized E. coli via LPS in an EDTA-inhibitable and temperature-sensitive fashion (49). At 37°C in the absence of HP, E. coli appear to be only weakly taken up by monocytes (Fig. 3 and Table II), and therefore it seems unlikely that CR3 alone can facilitate the complete transfer reaction. However, EDTA partially blocks removal of E. coli from E and almost completely inhibits monocyte uptake of E. coli previously bound to E via HP (Table V and Fig. 7). These results suggest that CR3 may play a role in transfer, possibly cooperating with FcR in facilitating binding and phagocytosis of E. coli.

As noted above, the results suggest that monocytes express a protease that can cleave E CR1 when the cells are in direct contact. Such close and sustained contact between naive E and monocytes (or fixed tissue macrophages) would be highly unlikely in the bloodstream. In the liver, the close proximity of E containing bound IC and fixed tissue macrophages may facilitate specific FcR-ligand interactions between the macrophages and substrates bound to E CR1. These interactions could promote sustained contact and allow for the proteolysis of E CR1 and release and uptake of the bound substrates. We note that all studies reported herein were performed on freshly isolated, undifferentiated monocytes; whether the details of the transfer reaction to more differentiated cells are comparable remains to be established.

The results of these in vitro studies reinforce and extend earlier in vivo findings, demonstrating that pathogens bound to E CR1 via HP are rapidly transferred to phagocytes, and then they are internalized and destroyed. These experiments, therefore, provide additional evidence for the potential of HP as a therapy for diseases mediated by blood-borne pathogens.

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References

BISPECIFIC Abs FACILITATE PHAGOCYTOSIS OF E. coli


