

Leukocyte Response Integrin and Integrin-associated Protein Act As a Signal Transduction Unit in Generation of a Phagocyte Respiratory Burst

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Summary

The leukocyte response integrin (LRI) is a phagocyte integrin which recognizes the basement membrane protein entactin and the synthetic peptide Lys-Gly-Ala-Gly-Asp-Val (KGAGDV). The function of LRI is intimately associated with that of a distinct membrane protein, integrin-associated protein (IAP), as antibodies which recognize IAP can inhibit all known functions of LRI. When adherent to a surface, the LRI ligands entactin and KGAGDV activate the respiratory burst in polymorphonuclear leukocytes (PMN) and monocytes, as do monoclonal antibodies (mAb) directed at either LRI or IAP. When added in solution, peptides and antibodies specific for LRI, and some, but not all, anti-IAP antibodies, can inhibit the respiratory burst activated by any of these surface-adherent ligands. Only monoclonal anti-IAP antibodies which can inhibit LRI function when added in solution are competent to activate the respiratory burst when adherent to a surface. KGAGDV peptide and anti-LRI added in solution can inhibit anti-IAP-stimulated respiratory burst. The LRI-IAP-initiated respiratory burst is independent of CD18, as judged by: (a) blockade of inhibition by anti-CD18 mAb with the protein kinase A inhibitor HA1004; (b) enhanced sensitivity of CD18-dependent respiratory burst compared with LRI/IAP-dependent respiratory burst to the tyrosine kinase inhibitors genestein and herbimycin; and (c) generation of a respiratory burst in response to KGAGDV, anti-LRI, and anti-IAP coated surfaces in PMN from a patient with LAD. Despite its apparent CD18 independence, LRI/IAP-initiated respiratory burst requires a solid phase ligand and is sensitive to cytochalasin B. These data suggest a model in which LRI and IAP act together as a single signal transduction unit to activate the phagocyte respiratory burst, in a manner that requires CD18-independent cell adhesion.

Circulating peripheral blood monocytes and neutrophils (PMN)¹ are in a resting state, consuming little energy or oxygen, minimally phagocytic, and poorly adherent. In contrast, at extravascular sites of inflammation, phagocytes achieve an "activated" phenotype, with greatly increased oxygen consumption, generation of toxic oxygen metabolites, and markedly increased phagocytic and adhesive potential. A variety of molecules found at inflammatory sites may signal this switch to the activated, inflammatory phenotype, among which are chemotaxins, arachidonate metabolites, and lysophospholipid. Extracellular matrix proteins, such as fibronectin and laminin, also are potent inducers of the inflammatory

phenotype. These proteins can stimulate enhanced phagocytosis directly, whether immobilized on a surface or in solution (1-3) and, on a surface, can sensitize cells for generation of the respiratory burst (4, 5). Teleologically, these effects of phagocyte interaction with extracellular matrix can be envisioned as signals to the cell that it has left the vasculature and entered tissue where maximum inflammatory and host defense functions are required (6). Many of these effects of extracellular matrix on phagocyte biology require the contribution of β_2 (CD18)-containing integrin receptors (4, 5, 7). Recently, a leukocyte integrin receptor for multiple extracellular matrix proteins has been described which mediates activation of phagocytic cells from the resting to the inflammatory phenotype but is not a β_2 integrin (1, 8). This integrin receptor, termed the leukocyte response integrin (LRI) is immunologically related to the β_3 integrin family. LRI recognizes the Arg-Gly-Asp sequence in a number of extracellular matrix proteins and via this interaction signals activation of phagocytic function (1, 8), adhesion, and chemotaxis

¹ Abbreviations used in this paper: Br-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; HPO, hydrogen peroxide oxidoreductase; IAP, integrin-associated protein; KRPG, Krebs buffer with glucose; LRI, leukocyte response integrin; opd, *o*-phenylenediamine; PLL, poly-L-lysine; PMN, peripheral blood neutrophils; RM, reaction mixture.

(9). LRI preferentially recognizes a variant of the Arg-Gly-Asp sequence, Lys-Gly-Ala-Gly-Asp-Val (KGAGDV), which is not a ligand for any other known integrin (8). All known functions of LRI, including binding of multivalent KGAGDV ligands, can be specifically inhibited by antibodies to a distinct 50-kD single chain membrane protein termed integrin-associated protein (IAP) (2, 8). This has led to the hypothesis that IAP is a component of the signal transduction cascade for phagocyte activation beginning with LRI ligation (10). However, experimental systems to test this hypothesis have been lacking.

In the current work, we have developed an experimental system to explore the signal transduction cascade initiated by LRI ligation. To do this, we have examined assembly of the NADPH oxidase in monocytes and PMN in response to ligation of LRI or IAP. We show that ligation of LRI by surface-bound ligands and antibodies will directly activate the respiratory burst in PMN and monocytes. Activation can be inhibited by LRI ligands or antibodies added as competitors in solution. mAbs recognizing some, but not all, epitopes of IAP are equally potent inhibitors of the LRI-initiated respiratory burst when added in solution. These same antibodies against IAP will activate the respiratory burst when attached to a surface. LRI-directed ligands and antibodies in solution can inhibit this anti-IAP mAb initiated respiratory burst. These data demonstrate that LRI and IAP act as a signal transduction unit in the generation of a phagocyte respiratory burst. This suggests that IAP may be analogous to the "extra chain" of cytokine receptors: a nonligand-binding component which influences both ligand affinity and signal transduction (11-15). The signal transduction cascade initiated by LRI/IAP can be distinguished pharmacologically from the CD18-dependent activation pathway. These data demonstrate that there exists a novel integrin-dependent mechanism for activation of PMN at sites of inflammation and infection.

Materials and Methods

Reagents. The following reagents were purchased: Scopoletin, poly-L-lysine (PLL), PMA, *o*-phenylenediamine (opd), cytochalasin B, 8-bromoadenosine 3':5''-cyclic monophosphate (Br-cAMP), hydrogen peroxide oxidoreductase (HPO), and Ficoll-Hypaque were from Sigma Chemical Co. (St. Louis, MO); protein A was from Pharmacia Fine Chemicals (Piscataway, NJ); H-7 and HA1004 were from Seikagaku America, Inc. (St. Petersburg, FL); genestein and herbimycin were from GIBCO BRL (Gaithersburg, MD); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Amersham Corp. (Arlington Heights, IL); and dimethylsulfoxide was from Aldrich Chemical Co. (Milwaukee, WI).

Buffers. PBS was from Biowhittaker (Walkersville, MD). Krebs-Ringer buffer (KRPG) was 145 mM NaCl, 4.86 mM KCl, 1.22 mM MgSO₄, 5.7 mM Na₂HPO₄, 0.54 mM CaCl₂, and 5.5 mM glucose, pH 7.4.

Ligands. Fibronectin was from the New York Blood Center (New York, NY); mouse laminin, purified from the EHS sarcoma, was the kind gift of Dr. Hynda Kleinman (National Institute of Dental Research/National Institutes of Health, Bethesda, MD); recombinant, baculovirus-produced entactin was from (Upstate Biotechnology Inc., Lake Placid, NY); the branched peptides KGAGDV

and KGALEV were prepared using fmoc chemistry as previously described (2, 8).

Monoclonal Antibodies. The following mAbs were used in these studies: IB4 (anti-CD18) (16); 3D9 (anti-CD 35) (17); W6/32 (anti-HLA) (18); 7G2 (anti-β₃ [CD61]) (1); and anti-IAP mAb B6H12, 2D3, and 1F7 (1, 2). IB4 and 3D9 IgG were purified from ascites using octanoic acid as described (19). W6/32, 7G2, B6H12, and 2D3 IgG were prepared using a bioreactor (Amicon Corp., Danvers, MA) according to the manufacturer's instructions. SDS-PAGE of all purified IgG preparations showed them to be >90% IgG.

Phagocyte Isolation. Human PMN were isolated by dextran sedimentation and Ficoll-Hypaque density centrifugation as described (20). PMN were obtained from a β₂ integrin-deficiency patient followed at Baylor College of Medicine (Waco, TX) (patient 10 [21]). The patient's blood and a normal control were transported and the PMN prepared as described (22). Peripheral blood monocytes were isolated by countercurrent elutriation, exactly as previously described (23).

Preparation of Ligand- and mAb-coated Plates. 96-well tissue culture plates (Costar, Cambridge, MA) were coated with protein A and then mAb essentially according to the method of Berton et al. (7). Briefly, PLL-coated wells were incubated with 100 μg/ml protein A. After washing, the protein A-coated wells were incubated with various mAb at 20 μg/ml for 4 h or overnight at 4°C. In preliminary experiments, binding of all the mAb used in this study to these wells was evaluated by ELISA using goat anti-mouse IgG to detect bound antibody. As described by Berton et al. (7), protein A-coated wells bound significantly more mAb than uncoated wells, regardless of the isotype of the mAb (see Fig. 1). For the mAb used in this study, there was no reproducible difference between protein A and protein G coating. At 20 μg/ml, all antibodies bound to protein A-coated wells equivalently (Fig. 1).

To coat wells with protein or peptide ligands, Immulon 2 plates (Dynatech, Chantilly, VA) were incubated with 300 μg/ml entactin, 50 μg/ml fibronectin or laminin, or 150 μg/ml synthetic peptide in PBS at 4°C overnight. After washing with PBS, the plates were blocked with 2 mg/ml casein at room temperature for 2 h and then with 10% heat-inactivated FCS in PBS for 40 min.

H₂O₂ Assay. The microwell H₂O₂ assay was adapted from the method of De la Harpe and Nathan (24), as modified by Berton et al. (7). Briefly, 50,000 PMN or monocytes in 20 μl KRPG were added to wells that already contained 80 μl of a reaction mixture (RM) for the detection of products of the respiratory burst. RM consisted of 37.5 μM scopoletin, 1.25 mM NaN₃, 1.25 U/ml HPO in KRPG, which also contained potential activators or inhibitors of the respiratory burst. After addition of the cells, generation of H₂O₂ was detected by the decrease in fluorescence of scopoletin as measured on a Cytofluor 2300 microtiter plate fluorimeter (Millipore Continental Water Systems, Bedford, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Both excitation and emission filters had a nominal band pass of 40 nm. Measurement of fluorescence was performed immediately and then every 15 min during kinetic assays and at the end of 60 min for endpoint assays. During the intervals between measurements, plates were covered with foil and kept at 37°C. Specific release of H₂O₂ was determined by comparison to a standard curve, as previously described (24). For each experimental group data were collected from triplicate wells.

Pretreatment of PMN. PMN at 2.5 × 10⁶ cells/ml in KRPG were pretreated with 2.5 μg/ml of mAb or 100 μg/ml of synthetic peptide at 4°C for 15 min. Without washing, PMN were added to antibody- or ligand-coated plates containing RM. For treatment with pharmacologic agents, PMN were preincubated with HA1004

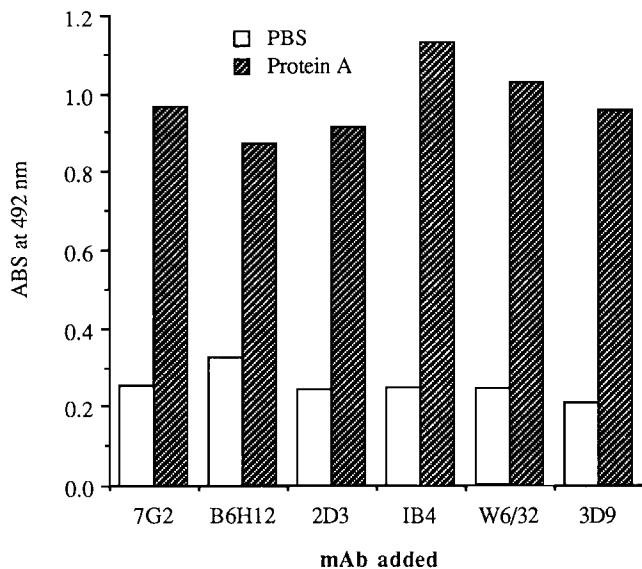


Figure 1. mAb binding to protein A immunized on tissue culture plates. The binding of the various mAbs used in this study to wells coated with protein A (▨) is compared to wells incubated with PBS alone (□). Antibody binding was detected in both cases with HRP-linked goat anti-mouse IgG. Protein A coating leads to increased binding of all mAb to the wells, and approximately equivalent binding of all mAb regardless of IgG subtype.

at 1 μ M, herbimicin at 10 μ g/ml, genestein at 50 μ g/ml, H-7 at 300 nM, or cAMP at 100 μ M, at 37°C for 15 min, and then added to RM containing the same concentration of the indicated agent. In experiments examining the effects of cytochalasin B, PMN were pretreated with 2.5 μ g/ml cytochalasin B and added to RM containing no additional drug. In some experiments, PMA (50 ng/ml) was added to RM simultaneously with PMN.

Results

Adhesive LRI Ligands Activate the Respiratory Burst in PMN and Monocytes. LRI on PMN binds the basement membrane protein entactin (9) and recognizes the peptide sequence KGAGDV (8). We examined whether these ligands could activate the respiratory burst when bound to a surface. Each ligand led to significant generation of H₂O₂ in the microwell assay by both PMN (Fig. 2) and monocytes (Fig. 3). In contrast, as previously described (4), the extracellular matrix proteins fibronectin and laminin did not elicit a respiratory burst, nor did the ligand-related control peptide KGALEV. The kinetics of entactin- and KGAGDV-stimulated respiratory burst were similar and did not show the lag phase characteristic of TNF- α or F-Met-Leu-Phe-stimulated adherent PMN (4, 5). The respiratory burst stimulated by these LRI ligands was inhibited by mAb 7G2, directed toward LRI, and mAb B6H12, directed toward IAP. The mAb 2D3, which recognizes IAP but unlike B6H12 does not inhibit LRI-dependent enhancement of phagocytosis or KGAGDV ligand binding (2, 8), also did not inhibit LRI-dependent respiratory burst (Fig. 4). mAb W6/32 (anti-HLA) and 3D9 (anti-CR1) also had no effect on peptide- or entactin-stimulated respiratory burst, even though both antibodies recognize cell surface receptors on PMN and monocytes (Fig. 4).

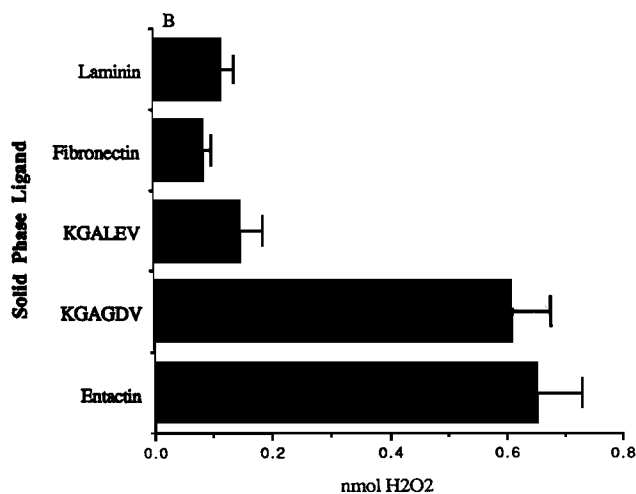
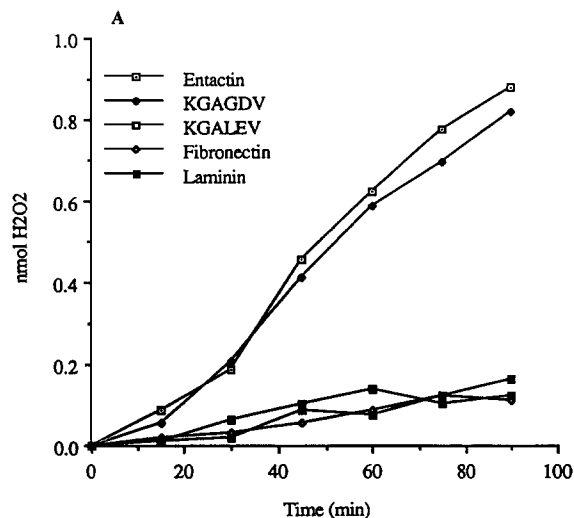


Figure 2. LRI ligands stimulate the respiratory burst in PMN. The generation of H₂O₂ by PMN incubated in wells coated with various extracellular matrix ligands or controls is compared kinetically (A) and at 60 min (B). The LRI ligands entactin and KGAGDV peptide activate a PMN respiratory burst, while the proteins laminin and fibronectin, as well as the control peptide KGALEV, do not. (A) A single experiment performed in triplicate. (B) Data shown are the mean \pm SEM of measurements at 60 min from three independent experiments, each performed in triplicate. Maximal respiratory burst stimulated with 50 ng/ml PMA was 2.5 ± 0.4 nmol H₂O₂ at 60 min.

Anti-LRI and Anti-IAP Antibodies Activate the Respiratory Burst. Recently, Berton et al. (7) showed that surface adherent anti-CD18 mAb could activate the PMN respiratory burst as a substitute for ligand. Surface-bound anti-LRI mAb 7G2 activated both PMN (Fig. 5) and monocyte (data not shown) respiratory burst to similar levels as the anti-CD18 mAb IB4. Anti-IAP mAb B6H12-stimulated H₂O₂ production by phagocytes to the same extent and with the same kinetics as the anti- β_3 mAb 7G2 (Fig. 5). The noninhibitory anti-IAP mAb 2D3, which recognizes a distinct epi-

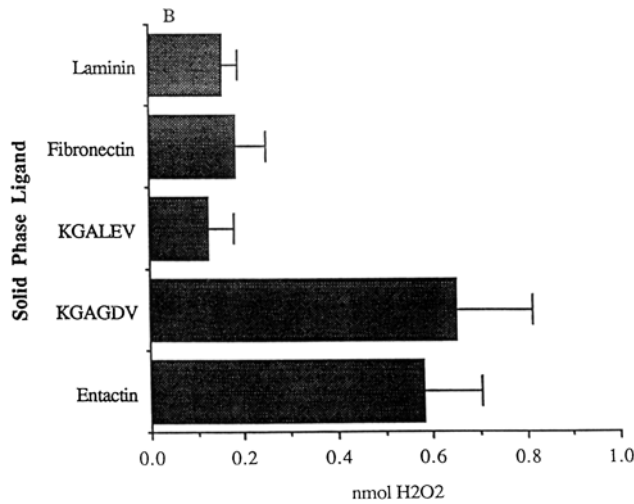
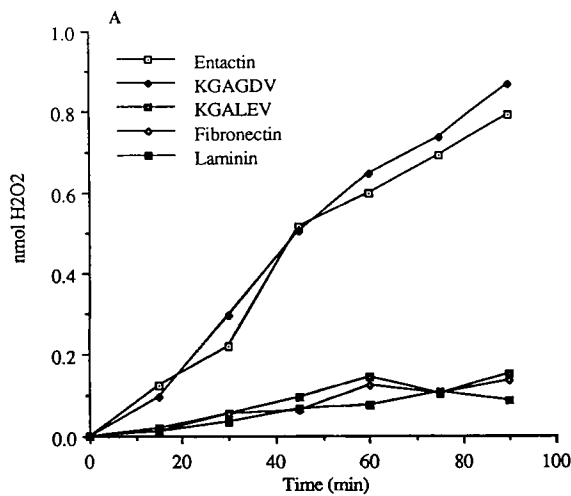


Figure 3. LRI ligands stimulate the respiratory burst in monocytes. The generation of H₂O₂ by freshly isolated peripheral blood monocytes incubated on wells coated with various extracellular matrix ligands and controls is compared kinetically (A) and at 60 min (B). Entactin and KGAGDV peptide stimulate respiratory burst activity in monocytes, but laminin, fibronectin, and KGALEV do not. (A) A single experiment performed in triplicate is shown. (B) Data shown are the mean \pm SEM of measurements at 60 min from three independent experiments, each performed in triplicate.

tope from B6H12 (2), did not activate the respiratory burst in the same assay, even though it recognizes IAP with similar affinity to B6H12 (2, 25). 1F7, a second inhibitory anti-IAP mAb, activated the PMN respiratory burst to the same extent and with similar kinetics as B6H12 (data not shown). Consistent with previous data (7), the anti-HLA mAb W6/32 did not activate the respiratory burst in either monocytes or PMN, nor did the anti-CR1 mAb 3D9.

Next, we examined the ability of LRI ligands, and anti-LRI or anti-IAP mAb, added in solution, to inhibit respiratory burst. When added in solution, mAb 7G2 (anti- β_3) and B6H12 (anti-IAP), as well as the peptide KGAGDV, inhibited 7G2-stimulated respiratory burst (Fig. 6 A). These reagents

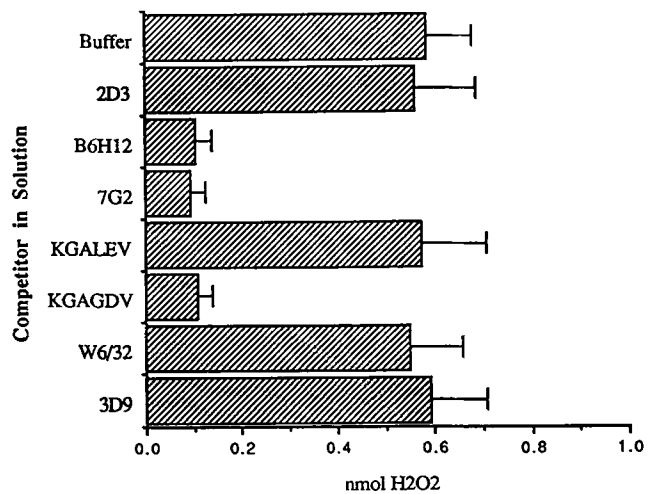


Figure 4. Inhibition of the KGAGDV-stimulated respiratory burst. PMN, pretreated with buffer (control), various mAb at 2.5 μ g/ml, or peptides at 100 μ g/ml for 15 min at 4°C, were incubated in wells coated with KGAGDV peptide. The generation of H₂O₂ was quantitated at 60 min. Data shown are the mean \pm SEM of three independent experiments performed in triplicate. 7G2, B6H12, and KGAGDV peptide inhibit the respiratory burst stimulated by adherent KGAGDV, whereas W6/32, 3D9, and the noninhibitory anti-IAP mAb 2D3 do not.

had no effect on anti-CD18-stimulated respiratory burst (Fig. 6 B). As controls, the irrelevant anti-HLA mAb W6/32, the noninhibitory anti-IAP mAb 2D3, and the inactive peptide KGALEV were used. None affected H₂O₂ generation by either stimulus. These data show that anti-LRI, anti-IAP, and the LRI ligand KGAGDV specifically inhibit the respiratory burst stimulated by surface-adherent LRI ligands and antibodies, presumably through competition with the substrate-bound activators.

LRI Ligands and Anti-LRI Antibodies Inhibit the Respiratory Burst Stimulated by Anti-IAP Antibodies. The ability of B6H12 to stimulate the respiratory burst directly when attached to a surface demonstrated that IAP was directly involved in the activation pathway. This observation also allowed a distinction among possible mechanisms of signal transduction through LRI and IAP. If IAP were activated after ligand binding to LRI, soluble LRI ligands and antibodies should not inhibit direct activation of respiratory burst by anti-IAP antibody. Alternatively, if IAP and LRI were components of a single signal transduction complex, LRI ligands in solution should compete with plate bound anti-IAP mAb and inhibit generation of the respiratory burst as they did when KGAGDV or 7G2 was the stimulating ligand in the solid phase. Indeed, KGAGDV and 7G2 in solution inhibited B6H12-stimulated respiratory burst (Fig. 7). These data are consistent with the hypothesis that LRI and IAP act as a signal transduction complex and are inconsistent with a hypothesis in which IAP activation is downstream from LRI ligation.

Anti-CD18 Inhibits LRI/IAP-stimulated Respiratory Burst via cAMP. As ligation of the CD18 receptors LFA-1 and p150,95 has been shown to activate the respiratory burst (7), it was necessary to determine whether CD18 integrins had a role

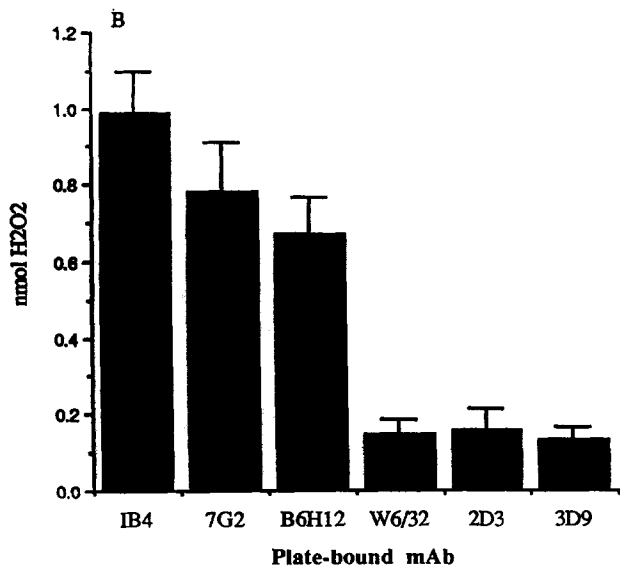
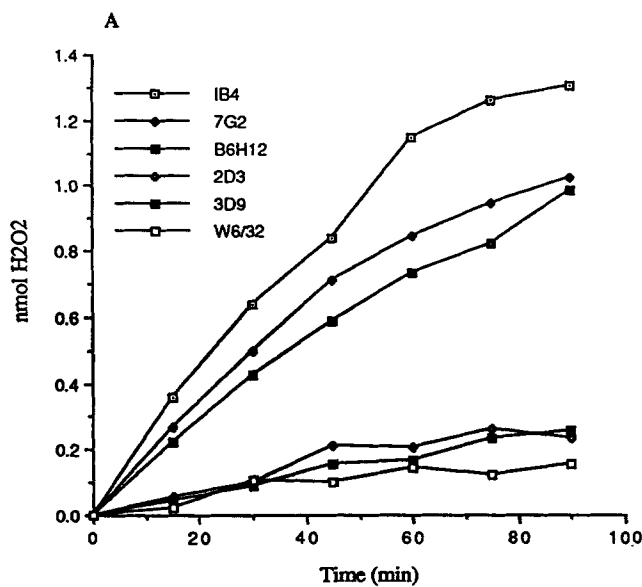


Figure 5. Surface-bound 7G2 and B6H12 stimulate the respiratory burst in PMN. The generation of H₂O₂ by PMN incubated in wells coated with various mAb is compared kinetically (A) and at 60 min (B). mAb IB4 stimulates the PMN respiratory burst, as reported (7). 7G2 and B6H12 also stimulate a respiratory burst, whereas mAb which bind to PMN but recognize unrelated antigens (3D9 and W6/32) do not. Surprisingly, the anti-IAP mAb 2D3, which is not inhibitory when added in solution, cannot stimulate a respiratory burst when adherent to a protein A-coated surface. (A) A single experiment performed in triplicate is shown. (B) Data shown are the mean \pm SEM of measurements at 60 min from three independent experiments, each performed in triplicate.

in respiratory burst activation via the LRI/IAP complex. The anti-CD18 mAb IB4 in solution inhibited 7G2-, B6H12-, as well as IB4-stimulated respiratory burst (Figs. 6 and 8 A). These data suggested the possibility that CD18-mediated cell adhesion and spreading might be necessary for LRI/IAP-

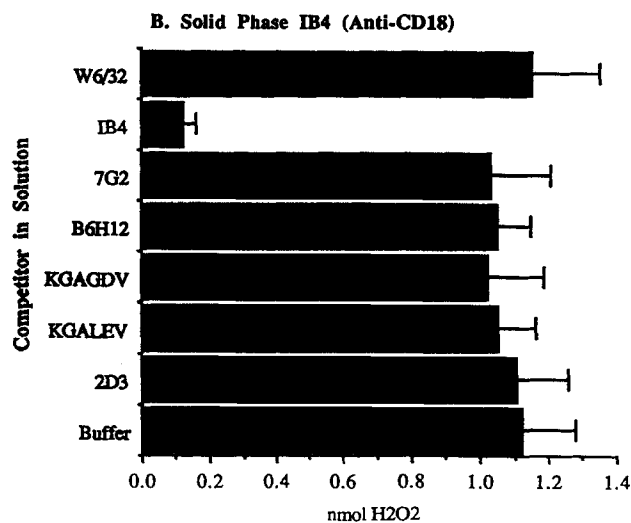
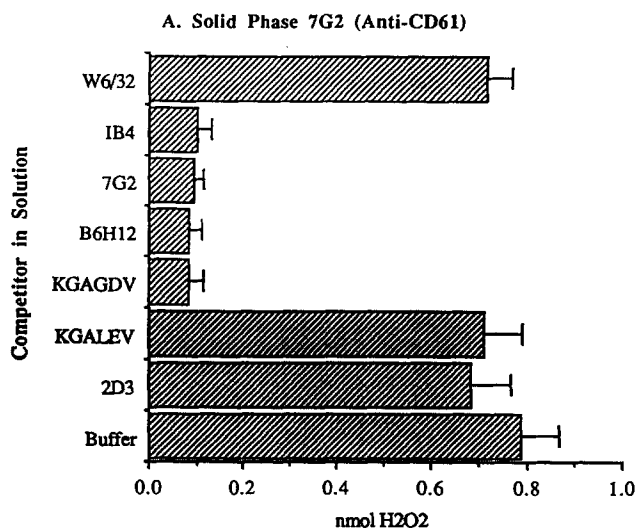


Figure 6. Inhibition of mAb-stimulated respiratory burst. The ability of various mAb and peptides to inhibit the respiratory burst generated by PMN adhesion to plate-bound 7G2 (A) and IB4 (B) were compared. 7G2, B6H12, and KGAGDV in solution all inhibited 7G2-stimulated respiratory burst, whereas controls KGALEV, 2D3, and W6/32 did not (A). None of these reagents inhibited IB4-stimulated respiratory burst (B). IB4 inhibited both 7G2- and IB4-stimulated respiratory burst. Data shown are the mean \pm SEM of measurements at 60 min from three independent experiments, each performed in triplicate.

stimulated respiratory burst. Alternatively, IB4 in solution might have inhibited because it perturbed normal signal transduction. In accord with the latter possibility, the inhibitory effect of IB4 on B6H12- and 7G2-stimulated respiratory burst was blocked entirely by the protein kinase A inhibitor HA1004 (Fig. 8 A). In contrast, the equally potent inhibition by IB4 in solution on the effect of solid phase IB4 was unaffected by HA1004. The effect of IB4 on LRI-IAP-activated H₂O₂ production was consistent with the previous observation that IB4 stimulates cAMP accumulation in PMN (22). Activation of the respiratory burst by many agonists is inhibited

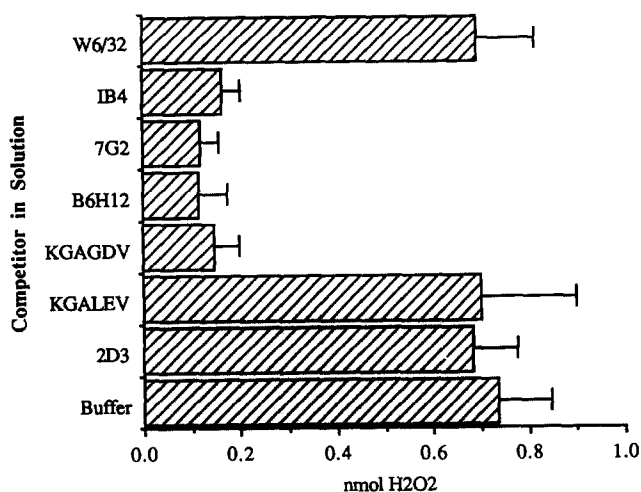


Figure 7. Inhibition of B6H12-stimulated respiratory burst. The ability of various mAb and peptides to inhibit the respiratory burst generated by PMN adhesion to plate-bound B6H12 was tested. 7G2 and KGAGDV in solution both inhibited B6H12-stimulated respiratory burst, whereas W6/32, 2D3, and KGALEV did not. Data shown are the mean \pm SEM of measurements at 60 min from three independent experiments, each performed in triplicate.

by increases in intracellular cAMP (26). We tested the effect of elevation of intracellular cAMP on mAb-stimulated respiratory burst by adding to the reaction wells Br-cAMP, which can cross the plasma membrane and increase cytoplasmic cAMP concentration. LRI/IAP-stimulated respiratory burst was much more sensitive to exogenous addition of Br-cAMP than CD18-dependent H₂O₂ production. At 100 μ M Br-cAMP, LRI/IAP-stimulated respiratory burst was almost completely inhibited, whereas CD18-dependent respiratory burst was inhibited by only 16.5% (Fig. 8 A).

We also examined whether PMN from a well-studied leukocyte adhesion deficiency (LAD) patient with the severe phenotype (21) could activate the respiratory burst in response to KGAGDV-, 7G2-, or B6H12-coated surfaces, the LAD PMN made $76 \pm 3\%$ of the H₂O₂ made by normal PMN. This demonstrated that CD18 was not necessary for the LRI/IAP-stimulated respiratory burst. The 7G2- and B6H12-stimulated H₂O₂ production was unaffected by IB4 in solution, whereas both KGAGDV and B6H12 mAb in solution inhibited the LAD PMN respiratory burst (Fig. 8 B). The 7G2- and B6H12-stimulated respiratory burst of LAD PMN was inhibited by Br-cAMP. Taken together, these data demonstrate that activation of respiratory burst through LRI/IAP is independent of CD18, but can be modulated by anti-CD18-initiated alterations in intracytoplasmic cAMP concentration.

To determine the role of the actin cytoskeleton in LRI/IAP-dependent H₂O₂ generation, cytochalasin-B-treated PMN were incubated in 7G2- and B6H12-coated wells. Cytochalasin B markedly inhibited respiratory burst activation, as was also true for CD18-dependent PMN activation (Fig. 9). In contrast, phorbol ester-stimulated respiratory burst was unaffected

by cytochalasin B treatment (Fig. 9). These data demonstrate that the actin cytoskeleton has a necessary role in integrin-dependent assembly of the respiratory burst oxidase.

Regulation of IAP/LRI-dependent Respiratory Burst Differs from the CD18-dependent Respiratory Burst. The difference in response between LRI/IAP- and CD18-initiated respiratory burst to Br-cAMP suggested that the mechanism for signaling oxidase assembly might differ between the two integrins. Because tyrosine phosphorylation has been implicated in generation of the respiratory burst (27–29), cells were treated with the tyrosine kinase inhibitors genestein and herbimycin. As shown in Fig. 9, these kinase inhibitors effectively blocked CD18-dependent respiratory burst, but had no effect on LRI/IAP-stimulated H₂O₂ production. The PKC inhibitor H7 effectively blocked respiratory burst from either CD18 or LRI/IAP stimulation, consistent with a role for PKC phosphorylation of cytosolic components during membrane translocation and assembly of the oxidase subunits (30). LRI/IAP-stimulated respiratory burst of LAD PMN was also inhibited by H7 but unaffected by herbimycin (data not shown). Thus, LRI/IAP-mediated respiratory burst is inhibited by cAMP, but insensitive to protein tyrosine kinase inhibitors, whereas CD18-dependent respiratory burst has the opposite phenotype. These data demonstrate that LRI and CD18 integrins activate the NADPH oxidase by distinct but overlapping signal transduction pathways.

Discussion

In this work we have examined the ability of the LRI/IAP signal transduction pathway to stimulate phagocyte respiratory burst and have used this function to examine the interaction of LRI and IAP. When adherent to a surface, the LRI ligands entactin and KGAGDV can activate the respiratory burst in PMN and monocytes. This makes these ligands quite different from other integrin ligands which do not activate the respiratory burst on their own (4, 31). Although the reason for this is unknown at present, respiratory burst activation by these ligands is consistent with the observation that LRI binding of entactin and KGAGDV does not require prior PMN activation (9), unlike β_1 or β_2 integrin-mediated adhesion. These other leukocyte integrins are thought to exist in a low affinity state on resting cells and to achieve substantial ligand binding affinity only after the cells have received an additional activating signal (32, 33). The discovery that this is not the case for LRI has led to the hypotheses that LRI is constitutively active and that LRI-entactin interaction represents a very early step in leukocyte extravasation to sites of inflammation. The lack of a requirement for cell activation to achieve RGD-dependent ligand binding by LRI may explain both the direct activation of the respiratory burst by LRI ligands and the lack of the lag phase which has been observed during the β_2 -dependent respiratory burst elicited by TNF- α or F-Met-Leu-Phe in PMN adherent to extracellular matrix proteins.

In the fluid phase, LRI ligands cannot activate the respiratory burst, even when they are multivalent, as in the branched KGAGDV peptide. In this regard, activation of the respira-

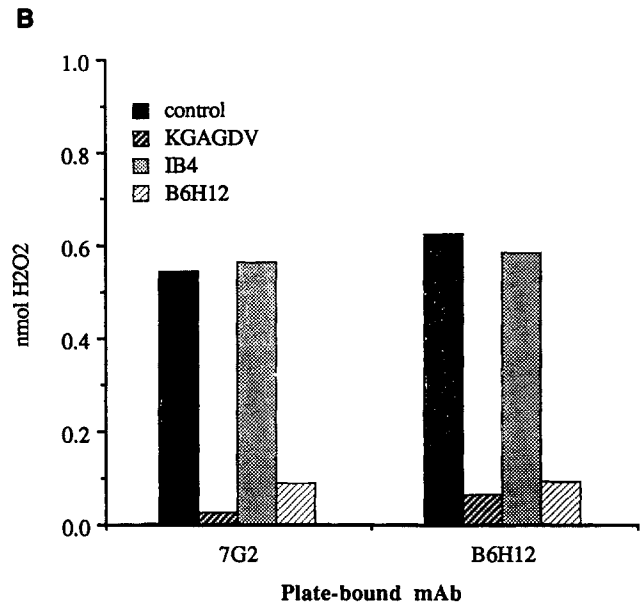
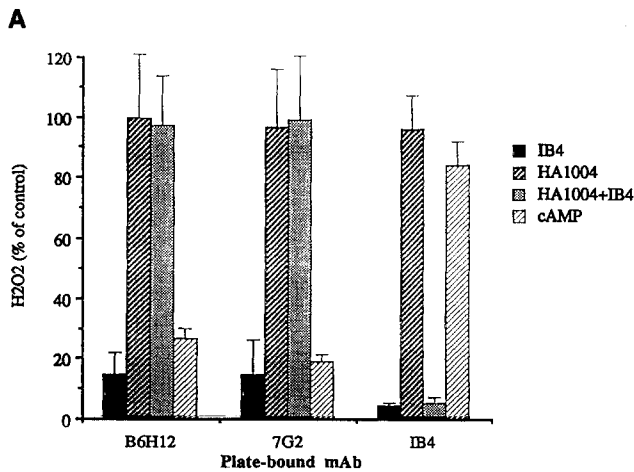


Figure 8. Effect of HA1004 on IB4 inhibition of LRI/IAP-stimulated respiratory burst and LRI/IAP-stimulated respiratory burst in LAD PMN. (A) The effect of 1 μ M HA1004, a cAMP-dependent kinase inhibitor, on IB4 inhibition of 7G2- and B6H12-stimulated respiratory burst was tested. Whereas HA1004 had no effect on 7G2- or B6H12-stimulated respiratory burst, it completely reversed the IB4 inhibition of the H_2O_2 production stimulated by these mAb. This is consistent with previous data that IB4 can stimulate cAMP generation by PMN (22). HA1004 had no effect on IB4 inhibition of IB4-stimulated respiratory burst. The respiratory bursts stimulated by both B6H12 and 7G2 are sensitive to 100 μ M Br-cAMP, whereas that stimulated by IB4 is not. At 500 μ M Br-cAMP also inhibited the IB4-stimulated respiratory burst (data not shown). Data shown are the mean \pm SEM of measurements done at 60 min from three independent experiments, each done in triplicate. (B) Respiratory burst stimulated by plate-bound 7G2 and B6H12 in LAD PMN. LAD PMN were incubated with LRI/IAP ligands and tested for respiratory burst and inhibition by fluid phase ligands as in Fig. 6. KGAGDV peptide and B6H12 mAb in the fluid phase inhibit the respiratory burst from LAD PMN, but IB4 does not. W6/32 (anti-HLA)- and 2D3 (noninhibitory anti-IAP)-coated surfaces did not stimulate a respiratory burst in LAD PMN (data not shown). Data are from a single experiment performed in triplicate.

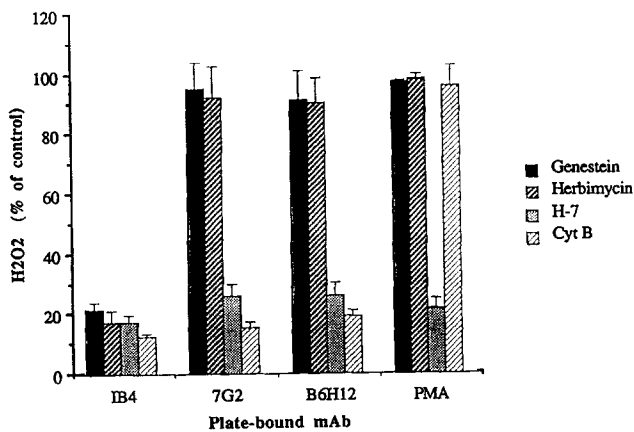


Figure 9. Pharmacologic agents distinguish between LRI/IAP- and CD18-dependent respiratory burst. The effects of cytochalasin B (2.5 μ g/ml), and the protein kinase inhibitors genestein (50 μ g/ml), herbimycin (10 μ g/ml), and H-7 (300 nM) on mAb-stimulated respiratory burst were examined. Cytochalasin B and H-7 inhibited both LRI/IAP- and CD18-dependent pathways for generation of the respiratory burst. In contrast, the tyrosine kinase inhibitors genestein and herbimycin affected CD18-dependent H_2O_2 production preferentially. The H-7 dose used to inhibit PMA-stimulated respiratory burst was 650 nM; for the other stimuli 300 nM H-7 was used. Data shown are the mean \pm SEM of measurements done at 60 min from three independent experiments, each done in triplicate.

burst differs from stimulation of Fc receptor-mediated phagocytosis, which can be activated by fluid phase LRI ligands (1, 2, 8, 22). This suggests that LRI ligation is not sufficient to activate the respiratory burst, and that the cytoskeletal rearrangements that accompany adhesion provide additional required signals. It is possible that the difference between this assay system and stimulated phagocytosis is that IgG Fc receptor ligation during phagocytosis provides some additional biochemical signals for cell activation. Adhesion itself may play a similar role during generation of the respiratory burst. For many cells, including PMN, integrin-mediated adhesion is known to have this priming or enhancing role on signal transduction pathways (4, 5, 26, 27, 34, 35).

Some anti-LRI and anti-IAP antibodies are equally capable as LRI ligands for activating the respiratory burst. Only anti-LRI and anti-IAP antibodies which when in solution can inhibit ligand activation are capable of activating phagocytes themselves when surface adherent. This suggests that simple aggregation of these antigens on the adherent surface of the cells is not sufficient to activate the respiratory burst. Rather, some essential signal must be provided by the surface-bound antibodies. Possibly, the antibodies stabilize a conformation of LRI or IAP capable of transducing a signal when aggregated. This hypothesis is consistent with data showing

that integrins (36–40) and IAP (25) can exist in different conformations in the plasma membrane. LRI ligands and anti-LRI antibody in solution inhibited the respiratory burst generated from anti-IAP-coated surfaces. This suggests that direct interaction with IAP does not bypass the integrin receptor in the signal transduction pathway for phagocyte activation. Rather, inhibitory reagents directed at either LRI or IAP can prevent activation stimulated by ligation of the other component. These data suggest a model in which LRI and IAP act as a signal transduction unit. This is consistent with a physical interaction between LRI and IAP as has been shown for $\alpha_v\beta_3$ and IAP (2). This hypothesis is analogous to that suggested for signal transduction from several cytokine receptors which interact with additional, nonligand binding plasma membrane proteins. Interaction with these additional components not only regulates ligand affinity, but also is required for cytokine effects on the target cells (11–15, 41, 42). Thus, the role for IAP may be to regulate ligand binding and integrin-mediated signal transduction for receptors of the LRI/ β_3 family.

Finally, these data demonstrate that the adhesion-dependent respiratory burst induced by the LRI/IAP complex is CD18 independent and proceeds by a signal transduction pathway distinct from that activated by CD18 ligation. Whereas CD18-independent adhesion in PMN has been described, its biological role has been unexamined until now (9, 43, 44). Soluble anti-CD18 fails to inhibit the LRI/IAP-dependent respiratory burst in LAD PMN and in normal PMN in the presence of the protein kinase A inhibitor HA1004. Since HA1004 does not block CD18-dependent adhesion (Fig. 8 A) or phagocytosis (22), these experiments show that, although LRI/IAP-generated H_2O_2 production requires adhesion, it is CD18 in-

dependent. This is consistent with previous data that PMN adhesion to the LRI ligand entactin is CD18 independent (9). Moreover, the signal transduction pathway for LRI/IAP stimulation of the respiratory burst can be distinguished from CD18-dependent respiratory burst. The LRI/IAP-dependent oxidative burst is inhibited by exogenous cAMP at concentrations that do not affect CD18-induced H_2O_2 production. In contrast, the CD18-induced respiratory burst is inhibited by low concentrations of tyrosine kinase inhibitors, which do not affect the LRI/IAP-induced respiratory burst at all. Taken together, these experiments demonstrate immunologically and pharmacologically that the LRI/IAP-induced respiratory burst, although dependent on cell adhesion, is independent of CD18.

In conclusion, this work has elucidated a novel pathway for signal transduction and phagocyte activation by extracellular matrix. This pathway requires not only ligand binding by an integrin, the LRI, but also an associated nonintegrin protein which is an integral part of the signal transduction complex. Interestingly, this complex can bind to the basement membrane protein entactin without prior cell activation, a feature shared by no other leukocyte integrin, including CD18 integrins (43). This feature, and the fact that interaction of PMN with 7G2- or B6H12-coated surfaces activates CD18 integrins (45) suggest that when the endothelial layer is not intact or does not make a tight junction, ligand binding by LRI/IAP may be a very early step in leukocyte activation during diapedesis into sites of inflammation and infection. The LRI/IAP signal transduction pathway may represent an important and specific target for intervention in the inflammatory process.

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