that lacks genes encoding the enzymes for most biosynthetic pathways. A consequence of this evolution to obligate parasitism has been the elimination of the requirement for Fe. This adaptation by *B. burgdorferi* has been successful, and other pathogenic bacteria with limited genomes, such as *T. pallidum* (950 Mb) (*19*) and *Mycoplasma pneumoniae* (650 kb) (*20*), may have adopted similar approaches to avoid host Fe limitation.

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Intracellular Parasitism by the Human Granulocytic Ehrlichiosis Bacterium Through the P-Selectin Ligand, PSGL-1

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Human granulocytic ehrlichiosis (HGE) is a febrile tick-borne illness caused by a recently discovered intracellular bacterium remarkable for its tropism for professionally phagocytic neutrophils. Monoclonal antibodies against the P-selectin binding domain of the leukocyte P-selectin glycoprotein ligand, PSGL-1, prevented HGE cell binding and infection, as did enzymatic digestion of PSGL-1. Furthermore, simultaneous neoexpression in nonsusceptible cells of complementary DNAs for both PSGL-1 and its modifying α-(1,3) fucosyltransferase, Fuc-TVII, allowed binding and infection by HGE. Thus, the HGE bacterium specifically bound to fucosylated leukocyte PSGL-1. Selectin mimicry is likely central to the organism's unique ability to target and infect neutrophils.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne infection that causes an acute febrile illness, which is often severe, and frequently coinfects individuals with Lyme disease (*Borrelia burgdorferi*), also carried by *Ixodes* ticks (*1*, *2*). In the few years since its recognition, the disease has been reported in several hundred individuals, primarily in the regions of the north central and northeastern United States, where Lyme disease is endemic, and seropositivity has been reported in several other countries. The etiologic agent is an obligate intracellular bacterium found in the peripheral blood granulocytes of infected patients and is closely related, or identical, to *Ehrlichia equi* and *E. phagocytophila* (*1*, *3*), which infect horses and ruminants, respectively. Several pathogens of humans have life cycles that involve permissive infection of monocytes, but the HGE agent is noteworthy in its tropism for and growth within professionally phagocytic granulocytes. The as of yet unnamed etiologic organism of HGE was recently isolated with the promyelocytic leukemia cell line, HL60, and it has since been shown that the organism can replicate in other myeloid cell lines and in normal human bone marrow progenitor cells (*3*–*5*). The tropism of this organism for leukocytes and its ability to evade normal phagocytic pathways and lysosomal fusion (*6*) suggest that the organism uses specific receptor-mediated pathway(s) for cellular adhesion and entry.

Although intracellular infection by bacteria is an area of intense investigation, the entry of pathogens into permissive pathways in normally hostile phagocytic cells remains poorly understood (*7*). Some organisms infect a variety of cells and bind to molecules that are widely distributed in the host (e.g., *Yersinia* species and *Toxoplasma gondii* to β-1 integrins) (*8*, *9*). Others are able to use specific leukocyte molecules in their adhesion to monocytes (e.g., *Legionella pneumophila*, *Leishmania donovanii*, and *Mycobacterium tuberculosis* through binding to complement receptors) (*10*, *11*). The receptors responsible for ehrlichial binding and entry into host cells were unknown.

Leukocyte cell surface expression of the sialyl Lewis x tetrasaccharide (sLe^x or CD15s) and the expression of the α-(1,3) fucosyltransferase, Fuc-TVII, responsible for its terminal fucosylation are linked to cell susceptibility to HGE infection (*12*). However, Fuc-TVII has multiple substrates, sLe^x itself modifies a wide variety of proteins and lipids, and antibodies to sLe^x (anti-sLe^x) did not block HGE adhesion to susceptible cells (*12*), suggesting that sLe^x alone is not a specific receptor for the organism. We hypothesized that the HGE receptor is a Fuc-TVII–modified cell surface glycoprotein expressed on neutrophils. The linkage of infection to the expression of sLe^x also suggested that the HGE receptor might be a selectin ligand.

We examined the effects of monoclonal antibodies (mAbs) against sLe^x itself and known sLe^x-modified or related mucin-modified proteins, including CD43, CD16, CD24, and the major identified selectin ligand of neutrophils (*13*, *14*), PSGL-1, on HGE binding (*15*). As shown by fluorescence-activated cell sorting (FACS) analysis (*16*) of binding of fluoresceinated bacteria, mAb PL1, which is directed against the NH_2-terminus of PSGL-1 (residues 8 to 21) and blocks P-selectin binding to cells (*17*), blocked the binding of HGE to HL60 cells in a dose-dependent manner (Fig. 1A). These results were confirmed with three different, geographically diverse strains of HGE. mAb KPL-1, which binds to the tyrosine sulfate motif of PSGL-1 and blocks binding to P-selectin (*18*), also blocked binding of bacteria to HL60 cells, whereas mAb PL2, which binds to a membrane proximal epitope on PSGL-1 and does not block binding to P-

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selectin (*17*), did not. mAb PL1 also blocked HGE binding to human peripheral blood neutrophils (Fig. 1B). In contrast, numerous other mAbs, including three directed against the β-1 integrin subunit CD11b (e.g., Leu 15; Fig. 1B), as well as isotype control immunoglobulins, had little or no effect.

To confirm that these results represent inhibition of specific binding, we performed identical studies by indirect immunofluorescent microscopy (IFA) allowing the direct visualization and enumeration of adherent bacteria (*12*). mAbs KPL-1 and PL1 prevented HGE binding to either HL60 cells or neutrophils. For example, HL60 cells in the presence of control immunoglobulin G, subclass 1 (IgG1), bound a median of 5.0 bacteria per cell (Fig. 2A, mean 5.0) compared with 1 bacteria per cell bound (mean 1.1) in the presence of KPL-1 (10 μg/ml) (Fig. 2C) ($P < 0.001$, Wilcoxan sum of ranks test).

If binding to PSGL-1 by HGE is required for bacterial entry, then the prevention of adhesion by blocking mAbs would be expected to reduce intracellular infection. Preincubation of HL60 cells with KPL-1 (10 μg/ml), but not control IgG1, prevented infection, with a mean of 0.1% of KPL-1–treated cells infected when observed 48 hours later (Fig. 2D) compared with 87% of control cells (Fig. 2B; $P < 0.001$, Fisher Exact Test). Similarly, incubation of granulocytes with KPL-1 (10 μg/ml) prevented infection by cell-free HGE bacteria (1% of cells compared with 18.3% infected in IgG1 controls at 48 hours; $P < 0.001$). HGE is probably transmitted directly from cell to cell, and such transfer in vitro is more efficient than the use of cell-free bacteria. Hence, we investigated whether KPL-1 could prevent cell-to-cell transfer from highly infected HL60 cells. Again, treatment of neutrophils with anti–PSGL-1 mAb reduced subsequent infection, even by large numbers of highly infected HL60 cells (38.5% of control IgG1-treated neutrophils infected compared with 4% of those treated with KPL-1; $P < 0.001$).

To extend our findings with mAbs, we used neoexpression of PSGL-1 on a normally nonexpressing leukocyte cell line to discover whether or not HGE would then bind to and enter such cells. Given our previous findings that cellular susceptibility to HGE infection correlates with sLe^x expression, we also investigated whether Fuc-TVII, which is needed for sLe^x synthesis in leukocytes (*19*), is required to glycosylate PSGL-1 for effective HGE binding to occur.

The B lymphoblastoid cell line BJAB (*20*) expresses neither PSGL-1 nor Fuc-TVII/sLe^x and does not significantly bind to or become infected by the HGE agent. Using cDNA for PSGL-1 or Fuc T-VII, we generated stable transfectants of BJAB that expressed either PSGL-1 or Fuc-TVII/sLe^x, or both (*21*). These transfectants were then each challenged with bacteria, and their ability to bind organisms and become infected was determined. Untransfected BJAB cells rarely bound organisms (mean 0.4 and median 0 bacteria per cell) and did not become infected (Fig. 3, A and B). Transfectants expressing PSGL-1 alone also did not bind bacteria or become significantly infected. Transfectants expressing Fuc-TVII alone bound slightly more bacteria (e.g., mean of 1.1 organisms per cell, median of 1; P = NS) than parental BJAB cells but did not become susceptible to infection. However, dual transfectants expressing both PSGL-1 and Fuc-TVII bound HGE (mean 3.4 and median 2 organisms per cell; $P < 0.001$ compared with parental BJAB cells; Fig. 3C) and became infected (mean 79% at 48 hours, compared with 0.5% of parental BJAB; $P < 0.001$; Fig. 3D). Furthermore, HGE binding to and subsequent infection of the dual transfectants could be prevented by preincubating cells with the anti–PSGL-1 mAb KPL-1. Identical results were obtained by FACS analysis of binding of fluorescent bacteria to parental and transfected cells. Thus, coexpression of PSGL-1 and Fuc-TVII was necessary and sufficient for HGE binding to and infection of BJAB cells.

The binding of PSGL-1 to P- and L-selectins is abolished by proteolytic cleavage by the *O*-sialyloglycoprotease of *Pastuerella hemolytica*. We examined whether pretreatment of cells with this enzyme would reduce HGE binding (*22*). Pretreatment of the PSGL-1– and sLe^x-expressing dual transfectant BJAB cells with this enzyme at concentrations of 0.06 to 0.24 mg/ml substantially reduced HGE binding in a dose-dependent manner (e.g., median of 1 bacteria per cell bound after pretreatment with 0.24 mg/ml compared with 7.5 bacteria per cell in controls; $P < 0.001$). Finally, to examine the possibility that the HGE bacterium might have itself acquired a known selectin [either the gene(s) or the selectin moieties themselves during growth in or exit from the cell] that binds to leukocyte PSGL-1, we pretreated bacteria with mAbs against P-, L-, or E-selectins as well as with a polyclonal antibody against P-selectin in an attempt to either block cell binding or to stain selectin(s) on the surface of HGE organisms (*15*). These antibodies had no effect on HGE binding and did not bind to components of the HGE bacteria.

The evidence presented here demonstrates that the agent of HGE uses PSGL-1 as its leukocyte receptor or as a key initial receptor component. Bacterial binding and infection

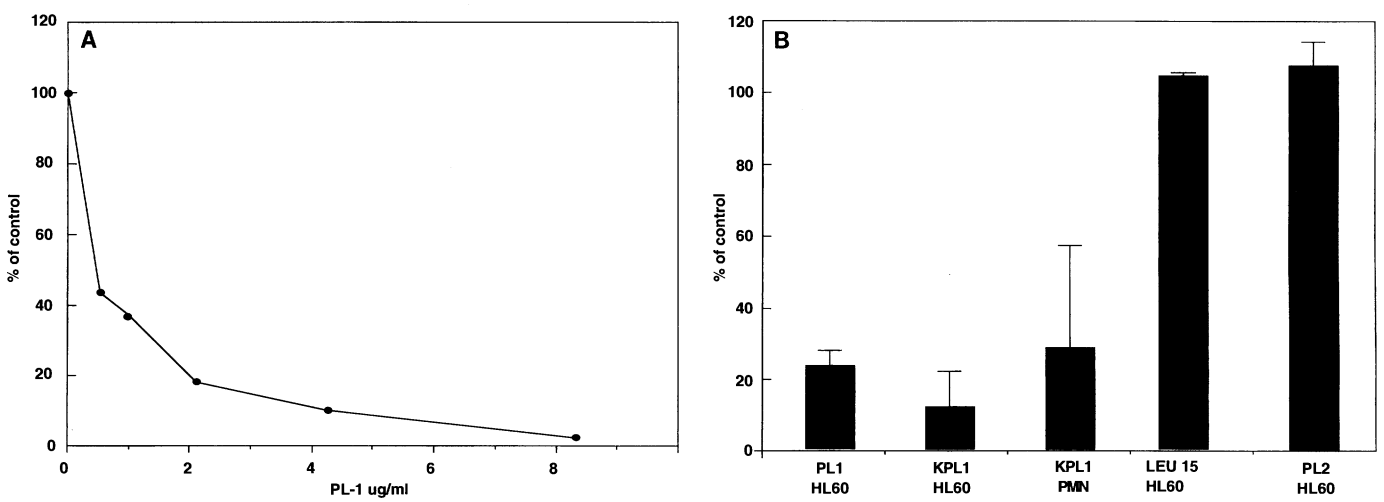

Fig. 1. mAbs against the P-selectin binding domain of P-selectin glycoprotein ligand-1 (PSGL-1) block binding of the HGE bacterium to susceptible cells. (**A**) Dose-response curve of inhibition of binding of fluoresceinated HGE bacteria (expressed as the percentage of control binding observed in the presence of isotype control IgG1) to HL60 cells by anti–PSGL-1 mAb PL1. Marked inhibition was observed at concentrations as low as 2 μg/ml. (**B**) mAbs PL1 and KPL-1 (10 μg/ml), which both bind to the PSGL-1 P-selectin binding site, block HGE binding to HL60 cells and human blood polymorphonuclear neutrophils (PMN), whereas mAb PL2, against a nonbinding PSGL-1 domain (see text), and the anti-CD11b mAb Leu 15 do not.

were prevented by mAbs against the selectin binding region of PSGL-1, and binding was reduced by proteolytic destruction of PSGL-1. The nonpermissive BJAB cell line, when transfected to express both PSGL-1 and Fuc-TVII/sLe^x, avidly bound HGE and became infected. Proper glycosylation of PSGL-1 is likely essential for this binding to occur, as evidenced by the failure of BJAB cells expressing PSGL-1 without Fuc-TVII to bind the HGE agent and by previous studies showing that Fuc-TVII/sLe^x expression correlates with HGE binding to hematopoietic cell lines (*5*, *12*). The neoexpression of Fuc-TVII/sLe^x alone, without PSGL-1, slightly enhanced HGE binding (but did not allow infection). In these respects, and in the failure of the PL2 mAb to block binding, the organism's interactions with leukocytes and related cell lines closely resemble the interaction of P-selectin with PSGL-1.

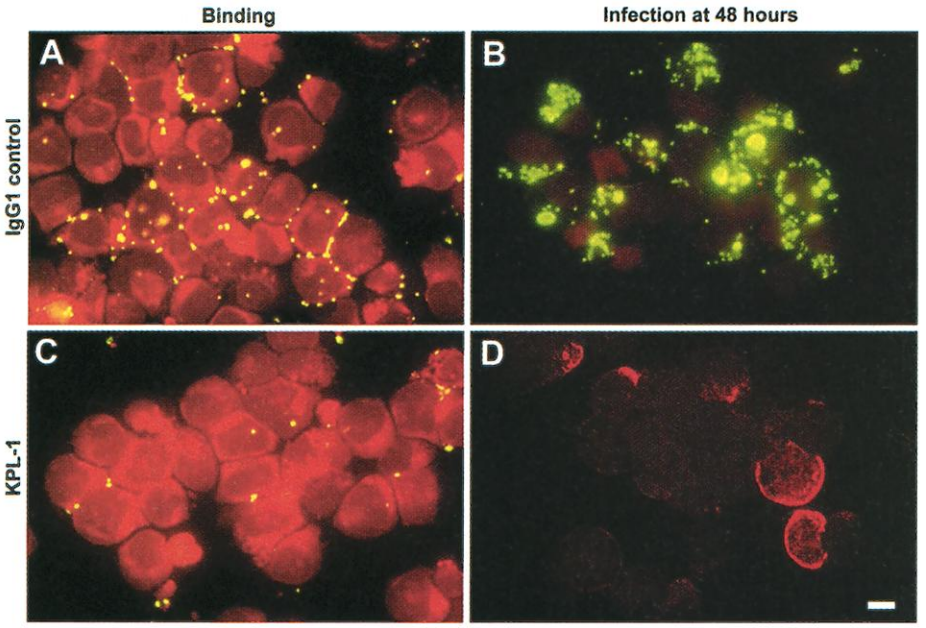

Fig. 2. Inhibition of HGE binding and infection by anti–PSGL-1. HL60 cells were preincubated for 1 hour at 37°C with isotype control IgG1 (**A** and **B**) or with the anti–PSGL-1 mAb KPL-1 (**C** and **D**) and then challenged with HGE (*13*). (A) and (C) show cells removed and washed immediately after challenge to assess bacterial binding. Bacteria (labeled yellow-green by indirect immunofluorescence) avidly bind to the HL60 cell surface (A), but this binding is prevented by KPL-1 (C). After 48 hours of cultivation, samples from the same experiments were evaluated for the presence of intracellular bacterial colonies indicative of bacterial replication. The majority of cells preincubated with control antibody are highly infected (B), but there is no infection of the cells pretreated with KPL-1 (D) (scale bar, 10 μm).

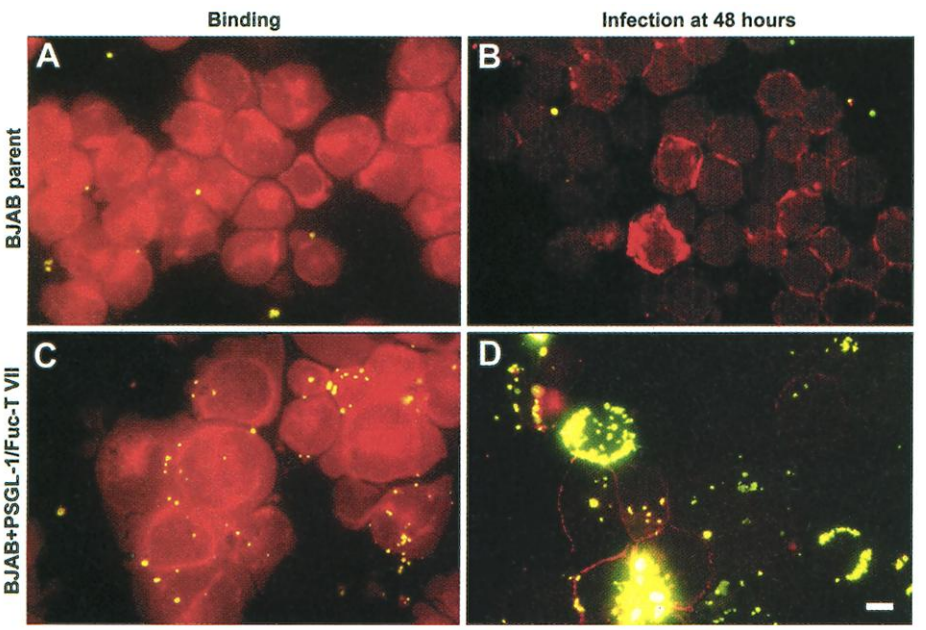

Fig. 3. Neoexpression of PSGL-1 and sLe^x confers on BJAB cells the ability to bind the HGE bacterium and become infected. The B lymphoblastoid cell line, BJAB, does not normally express either PSGL-1 or the α-(1,3) fucosyltransferase, Fuc-TVII, which fucosylates sLe^x, a tetrasaccharide that modifies PSGL-1. Parental BJAB cells neither bind HGE bacteria (**A**) nor become infected (**B**). However, BJAB cells stably transfected to express both PSGL-1 and Fuc-TVII bind HGE (**C**) and become infected (**D**) (scale bar, 10 μm).

Specific cell surface receptors for intracellular organisms have seldom been identified, none previously on granulocytes. The avidity of HGE for α-(1,3) fucosylated PSGL-1 is likely important in its tropism for phagocytes, which richly express this glycosylated molecule on their surface (*13*, *18*). Engagement of PSGL-1 by P-selectin is known to induce signal transduction (*23*, *24*), and such events have been shown to be important in the entry into cells of such pathogens as *Yersinia* and *Salmonella* species (*25*, *26*). That signal transduction occurs after HGE binding is supported by our recent findings that HGE infection markedly up-regulates β-chemokine as well as tissue factor secretion (*27*, *28*). Whether PSGL-1–mediated signaling by HGE directly results in endocytosis of the bacterium or triggers changes in expression of other molecules to allow subsequent entry is unknown. However, HGE binding to PSGL-1 likely allows it either to escape or modify a neutrophil phagocytic pathway and thus survive and multiply. Better understanding of this pathway could help define new leukocyte mechanisms that might be manipulated to up- or down-regulate phagocyte responses. Studies to identify the bacterial ligand for PSGL-1 also have important implications. This ligand represents a potential vaccinogen against HGE infection and could also provide a molecular model for the development of compounds to serve as nonselectin ligands for PSGL-1 that could then be used as pharmacologic inhibitors of deleterious cell adhesion–mediated events (*29*).

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16. Fluorescent HGE was prepared from infected HL60 cultures incubated with the vital fluorescent dye CellTracker Green (Molecular Probes, Eugene, OR) (C. Nelson, M. Herron, J. L. Goodman, unpublished data). HGE bacteria were liberated by passage through a 25-gauge needle and washed twice in phosphate-buffered saline (PBS). Fluorescent HGE from 1 to 2 million infected HL60 was incubated for 30 min with 250,000 target cells at 22°C in 25 μl of PBS. Binding was stopped, and the cells and bacteria were fixed by addition of 0.25 ml of 1% paraformaldehyde in PBS. Cell surface binding of labeled HGE was assessed by FACS analysis of the mean cellular fluorescence of 5000 cells and expressed as the percentage of a control with no antibodies added, with subtraction of nonspecific background determined with a baseline control in which binding had been stopped by fixation immediately upon addition of HGE to target cells. This method was shown to provide results that linearly correlate with binding as determined by direct visualization by IFA.
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The Prefrontal Cortex: Response Selection or Maintenance Within Working Memory?

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It is controversial whether the dorsolateral prefrontal cortex is involved in the maintenance of items in working memory or in the selection of responses. We used event-related functional magnetic resonance imaging to study the performance of a spatial working memory task by humans. We distinguished the maintenance of spatial items from the selection of an item from memory to guide a response. Selection, but not maintenance, was associated with activation of prefrontal area 46 of the dorsal lateral prefrontal cortex. In contrast, maintenance was associated with activation of prefrontal area 8 and the intraparietal cortex. The results support a role for the dorsal prefrontal cortex in the selection of representations. This accounts for the fact that this area is activated both when subjects select between items on working memory tasks and when they freely select between movements on tasks of willed action.

It has been controversial whether the dorsal prefrontal cortex is involved in the maintenance of working memory (*1*) or in the selection of responses (*2*, *3*). The first hypothesis accounts for the fact that in monkeys, there are cells in the dorsal prefrontal cortex that continue to fire during the delay on a working memory task (*4*). There is also activity in this area when humans perform working memory tasks (*5*, *6*), though there is no agreement as to whether to emphasize its role in the maintenance of information (*4*) or in the manipulation or monitoring of that information (*7*). However, there is also activity in the prefrontal cortex when humans freely select between manual or verbal responses (*8*). It could be argued that on such "free selection" tasks, the participants maintain a record of their responses on previous trials and that the activity can therefore still be related to working memory. But it has recently been shown that transcranial magnetic stimulation over the dorsal prefrontal cortex interferes with free selection of finger response even when there is no memory load (*9*). We have tried to reconcile these facts by using functional magnetic resonance imaging (fMRI) to measure activity in the dorsal prefrontal cortex during an experiment on working memory.

We used event-related fMRI to distinguish delay-related activity during the maintenance of items in memory ("set activity") from the transient activity related to selection of a single item from memory in that same trial. During working memory trials, the study participants remembered three spatial locations for up to 18 s (Fig. 1). They then selected the location of just one of these items to guide a response using a joystick. During the delay, the participants maintained the items in memory without requiring manipulation, monitoring, or preparation of their responses. They could not select the appropriate remembered location until the end of the working memory delay, and the stimulus locations changed at random from trial to trial. The control trials included similar stimuli and motor responses, but the participants were not required to remember or select spatial locations. We deliberately avoided the use of verbal material because we wished to ensure that the participants maintained items in memory during the delay without articulatory rehearsal.

A general linear model was applied to the time course of activation of each voxel (*10*). The model included separate covariates for transient neuronal activations in response to stimulus presentation, motor response, the selection from memory at the end of memory trials, sustained activation during working

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