Published in final edited form as:

Int J Antimicrob Agents. 2012 July ; 40(1): 75–79. doi:10.1016/j.ijantimicag.2012.03.007.

Human β -defensin-3 alters, but does not inhibit, the binding of *Porphyromonas gingivalis* haemagglutinin B to the surface of human dendritic cells

Jonathan R. Van Hemert^a, Erica N. Recker^a, Deborah Dietrich^a, Ann Progulske-Fox^b, Zoya B. Kurago^c, Katherine S. Walters^d, Joseph E. Cavanaugh^e, and Kim A. Brogden^{f,*}

^aDows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA 52242, USA

^bCenter for Molecular Microbiology and Department of Oral Biology, University of Florida, Gainesville, FL 32610, USA

^cDepartment of Oral & Maxillofacial Pathology, Radiology and Medicine, New York University College of Dentistry, New York, NY 10010, USA

^dCentral Microscopy Research Facility, The University of Iowa, Iowa City, IA 52242, USA

^eDepartment of Biostatistics, College of Public Health, The University of Iowa, Iowa City, IA 52242, USA

^fDepartment of Periodontics and Dows Institute for Dental Research, N447 DSB, College of Dentistry, 801 Newton Road, The University of Iowa, Iowa City, IA 52242, USA

Abstract

Human β -defensin-3 (HBD3) is a small, cationic, host defence peptide with broad antimicrobial activities and diverse innate immune functions. HBD3 binds to many microbial antigens and, in this study, we hypothesised that the known binding of HBD3 to *Porphyromonas gingivalis* recombinant haemagglutinin B (rHagB) alters, but does not inhibit, the binding of rHagB to human dendritic cells. To test this, human myeloid dendritic cells were incubated for 5 min with rHagB, HBD3 + rHagB (10:1 molar ratio), HBD3 or 0.1 M phosphate-buffered saline (PBS) (pH 7.2) and were then rapidly fixed and processed for confocal microscopy and ultramicrotomy. rHagB and HBD3 could be detected with primary monoclonal mouse antibody to rHagB (MoAb 1858) or polyclonal rabbit antibody to HBD3 (P241) and secondary fluorescent-labelled anti-mouse or anti-rabbit antibodies (confocal microscopy) or protein A-colloidal gold (immunoelectron microscopy). In cells incubated with rHagB only, fluorescence and protein A-colloidal gold were seen at the cell surface and throughout the cytoplasm. In cells incubated with HBD3 + rHagB, fluorescence was observed only at the cell surface in a ‘string of pearls’

© 2012 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

Tel.: +1 319 335 8077; fax: +1 319 335 8895. kim-brogden@uiowa.edu.

Competing interests

None declared.

Ethical approval

Not required.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

configuration. Overall, these results suggest that HBD3 binding to rHagB alters, but does not inhibit, the binding of rHagB to human myeloid dendritic cells.

Keywords

Defensins; Human β -defensin-3; HBD3; *Porphyromonas gingivalis*; Haemagglutinin B; Dendritic cells; Confocal microscopy

1 Introduction

Host defence antimicrobial peptides are very prominent throughout the oral cavity, where they are thought to contribute to the overall immune homeostasis of the oral tissues [1]. One important host defence peptide is Human β -defensin-3 (HBD3). HBD3 is widely expressed in oral tissues [2–4] and is present in saliva and gingival crevicular fluid at concentrations up to 7.3 $\mu\text{g/mL}$ [5,6]. HBD3 contains 45 amino acid residues with a predicted monoisotopic mass of 5157.71 Da, has a net +11 charge with a theoretical isoelectric point (pI) of 10.08, has a β -sheet structure in solution, and forms dimers. It is induced by bacteria, bacterial products and the cytokines tumour necrosis factor- α (TNF α), interleukin 1- β (IL-1 β) and interferon- γ [2,7]. HBD3 has broad-spectrum antimicrobial activity against bacteria, fungi and enveloped viruses, regulates innate immune mechanisms and enhances adaptive immunity [8].

Porphyromonas gingivalis, a late coloniser in the polymicrobial oral biofilm, is a low-abundance yet dominant aetiological agent in the pathogenesis of periodontitis [9]. *Porphyromonas gingivalis* attachment to and invasion of oral and endothelial cells is facilitated by numerous fimbrial and haemagglutinin adhesins, including haemagglutinin B [10]. Haemagglutinin B is a major virulence determinant and is among the more closely studied haemagglutinins of *P. gingivalis*. Recombinant haemagglutinin B (rHagB) is a 350-amino acid residue protein with a mass of 49 kDa [10].

Previously, we observed using surface plasmon spectroscopy and enzyme-linked immunosorbent assay (ELISA) that HBD3 binds to immobilised rHagB, attenuates the IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF α responses induced by rHagB in human myeloid dendritic cell culture supernatants, and attenuates the extracellular signal-regulated kinases (ERK1/2) response in human myeloid dendritic cell lysates [11,12]. HBD3 is known to bind to many microbial antigens and, in this study, we hypothesised that the binding of HBD3 to rHagB alters, but does not inhibit, the binding of rHagB to the surface of human myeloid dendritic cells. The objective was two-fold: first, to demonstrate that rHagB binds to human myeloid dendritic cells; and second, to determine whether pre-incubation of HBD3 with rHagB alters the attachment of rHagB to dendritic cells.

2 Material and methods

2.1 2.1. Inocula and antibodies

First, 0.01 M phosphate-buffered saline (PBS) (pH 7.2) [0.01 M sodium phosphate buffer with 0.14 M NaCl (pH 7.2)] was prepared with pyrogen-free water and filter sterilised (0.22 μm filter; Millipore, Billerica, MA). It was found to contain 0.103 endotoxin units/mL (equivalent to 0.0103 ng/mL) by QCL-1000 assay (Cambrex, Walkersville, MD).

rHagB was prepared as previously described in other studies using *hagB* of *P. gingivalis* (1.4 kb) cloned into the vector pQE31 (QIAGEN Inc., Valencia, CA) and expressed in

Escherichia coli M15(pREP4)pQE-31-TX1 [10–12]. Briefly, cultured bacteria were pelleted by centrifugation and were lysed in 6 M guanidine-HCl, 0.1 M NaH₂PO₄ and 0.01 M Tris (pH 8.0) for 1.5 h. The lysate was clarified by centrifugation and the supernatant was passed through a Ni-NTA spin column (HisPur™; Pierce Biotechnology, Rockford, IL). The column was washed with 6 M guanidine-HCl (three times), 8 M urea in 0.01 M Tris with 0.1 M Na₂PO₄ (pH 8.0) (three times) and 8 M urea in 0.01 M Tris with 0.1 M Na₂PO₄ (pH 6.3) (three times) and bound rHagB was then eluted with 0.25 M imidazole in 0.01 M Tris, 0.5 M NaCl and 20% glycerol (pH 7.4) and dialysed against 0.01 M Tris with 0.5 M NaCl (pH 7.4) at 4 °C. The purity of rHagB was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and contained 1.5 pg of lipopolysaccharide per 1.0 µg of rHagB as determined by QCL-1000 assay. A 2× stock solution of 0.2 µM rHagB in 0.01 M PBS (pH 7.2) was prepared.

HBD3 was purchased from PeproTech Inc. (Rocky Hill, NJ). A 2× stock solution of 2.0 µM HBD3 in 0.01 M PBS (pH 7.2) was prepared.

Monoclonal mouse antibody to rHagB (MoAb 1858) was used and prepared as previously described in other studies [10]. Polyclonal rabbit antibody to HBD3 (P241) was purchased from PeproTech Inc. In preliminary tests, MoAb 1858 antibody was diluted 1:10, 1:20, 1:40, 1:100 and 1:200, and P241 antibody was diluted 1:10, 1:100, 1:300, 1:400, 1:500 and 1:1000; only results with final optimal dilutions are reported below. Goat anti-mouse antibody labelled with Alexa Fluor® 488 and goat anti-rabbit antibody labelled with Alexa Fluor® 568 were both used and diluted 1:500.

2.2 2.2. Confocal microscopy

Human myeloid dendritic cells (CC-2701; Lonza Walkersville Inc., Walkersville, MD) were thawed, suspended in lymphocyte growth media 3 (LGM-3) (Lonza Walkersville Inc.) and washed. Then, 600 µL of LGM-3 containing 6.3×10^4 viable cells was put into each of four chambers of Lab-Tek™ chamber slides (Thermo Fisher Scientific, Rockford, IL) and the cells were allowed to attach. After 2 h, medium and non-adherent cells were removed and the adherent cells were washed with 600 µL of 0.01 M PBS (pH 7.2) per chamber. Then, 600 µL of 0.1 µM rHagB, 0.1 µM rHagB + 1.0 µM HBD3 (incubated together at 37 °C for 30 min prior to use), 1.0 µM HBD3 or 0.01 M PBS (pH 7.2) was added to each of the four chambers, respectively, and was incubated at 37 °C. After 5 min, all test solutions were quickly removed by aspiration and the cells were washed three times with 600 µL of 0.01 M PBS (pH 7.2) per chamber and fixed in 600 µL of 4.0% paraformaldehyde for 10 min at room temperature.

To detect rHagB, fixed cells were washed three times for 5 min each with 600 µL of Wash Buffer consisting of Dulbecco PBS (pH 7.4) (DPBS) with 0.05% Tween 20 and then for 30 min with Blocking Solution consisting of DPBS with 0.05% Tween 20 and 10% normal goat serum. Then, 600 µL of MoAb 1858 antibody diluted 1:200 in Blocking Solution was added for 1 h. Cells were washed three times for 5 min each with 600 µL of Wash Buffer. Then, 600 µL of goat anti-mouse antibody labelled with Alexa 488 diluted 1:500 in DPBS was added for 30 min.

To detect HBD3, fixed cells were washed three times for 5 min each with 600 µL of Wash Buffer and 30 min with Blocking Solution. Then, 600 µL of P241 antibody diluted 1:400 in DPBS was added for 1 h. Cells were washed three times for 5 min each with 600 µL of Wash Buffer. Then, 600 µL of goat anti-rabbit antibody labelled with Alexa 568 diluted 1:500 in DPBS was added for 30 min.

Following the final incubation with secondary fluorescent-labelled anti-mouse or anti-rabbit antibodies, all cells were washed three times for 5 min each with 600 μ L of DPBS, counterstained for 5 min with 600 μ L of TO-PRO[®]-3 iodide (Life Technologies, Grand Island, NY) diluted 1:2000 in DPBS, washed three times for 5 min each with 600 μ L of DPBS, mounted with Vectashield[®] and examined with a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). All experiments were performed in triplicate and 668 saved images were printed for examination and analysis.

2.3 2.3. Immunoelectron microscopy

Human myeloid dendritic cells were cultured from peripheral blood monocytes of normal donors as previously described in other studies [12]. Briefly, 400 μ L of X-Vivo-15 (Lonza Walkersville Inc.) containing 2.1×10^5 cells was put into each of four polypropylene microcentrifuge tubes and the cells were pelleted by centrifugation at $800 \times g$ for 3 min at 4 °C. Solutions of rHagB and HBD3 were prepared and were incubated at 37 °C for 30 min prior to use. Then, 200 μ L of 0.1 μ M rHagB, 0.1 μ M rHagB + 1.0 μ M HBD3 (incubated together at 37 °C for 30 min prior to use), 1.0 μ M HBD3 or 0.01 M PBS (pH 7.2) was added to each of the four microcentrifuge tubes and gently mixed. Following incubation for 5 min at room temperature, dendritic cells were pelleted by centrifugation at $800 \times g$ for 3 min at 4 °C. The supernatant was removed and 200 μ L of ice-cold glutaraldehyde in 0.1 M cacodylate buffer was added. Following fixation for 1 h on ice, cells were processed for ultramicrotomy. rHagB was detected in cell sections with MoAb 1858 antibody and protein A–colloidal gold as previously described [13]. Sections were examined using a JEOL JEM-1230 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at 5000 $\times$ magnification. All experiments were performed in duplicate and 74 images were analysed using ImageJ software (US National Institutes of Health, Bethesda, MD) for specific labelling density (e.g. protein A–colloidal gold particles/ μ m² cell cytoplasm plus the number of gold particles up to 0.150 μ m from the membrane surface). Mean differences between groups were determined by a one-way analysis of variance (ANOVA) using JMP v9.0.2 (SAS Institute Inc., Cary, NC), and pairwise comparisons were done using the Tukey–Kramer HSD test. For all comparisons, a *P*-value of <0.05 was considered significant.

3 Results

3.1 3.1. Binding of recombinant haemagglutinin B and human β -defensin-3 to human myeloid dendritic cells

rHagB induces the production of chemokines and pro-inflammatory cytokines in human myeloid dendritic cells [12]. The first objective of this study was to demonstrate that rHagB binds to these cells and can be seen on fixed cells by confocal microscopy (Figs 1 and 2) or in cell sections by immunoelectron microscopy (Fig. 3). In dendritic cells incubated with rHagB, MoAb 1858 antibody and goat anti-mouse antibody labelled with Alexa 488, fluorescence was diffuse throughout the cytoplasm of the cell (Fig. 1a).

Dendritic cells incubated with 0.01 M PBS (pH 7.2), MoAb 1858 antibody and goat anti-mouse antibody labelled with Alexa 488 did not show any fluorescence (Fig. 1d). Likewise, dendritic cells incubated only with MoAb 1858 antibody, P241 antibody, secondary goat anti-mouse antibody labelled with Alexa 488, or goat anti-rabbit antibody labelled with Alexa 568, at all dilutions, did not show any fluorescence. In all instances, cells appeared normal and only the nuclei of cells treated with TO-PRO-3 could be seen.

Dendritic cells incubated with rHagB, MoAb 1858 antibody and protein A–colloidal gold had significantly more (*P* < 0.05) gold label/ μ m² of section than that seen in sections of dendritic cells incubated with HBD3 + rHagB, HBD3 or 0.01 M PBS (pH 7.2).

3.2 3.2. Human β -defensin-3-induced inhibition of recombinant haemagglutinin B binding to dendritic cell surfaces

HBD3 binds to immobilised rHagB [11,12] and the second objective of this study was to assess whether this association alters the binding of rHagB to dendritic cells. In dendritic cells incubated with HBD3 + rHagB, MoAb 1858 antibody and goat anti-mouse antibody labelled with Alexa 488, a unique 'string of pearls' fluorescence was seen on the periphery of the cell (Fig. 1b). Clearly, penetration of rHagB into the cell was arrested by prior incubation with HBD3.

In dendritic cells incubated with HBD3 + rHagB, P241 antibody and goat anti-rabbit antibody labelled with Alexa 568, fluorescence was diffuse throughout the cytoplasm of the cell resulting in brightly stained, individual cells (Fig. 2b). Dendritic cells incubated with rHagB, HBD3 or 0.01 M PBS (pH 7.2), P241 antibody and goat anti-rabbit antibody labelled with Alexa 568 did not show any fluorescence (Fig. 2a, 2c and 2d, respectively).

There were no differences in gold labelling densities of dendritic cells treated with HBD3 + rHagB, HBD3 or 0.01 M PBS (pH 7.2) after incubation with MoAb 1858 antibody or P241 antibody and protein A-colloidal gold.

4 Discussion

Previously, we observed that HBD3 directly binds to rHagB, attenuates a rHagB-induced ERK1/2 signalling pathway in human myeloid dendritic cells, and attenuates a select rHagB-induced pro-inflammatory cytokine response [11,12]. A first step in understanding this process, e.g. the objectives of this study, was to first determine whether rHagB binds to human myeloid dendritic cells and, second, to determine whether prior incubation of HBD3 with rHagB alters this attachment to dendritic cells in any way. In this study, we show both, i.e. the binding and penetration of rHagB to dendritic cells, and HBD3-altered binding with no penetration of cells.

Whilst both confocal microscopy and immunoelectron microscopy could be used to show that rHagB binds to dendritic cells (Figs 1a and 3a), only confocal microscopy could be used to show that HBD3 alters the binding of rHagB to dendritic cells (Fig. 1b). Moreover, only confocal microscopy could be used to show that HBD3 in the presence of rHagB could be detected in the cell cytoplasm (Fig. 2b). These differences could be related to the epitopes on HBD3 and rHagB recognised by MoAb 1858 and P241 antibodies. It is likely that MoAb 1858 antibody recognises an epitope on the surface of rHagB that is still exposed once rHagB has bound to the dendritic cell membrane.

The observation that HBD3, pre-incubated with rHagB, alters, but does not inhibit, the binding of rHagB to dendritic cells is logical and is supported by the previous observation that HBD3 selectively attenuates only IL-6, IL-10, GM-CSF and TNF α responses induced by rHagB and not all chemokine and cytokine responses in human myeloid dendritic cell culture supernatants, and only attenuates rHagB-induced MAPK signal pathway ERK1/2 responses and not related p38 and JNK pathway responses in human myeloid dendritic cell lysates [12]. Unfortunately, this issue will not likely be resolved by confocal and immunoelectron microscopy. Future work will focus on isolating the dendritic cell receptor for rHagB, assessing the epitope binding sites of HBD3 and MoAb 1858 antibody on rHagB, and assessing the role of HBD3 in altering rHagB binding to its isolated receptor.

HBD3 likely plays a role in this process. Recent work by Semple et al. showed that HBD3 rapidly associates with and enters Toll-like receptor 4-stimulated macrophages where it targets Toll-like receptor signalling pathways and inhibits the transcription of pro-

inflammatory genes, particularly those involved in the positive regulation of nuclear factor kappa B (NF κ B) [14]. In dendritic cells incubated with HBD3 + rHagB, P241 antibody and goat anti-rabbit antibody labelled with Alexa 568, fluorescence is stronger in the cytoplasm of brightly stained, individual cells (Fig. 2b). Why rHagB appears on the surface of these cells (Fig. 1b) yet HBD3 appears to be in the cytoplasm of these cells (Fig. 2b) is not yet known.

In summary, rHagB binds to human myeloid dendritic cells, quickly moving into the cytoplasm. Prior incubation of HBD3 with rHagB alters, but does not inhibit, rHagB attachment to the dendritic cell surface. Additional roles of HBD3 in this process are not yet known but may be related to the ability of HBD3 to rapidly associate with and enter cells where it targets signalling pathways.

Acknowledgments

The authors are very grateful to Patricia J. Conrad for preparation of Figs 1–3.

Funding

This work was supported by funds from the training grant NIH/NIDCR T32 DE014678 and the research grant NIH/NIDCR R01 DE014390 from the National Institute of Dental and Craniofacial Research of the US National Institutes of Health (Bethesda, MD). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Gorr SU. Antimicrobial peptides of the oral cavity. *Periodontol* 2000. 2009; 51:152–80. [PubMed: 19878474]
2. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human β -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem*. 2001; 276:5707–13. [PubMed: 11085990]
3. Dunsche A, Acil Y, Dommisch H, Siebert R, Schroder JM, Jepsen S. The novel human β -defensin-3 is widely expressed in oral tissues. *Eur J Oral Sci*. 2002; 110:121–4. [PubMed: 12013554]
4. Lu Q, Samaranayake LP, Darveau RP, Jin L. Expression of human β -defensin-3 in gingival epithelia. *J Periodontol Res*. 2005; 40:474–81. [PubMed: 16302926]
5. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, et al. Salivary antimicrobial peptide expression and dental caries experience in children. *Antimicrob Agents Chemother*. 2005; 49:3883–8. [PubMed: 16127066]
6. Dale BA, Tao R, Kimball JR, Jurevic RJ. Oral antimicrobial peptides and biological control of caries. *BMC Oral Health*. 2006; 6(Suppl 1):S13. [PubMed: 16934114]
7. Joly S, Organ CC, Johnson GK, McCray PB Jr, Guthmiller JM. Correlation between β -defensin expression and induction profiles in gingival keratinocytes. *Mol Immunol*. 2005; 42:1073–84. [PubMed: 15829297]
8. Dhople V, Krukemeyer A, Ramamoorthy A. The human β -defensin-3, an antibacterial peptide with multiple biological functions. *Biochim Biophys Acta*. 2006; 1758:1499–512. [PubMed: 16978580]
9. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskandari MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe*. 2011; 10:497–506. [PubMed: 22036469]
10. Song H, Belanger M, Whitlock J, Kozarov E, Prohaska-Fox A. Hemagglutinin B is involved in the adherence of *Porphyromonas gingivalis* to human coronary artery endothelial cells. *Infect Immun*. 2005; 73:7267–73. [PubMed: 16239522]
11. Dietrich DE, Xiao X, Dawson DV, Bélanger M, Xie H, Prohaska-Fox A, et al. Human α - and β -defensins bind to immobilized adhesins from *Porphyromonas gingivalis*. *Infect Immun*. 2008; 76:5714–20. [PubMed: 18852241]

12. Pingel LC, Kohlgraf KG, Hansen CJ, Eastman CG, Dietrich DE, Burnell KK, et al. Human β -defensin 3 binds to hemagglutinin B (rHagB), a non-fimbrial adhesin from *Porphyromonas gingivalis*, and attenuates a pro-inflammatory cytokine response. *Immunol Cell Biol.* 2008; 86:643–9. [PubMed: 18711400]
13. Heidari M, Hamir A, Cutlip RC, Brogden KA. Antimicrobial anionic peptide binds in vivo to *Mannheimia (Pasteurella) haemolytica* attached to ovine alveolar epithelium. *Int J Antimicrob Agents.* 2002; 20:69–72. [PubMed: 12127715]
14. Semple F, MacPherson H, Webb S, Cox SL, Mallin LJ, Tyrrell C, et al. Human β -defensin 3 affects the activity of pro-inflammatory pathways associated with MyD88 and TRIF. *Eur J Immunol.* 2011; 41:3291–300. [PubMed: 21809339]

Fig. 1.

Binding of recombinant haemagglutinin B (rHagB) detected on dendritic cells treated with (a) 0.1 μ M rHagB, (b) 1.0 μ M human β -defensin-3 (HBD3) + 0.1 μ M rHagB, (c) 1.0 μ M HBD3 or (d) 0.01 M phosphate-buffered saline (PBS) (pH 7.2) using primary monoclonal mouse antibody to rHagB (MoAb 1858) and secondary goat anti-mouse antibody labelled with Alexa Fluor[®] 488. Note the diffuse fluorescence throughout the cytoplasm of cells incubated with rHagB (a) and localised fluorescence on the periphery of cells incubated with HBD3 + rHagB (b), with a 'string of pearls' appearance, but no presence in cells incubated HBD3 (c) or 0.01 M PBS (d).

Fig. 2. Binding of Human β -defensin-3 (HBD3) detected on dendritic cells treated with (a) 0.1 μ M recombinant haemagglutinin B (rHagB), (b) 1.0 μ M HBD3 + 0.1 μ M rHagB, (c) 1.0 μ M HBD3 or (d) 0.01 M phosphate-buffered saline (PBS) (pH 7.2) using primary polyclonal rabbit antibody to HBD3 (P241) and secondary goat anti-rabbit antibody labelled with Alexa Fluor[®] 568. Note the intense fluorescence throughout the cytoplasm of cells incubated with HBD3 + rHagB (b) not present in cells incubated with rHagB (a), HBD3 (c) or 0.01 M PBS (d).

Fig. 3. Binding of recombinant haemagglutinin B (rHagB) detected on dendritic cells treated with (a) 0.1 μ M rHagB, (b) 1.0 μ M human β -defensin-3 (HBD3) + 0.1 μ M rHagB, (c) 1.0 μ M HBD3 or (d) 0.01 M phosphate-buffered saline (PBS) (pH 7.2) using primary monoclonal mouse antibody to rHagB (MoAb 1858) and protein A-colloidal gold. Note the significant ($P < 0.05$) increase in protein A-colloidal gold particles (arrows) in cells incubated with rHagB (a), but not present in cells incubated with HBD3 + rHagB (b), HBD3 (c) or 0.01 M PBS (d).