Immune Complex-stimulated Neutrophil LTB_4 Production Is Dependent on β_2 Integrins

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Abstract. The β_2 integrins (LFA-1, Mac-1, and p150,95) are critical for many adhesive functions of leukocytes. Although the binding of the IgG-opsonized particles occurs normally in the absence of β_2 integrins, phagocytosis of IgG-opsonized particles by activated neutrophils (PMN) requires these integrins. This observation suggests a role for β_2 integrins in phagocytosis subsequent to particle binding. To investigate the mechanism of involvement of β_2 integrins in IgG-mediated functions, we examined the role of β_2 integrins in adhesion to immune complex (IC)-coated surfaces. Initial adhesion and spreading on IC-coated surfaces were equivalent in control and β_2-deficient phagocytes. However, both genetically β_2-deficient PMN and PMN treated with the anti-β_2 mAb IB4 subsequently detached from the IC-coated surfaces. To determine whether biochemical consequences of IgG activation were also affected by β_2 deficiency, LTB_4 production in response to Fc receptor ligation was assessed. LTB_4 production by β_2-deficient PMN adherent to IC-coated surfaces was markedly decreased in comparison with control PMN. Importantly, LTB_4 production by PMN stimulated with fluid phase heat-aggregated IgG also required the β_2 integrins, showing that the defect was not a simple consequence of abnormal adhesion. In contrast, superoxide production by IC-adherent PMN was equivalent in control and β_2-deficient PMN. The initial rises in intracytoplasmic $[Ca^{2+}]_i$ in response to aggregated IgG also were unaffected by inhibition of β_2 integrins. These data show that lack of β_2 integrins does not inhibit all FcR-dependent signal transduction. Finally, LTB_4 production by normal PMN adherent to ICs was inhibited by antibodies to FcRII, but not FcRIII, showing that FcRII ligation was required for this effect. Together these data identify a role for the β_2 integrins in a signal transduction pathway leading to sustained adhesion and LTB_4 production in response to IC. Since both β_2 integrins and FcRII are required for these effects, the data further suggest cooperation between these receptors in generating PMN activation in response to IC stimulation.

THE β_2 integrins are a family of leukocyte receptors which includes LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). These heterodimeric glycoproteins share a common β chain (β_2), and each has a unique α chain (38, 42). A severe host defense defect, known as leukocyte adhesion deficiency (LAD),[1] is associated with the congenital deficiency or absence of the β_2 integrins (1, 2). These patients are subject to recurrent, life-threatening infections. The predominant in vivo abnormality in leukocyte function in LAD is the inability to accumulate neutrophils (PMN) at inflammatory sites.

Both LFA-1 and Mac-1 are involved in leukocyte binding to endothelial cells via recognition of intercellular adhesion molecule-1 (10, 27). LFA-1 also recognizes an additional endothelial receptor, intercellular adhesion molecule-2 (41). Alternative mechanisms of leukocyte endothelial binding are intact in β_2-deficient polymorphonuclear leukocytes (PMN), yet in most instances the β_2-deficient PMN remain unable to migrate to sites of inflammation (12, 40). In addition, β_2-deficient PMN demonstrate abnormal adhesion and spreading on many surfaces in vitro (5). The involvement of β_2 integrins in homotypic PMN aggregation and in phagocytosis of IgG- and C3b-opsonized particles are other examples of the widespread importance of β_2 integrins for leukocyte adhesion-dependent functions (7). The involvement of the β_2 integrin Mac-1 in phagocytosis is particularly intriguing because LAD PMN and monocytes can bind the IgG- and C3b-opsonized particles normally, yet are markedly abnormal in the ingestion of these particles (17). This observation suggests a role for Mac-1 in ingestion of IgG-opsonized particles at some step subsequent to particle binding. Although several studies have investigated the mechanism by which cell activation enhances β_2 integrin avidity for ligands (7, 18, 43), any role for these integrins in signal transduction leading to cell activation remains controversial. Because the defects in IgG-mediated phagocytosis that are

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1. *Abbreviations used in this paper*: FMLP, formyl-met-leu-phe; IC, immune complexes; LAD, leukocyte adhesion deficiency; PMN, polymorphonuclear leukocytes.

apparent in LAD cells might imply such a signal transduction role for these integrins, we have studied this system in some detail.

In this study we have investigated the mechanism of involvement of the β_2 integrins in PMN interactions with IgG-containing immune complexes (ICs). We have found that, although the initial adhesion and spreading of β_2-deficient PMN on IC-coated surfaces is normal, the β_2-deficient PMN are unable to progress to the stage of actin reorganization in which the PMN become "morphologically polarized," associated with a concentration of actin at the anterior border. Since chemoattractants are known to cause this morphologic polarization of PMN, and since LTB_4 is generated by IC-stimulated PMN, we investigated the role of the β_2 integrins in IC-stimulated LTB_4 production. We have found that LTB_4 generation by PMN adherent to IC-coated surfaces, or stimulated by fluid phase aggregated IgG involves the β_2 integrins. This demonstration of a role for the β_2 integrins in receptor-mediated LTB_4 generation suggests the intriguing hypothesis that a deficiency in LTB_4 generation may contribute to the chemotactic defect in β_2-deficient PMN. Moreover, these data establish a role for β_2 integrins in signal transduction leading to PMN activation by ICs.

Materials and Methods

Cells

PMN from normal volunteers were isolated as described (15). PMN from two LAD patients, followed at Baylor College of Medicine, who have a complete absence of β_2 expression were obtained as described (17).

Monoclonal Antibodies

IB4 and 60.3 (both anti-β_2, IgG2a) were the generous gifts of Dr. Sam Wright (Rockefeller University, New York, NY) (44) and Dr. Patrick Beatty (University of Washington, Seattle, WA), respectively (3). The cell line that produces W632 (anti-HLA-I, IgG2a) was purchased from the American Type Culture Collection, Rockville, MD. IB4 and W632 were purified from ascites using protein A–Sepharose. $F(ab')_2$ fragments of IB4 were prepared as described (14). 3G8 $F(ab')_2$ (anti-FcRIII) and IV.3 Fab (anti-FcRII) were obtained from Medarex, Inc., West Lebanon, NH.

Adhesion Assay

Glass coverslips (13 mM) were treated with poly-L-lysine as described (14), then coated with 100 μg/ml BSA and followed by anti-BSA IgG (1.2–20 μg; Sigma Chemical Co., St. Louis, MO) to generate IC-coated surfaces. PMN (3×10^5) in 300 μl RPMI buffer were preincubated ±30 μg/ml of IB4 (anti-β_2 mAb) at 37°C for 15 min, followed by adhesion to IC-coated coverslips at 37°C in a 5% CO_2 incubator for the indicated times. Coverslips were then washed once with 37°C buffer, fixed with glutaraldehyde, stained with Giemsa, and mounted on slides with Permount (Fisher Scientific Co., Pittsburgh, PA). Adhesion was quantitated as the mean number of phagocytes adherent per 40× high power field.

F-Actin Staining

PMN were allowed to adhere to IC-coated coverslips for the indicated times at 37°C. Cells were then extracted for 30 s on ice with Triton buffer (10 mM PIPES, pH 6.8, 0.5% Triton, 300 mM sucrose, 100 mM KCl, 3 mM $MgCl_2$, and 10 mM EGTA), washed once with ice-cold Triton buffer, and fixed with 3% paraformaldehyde (50 mM KCl, 25 mM PIPES, pH 7.0, 10 mM $MgSO_4$, 5 mM EGTA, and 3% paraformaldehyde) for 20 min at room temperature. Cells were stained with 160 nM rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) in PBS for 20 min at room temperature. Coverslips were mounted on glass slides in 50% glycerol/50% PBS with 0.1 M propyl gallate. Finally, they were viewed with a Nikon epifluorescence photomicroscope.

LTB_4 Assay

Samples of 3×10^5 PMN were prepared in 300 μl of RPMI supplemented to 1 mM Ca^{2+} and Mg^{2+}. Monoclonal antibodies were centrifuged at 100,000 *g* for 10 min to remove aggregates before each experiment. After preincubation for 1 h at room temperature with and without ±5 μg/ml of the various mAb, PMN were incubated for the indicated times at 37°C in 24-well plates containing IC-coated coverslips. Plates were then placed on ice and supernatants were harvested and centrifuged at 800 *g* for 10 min. In samples containing 3G8, IV.3, or the respective controls the final concentration of azide was 0.0001%. Heat-aggregated IgG was prepared using human IgG as described (14). Aggregated IgG was added at 300 μg/ml to PMN in suspension at 1×10^6/ml immediately before incubation for 25 min at 37°C. The studies of the time course for LTB_4 production in response to aggregated IgG or formyl-met-leu-phe (FMLP) were done by adding 300 μg/ml aggregated IgG or 10^{-6} M FMLP to 3×10^5 PMN in the fluid phase in 24-well plates and incubating for the indicated times. LTB_4 in the supernatants was quantitated in duplicate by RIA as described (24).

Lysozyme Assay

Samples of 2×10^6 PMN were prepared in 400 μl RPMI supplemented to 1 mM Ca^{2+} and Mg^{2+}. After preincubation for 1 h at room temperature with 5 μg/ml of mAb, PMN were incubated with 10^{-6} M FMLP for 15 min at 37°C in 24-well plates. Lysozyme was quantitated in the supernatant as described (28).

Superoxide Assay

Superoxide anion was quantitated by cytochrome *c* reduction as described (16). PMN were preincubated for 15 min at 37°C ± 30 μg/ml of IB4 (anti-β_2). Reaction mixtures (0.5 ml of HBSS with 1.5 mM Ca^{2+} and 1.5 mM Mg^{2+} with 1% human serum albumin) were prepared on ice containing 3×10^5 PMN and 80 μM cytochrome *c* in 24-well plates coated with ICs as described above. Samples were prepared in duplicate. Additional duplicates containing 125 μg/ml of superoxide dismutase were also prepared. Samples were incubated for 20 min at 37°C. Supernatants were centrifuged at 4°C at 12,500 *g* for 5 min, followed by measurement of the OD at 550 nm. Superoxide levels are reported as the superoxide dismutase inhibitable nanomoles of cytochrome *c* reduced per 3×10^5 PMN/20 min (32).

Intracytoplasmic Ca^{2+} Concentration

PMN were loaded with 2 μM fura-2 (Molecular Probes, Inc.) as described (33). Intracytoplasmic Ca^{2+} concentration was determined in samples of 2×10^6 PMN/ml with a spectrofluorimeter (model F2000; Hitachi, Ltd., Tokyo, Japan) with a 37°C stirred cell as described (13).

Arachidonate Measurements

PMN were allowed to adhere to IC-coated coverslips under conditions identical to those used for evaluation of LTB_4 production. The buffer was the same except for the addition of 1 mg/ml of fatty acid–free BSA to serve as a trap for released arachidonate. After adhesion, supernatants from 3×10^6 PMN were pooled for each point. Arachidonate was extracted and assayed by mass spectrometry as described (25). Control PMN were treated with the isotype-matched mAb W632. Samples were assayed in duplicate.

Results

Sustained Adhesion to IC-coated Surfaces Requires β_2 Integrins

To begin to investigate the mechanism of involvement of the β_2 integrins in IgG-mediated functions we examined the adhesion of PMN to IC-coated surfaces. Initially we measured the number of cells adherent to the IC-coated surfaces at sequential time points, comparing control PMN and PMN treated with $F(ab')_2$ of the anti-β_2 mAb IB4 (Fig. 1 *A*). At the 5- and 12-min time points the same number of control and $F(ab')_2$-treated PMN were adherent. Control PMN continued to maintain adhesion to the IC-coated surface throughout a 40-min assay. In contrast, the $F(ab')_2$-treated cells

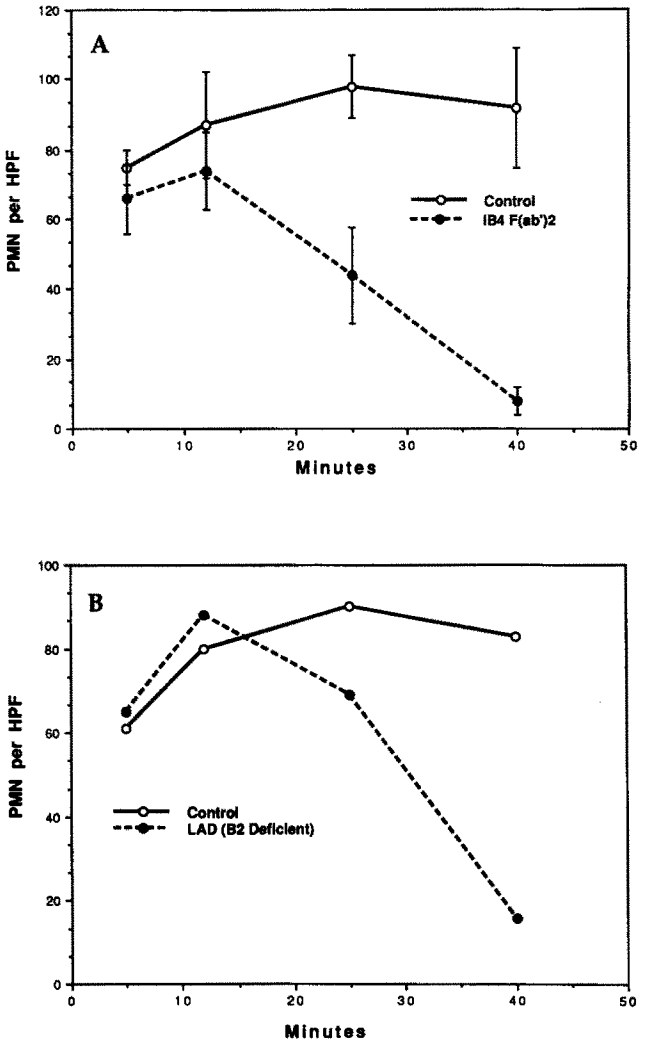

Figure 1. (*A*) PMN adhesion to IC-coated surfaces. PMN were preincubated with or without IB4 F(ab')$_2$ (anti-β_2 mAb) and then allowed to adhere to IC-coated surfaces for the indicated times. Control PMN maintain adhesion throughout the 40-min assay. In contrast, β_2 mAb-treated PMN initially adhere well, but subsequently detach from the surface. Adhesion is quantitated as the mean number of PMN/high power field ± SEM, $n = 3$. (*B*) Adhesion of genetically β_2-deficient PMN to IC-coated surface. Like the β_2 mAb-treated PMN, β_2-deficient PMN initially adhere well, but are unable to sustain adhesion to IC-coated surfaces. A representative experiment is shown; $n = 3$.

showed a progressive loss of adhesion, and few cells remained attached by 40 min. Genetically β_2-deficient PMN from a patient with LAD showed a similar normal early adhesion, but like the mAb-treated cells were unable to sustain adhesion to this surface (Fig. 1 *B*). Although there is no identified ligand for any β_2 integrin on an IC-coated surface, sustained adhesion to this surface requires β_2 integrins.

Actin Distribution in PMN Adherent to IC-coated Surfaces

To investigate why β_2 integrins are necessary for sustained adhesion to IC-coated surfaces, we examined the actin distribution of control PMN and genetically β_2-deficient PMN adherent to IC. After adhesion for 5 min to ICs both the control and β_2-deficient PMN had spread and showed a similar redistribution of actin to the periphery of the cell (Fig. 2). By the 12-min time point, the control PMN had become morphologically polarized (Fig. 2). These polarized cells had a crescentic shape with an anterior ruffling border and also central punctate actin staining. The control cells maintained this polarization and sustained adhesion throughout a 40-min assay. In contrast, although the β_2-deficient PMN remained adherent and spread after 12 min, these PMN were unable to progress to the stage of morphologic polarization. Subsequently, by the 40-min time point, the β_2-deficient PMN had contracted and detached from the plate (Fig. 2). In addition, PMN treated with the β_2 mAb IB4 showed a similar inability to progress to morphologic polarization, and by the late time points had contracted and detached from the IC-coated surface (data not shown). Therefore the β_2 integrins are required for the second stage of actin redistribution associated with morphologic polarization, and for sustained adhesion to IC-coated surfaces.

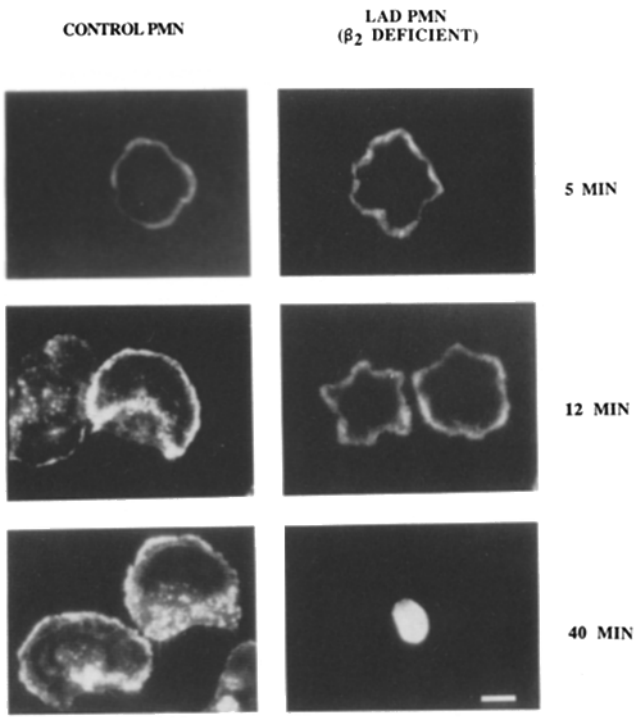

Figure 2. Actin distribution of PMN adherent to IC-coated surfaces. Control or genetically β_2-deficient PMN were allowed to adhere to IC-coated surfaces for the indicated times, and then stained with phalloidin to demonstrate the filamentous actin distribution. *5 min*, both control and β_2-deficient PMN are adherent and spread, with a similar peripheral distribution of f-actin. *12 min*, control PMN have developed a polarized morphology with an anterior ruffling border and posterior retraction fibers. β_2-deficient PMN remain adherent, but have failed to polarize. *40 min*, control PMN remain adherent and polarized. β_2-deficient PMN have contracted and are detaching from the surface.

LTB_4 Production by PMN Adherent to IC-coated Surfaces

LTB_4 is an arachidonic acid metabolite known to be produced by IC-stimulated PMN (9). LTB_4 is a potent chemoattractant, which can activate PMN with a resultant morphologic polarization (46). In adherent cells polarization is

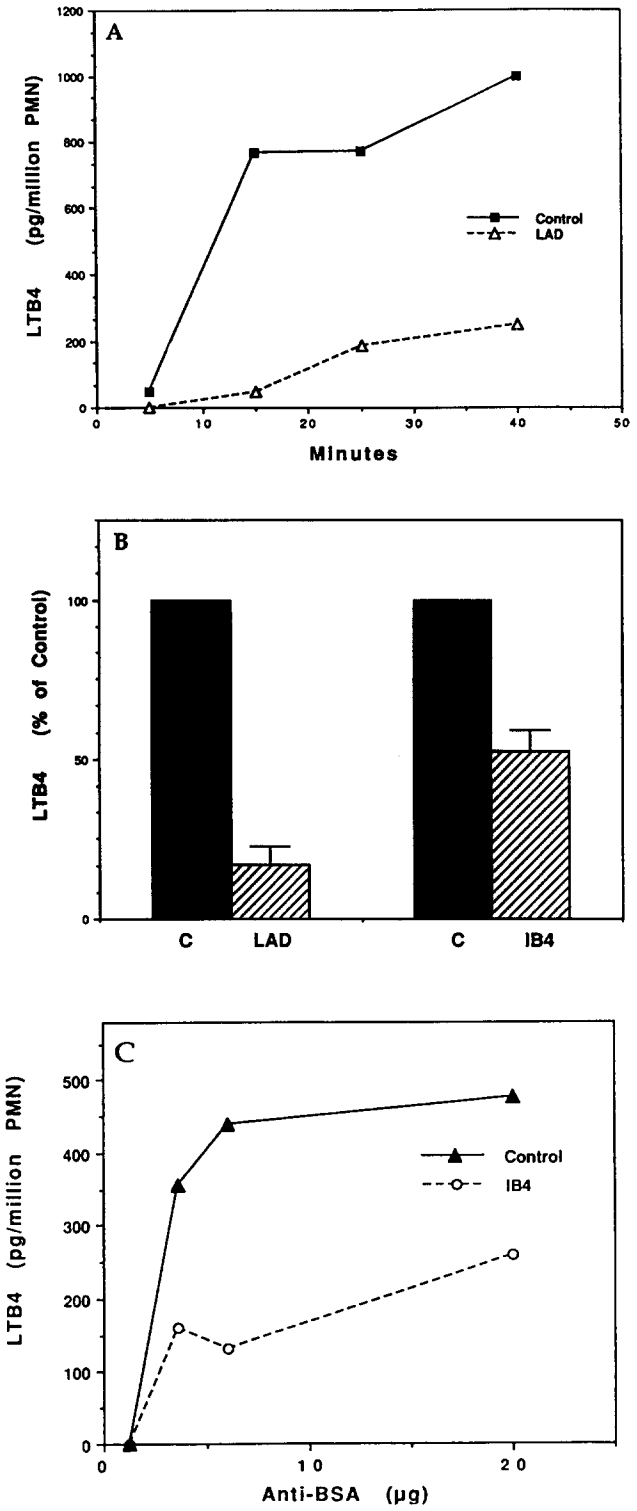

Figure 3. (*A*) LTB_4 production by control and genetically β_2-deficient PMN (LAD) adherent to IC-coated surfaces. LTB_4 was quantitated in the supernatant by RIA. LTB_4 production by the LAD cells was significantly lower than control PMN throughout a 40-min time course. A representative experiment is shown; $n = 3$. (*B*) LTB_4 production by PMN adherent to IC-coated surfaces is compared at the 15-min time point. The LTB_4 production by the genetically β_2-deficient PMN (LAD) is <20% of the amount produced by control PMN (presented as the mean ± SEM; $n = 3$). β_2 mAb (IB4, IgG2a)-treated PMN are compared with control PMN treated with an isotype-matched mAb (W632, anti-HLA I, IgG2a). LTB_4 production by the IB4-treated PMN is 52% of the amount produced by control PMN (presented as the mean ± SEM, $n = 6$). (*C*) LTB_4 production by PMN adherent to ICs (BSA/anti-BSA) made with increasing concentrations of anti-BSA ab. A representative experiment is shown. IB4 inhibits LTB_4 production at all concentrations tested that generate LTB_4 (% inhibition ± SEM, 3.6 μg: 62 ± 10%; 6 μg: 60 ± 8%; 20 μg: 52 ± 7%; $n = 4$). ICs used in *A* and *B* were made with 20 μg anti-BSA ab.

associated with both a ruffling border at the advancing edge and posterior retraction fibers. Since the β_2-deficient PMN were unable to progress to this morphologically polarized stage when adherent to ICs, LTB_4 production was assessed. Control and β_2-deficient PMN were allowed to adhere to IC-coated surfaces for the indicated times and the LTB_4 produced was measured (Fig. 3 *A*). LTB_4 production by the β_2-deficient PMN was markedly decreased at all time points. At 15 min, when adhesion was similar between LAD and controls, LTB_4 production by β_2-deficient PMN was <20% of the amount produced by control PMN (Fig. 3 *B*). Similarly, treatment of control PMN with IB4 (an anti-β_2 mAb) resulted in a 40–50% reduction in LTB_4 production (Fig. 3 *B*). Treatment of control PMN with 60.3, an alternative β_2 mAb, also decreased LTB_4 production by 40 ± 10% (SEM, $n = 3$). IB4 consistently inhibited LTB_4 production by PMN adherent to IC-coated surfaces over a range of IC densities (48–62%, Fig. 3 *C*). These data demonstrate that β_2 integrins are involved in LTB_4 production by PMN adherent to IC-coated surfaces. The addition of exogenous LTB_4 (10^{-5}–10^{-9} M) to the β_2-deficient PMN failed to correct the adhesion abnormality in the β_2-deficient PMN, suggesting that abnormalities in addition to LTB_4 synthesis may prevent normal adhesion and polarization in LAD cells.

Fluid Phase–Aggregated IgG-stimulated LTB_4 Production Is Also Dependent on β_2 Integrins

Adhesion and spreading of β_2-deficient PMN is abnormal on many different surfaces. This abnormal adhesion might provide an explanation for the lack of LTB_4 production in response to adherence to IC in LAD- or IB4-treated cells. We therefore tested whether the requirement for the β_2 integrins in LTB_4 production was unique to an IC-coated surface, or whether the β_2 integrins were also required for LTB_4 production initiated by a fluid phase IgG FcR ligand. Control and β_2-deficient PMN were stimulated with heat-aggregated IgG, and LTB_4 production was assayed by RIA. β_2-deficient PMN produced <5% of the amount of LTB_4 produced by aggregated IgG-stimulated control PMN (Fig. 4 *A*). Similarly, PMN pretreated with IB4 (anti-β_2 mAb) produced only 25% of the LTB_4 generated by control PMN (Fig. 4 *A*). The time course of LTB_4 production was examined for fluid phase aggregated IgG (Fig. 4 *B*). This demonstrates that LTB_4 is first measurable at the 15-min time point, and that IB4 inhibits LTB_4 production at both the 15- and 25-min time points.

To determine whether other stimuli for LTB_4 synthesis also required β_2 integrins, adherent and suspension PMN were incubated with 1 μM FMLP. PMN in suspension produced no LTB_4 in response to this high agonist concentration. However, adherent PMN produced 26 pg/million PMN

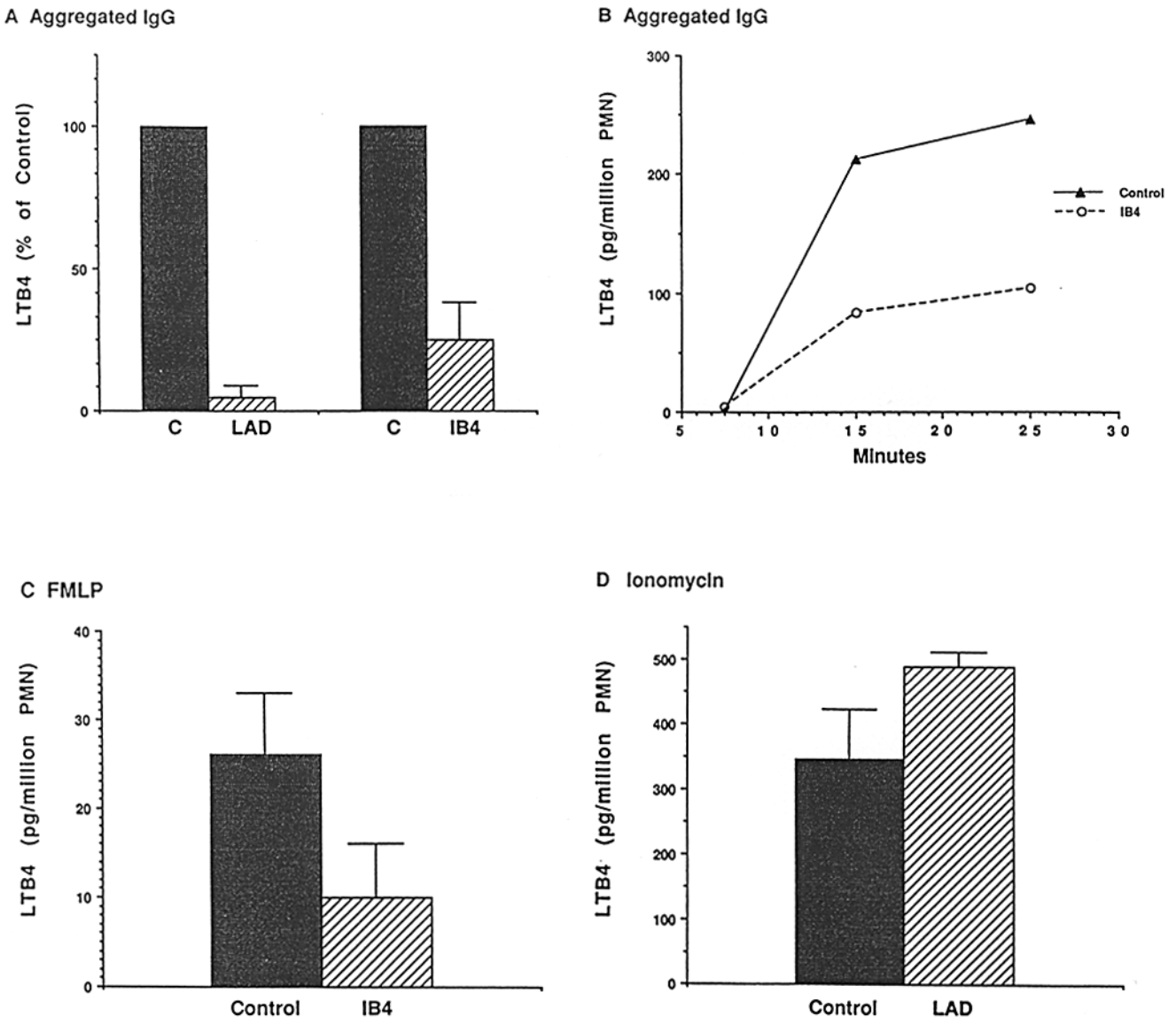

Figure 4. (A) LTB_4 production by PMN stimulated with fluid phase aggregated IgG. Genetically β_2-deficient PMN (LAD) produce only 4% as much LTB_4 as control PMN. (Mean LTB_4 production by control PMN was 151 pg/million PMN in 25 min; $n = 3$.) IB4 (anti-β_2)-treated PMN produce 25% as much LTB_4 as control PMN stimulated with aggregated IgG. A similar result was obtained using IB4 $F(ab')_2$ fragments ($n = 4$). (*B*) Time course of LTB_4 produced by PMN stimulated with fluid phase aggregated IgG. There is no detectable LTB_4 produced by 7.5 min after stimulation. IB4 inhibits LTB_4 production at 15 min (60 ± 7%) and 25 min (55 ± 5%). Data are % inhibition ± SEM; $n = 4$. (*C*) LTB_4 produced by PMN stimulated with FMLP. IB4 inhibits LTB_4 production by 60%; $n = 4$. (*D*) LTB_4 production by PMN stimulated with ionomycin. Control or β_2-deficient PMN were treated with 10 μM ionomycin for 10 min at 37°C. An equivalent amount of LTB_4 was produced by the control and the β_2-deficient PMN ($n = 3$). Data are presented as the mean ± SEM.

in response to 1 μM FMLP, and this synthesis was partially inhibited by IB4 mAb (Fig. 4 *C*). This suggests that PMN adhesion, partly mediated by β_2 integrins, may prime PMN for FMLP-induced synthesis of LTB_4. This may be analogous to the priming effect of β_2-dependent PMN adhesion on tumor necrosis factor-α-stimulated superoxide production (31).

Finally, the role of the β_2 integrins in ionomycin-stimulated LTB_4 production was examined. β_2-deficient PMN generated an equivalent amount of LTB_4 as ionomycin-stimulated control PMN (Fig. 4 *D*). Similarly, IB4 did not inhibit ionomycin-stimulated LTB_4 production. This shows that LTB_4 production stimulated by a Ca^{2+} ionophore is independent of the β_2 integrins, but IgG- and FMLP-stimulated LTB_4 production requires β_2 integrins, even when the stimulus is in suspension.

We also investigated prostaglandin E_2 production and arachidonic acid release in IC-adherent PMN. We could not detect prostaglandin E_2 synthesis, even in normal PMN. IB4-treated PMN released 89% as much arachidonate in response to IC as control, W632-treated PMN (72 ng/10^6 IB4 treated cells; 81 ng/10^6 w632 treated cells; $n = 2$ for each group). Thus, there is ~200-fold more arachidonate released as LTB_4 produced.

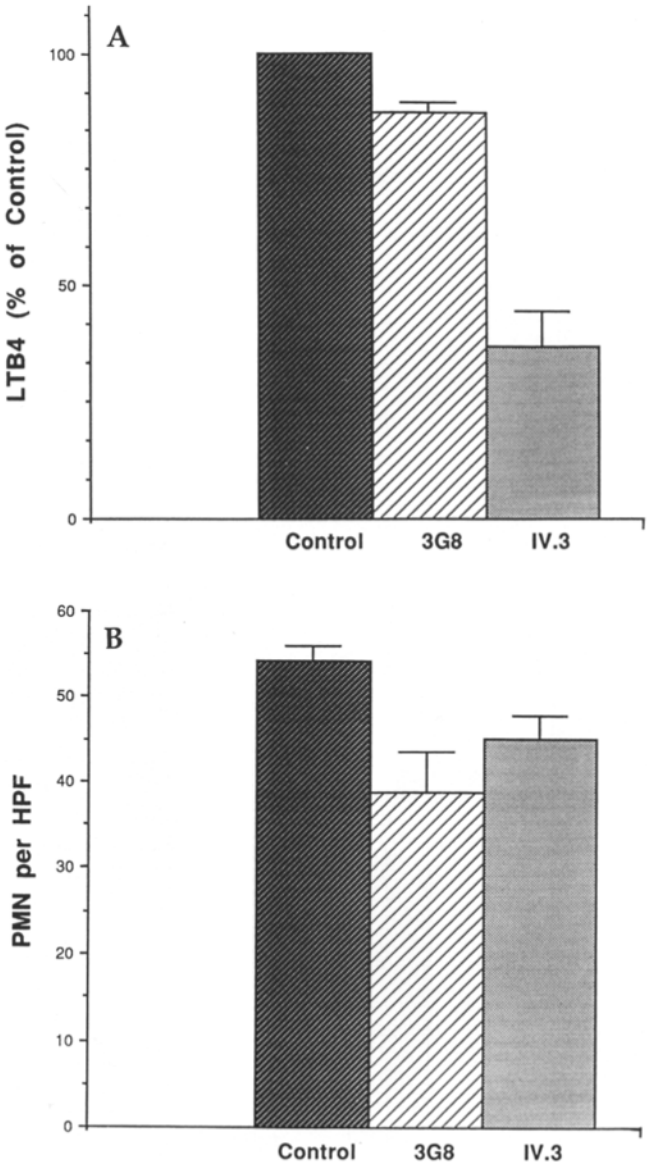

Figure 5. Role of FcRs in LTB_4 production by PMN adherent to IC-coated surfaces. (*A*) LTB_4 production. (*B*) Adhesion to ICs. PMN were pretreated with 5 μg/ml of 3G8 $F(ab')_2$ (anti-FcRIII) or IV.3 Fab (anti-FcRII), and then allowed to adhere to IC-coated surfaces. LTB4 production by 3G8-treated PMN was decreased by 13% compared with control PMN, while 3G8 decreased adhesion by 29%. In contrast, IV.3-treated PMN showed a 63% reduction in LTB_4 production, while adhesion was decreased 9%. This suggests that FcRII is involved in LTB_4 production, since LTB_4 is inhibited to a much greater extent than adhesion by IV.3. Data are presented as the mean ± SEM, and assays were done in duplicate; $n = 3$. (As a control for nonspecific effects of IV.3 and 3G8, superoxide production in response to PDBu and FMLP were assayed. These mAbs had no affect on superoxide production in response to these stimuli.)

IgG FcRII Is Involved in LTB_4 Production by PMN Adherent to IC-coated Surfaces

PMN express both IgG FcRII, a transmembrane receptor family, and the glycophosphatidyl inositol–linked form of IgG FcRIII (34). Both receptors have been reported to be involved in LTB_4 production (9). We have investigated which

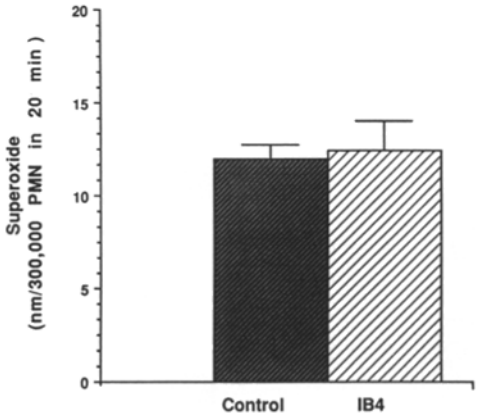

Figure 6. Superoxide production by PMN adherent to IC-coated surfaces. Superoxide is quantitated by cytochrome *c* reduction in the supernatant of PMN adherent to IC-coated surfaces for 20 min. Control and IB4 (anti-β_2)-treated PMN produce equivalent amounts of superoxide. (Presented as the mean nanomoles of superoxide per 3×10^5 PMN in 20 min ± SEM. Assay was performed in duplicate; $n = 3$).

FcR(s) are involved in LTB_4 production after adhesion to IC-coated surfaces. For these studies PMN were pretreated with Fab of mAb IV.3 (anti-FcRII) or $F(ab')_2$ of 3G8 (anti-FcRIII) and allowed to adhere to ICs. The PMN treated with IV.3 demonstrated a 65% decrease in LTB_4 production (Fig. 5 *A*), while PMN adhesion was decreased less than 10% (Fig. 5 *B*). The marked reduction in LTB_4 production in the absence of a significant effect on PMN adhesion suggests that FcRII is involved in LTB_4 production by PMN adherent to IC-coated surfaces. The requirement for both FcRII and β_2 integrins in IC-stimulated LTB_4 production suggests a cooperative interaction between the β_2 integrins and FcRII. In contrast, 3G8 (anti-FcRIII)-treated PMN showed a 13% reduction in LTB_4 production (Fig. 5 *A*), which was even less than the decrease in adhesion (29%) associated with this mAb (Fig. 5 *B*). The equivalent small decreases in LTB_4 production and adhesion suggest that FcRIII is not involved in LTB_4 production in PMN adherent

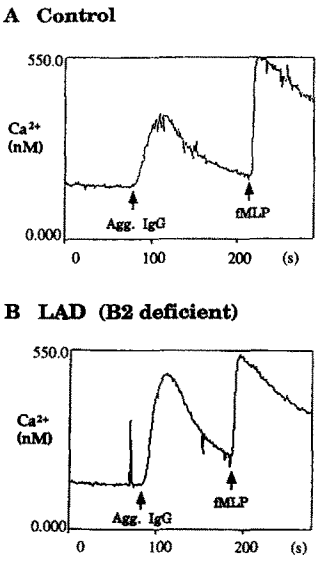

Figure 7. Aggregated IgG-induced rise in $[Ca^{2+}]_i$. PMN were loaded with F/ura-2 and the $[Ca^{2+}]_i$ was monitored continuously using a spectrofluorimeter. The addition of aggregated IgG (300 μg/ml) resulted in a 200-nM rise in $[Ca^{2+}]_i$ in control PMN. Aggregated IgG induced a 300-nM rise in $[Ca^{2+}]_i$ in the β_2-deficient PMN in the representative experiment shown. The aggregated IgG-induced rise in $[Ca^{2+}]_i$ in β_2-deficient PMN was consistently equivalent to or greater than control PMN; $n = 3$. FMLP was added subsequently and also resulted in an equivalent rise in $[Ca^{2+}]_i$ in both cell types.

to IC-coated surfaces. The combination of both FcRII and FcRIII mAbs completely abolishes adhesion of PMN to the IC-coated surface while reducing LTB_4 production by 79% (data not shown). The reduction in adhesion in conjunction with LTB_4 production makes it difficult to exclude definitively a supplemental role for FcRIII in LTB_4 production.

Some IgG- and FMLP-stimulated Effects on PMN Do Not Require β_2 Integrins

To determine whether all signal transduction via FcRs was abnormal in β_2-deficient PMN adherent to ICs, superoxide production was measured. Control and IB4 (anti-β_2 mAb)-treated PMN demonstrated an equivalent production of superoxide after adhesion to ICs (Fig. 6). Second, we measured lysozyme a marker of granule secretion. We could not detect lysozyme release in IC-adherent normal PMN. Lysozyme was released in response to FMLP by adherent PMN. IB4 did not affect release (lysozyme release in IB4-treated PMN was 104% of control PMN, $n = 3$). Thus, for both IC-induced superoxide production and FMLP-induced secretion, there is no apparent role for β_2 integrins.

Finally, we measured $[Ca^{2+}]_i$ in response to aggregated IgG and FMLP. Since the generation of LTB_4 requires release both of arachidonic acid and activation of 5-lipoxygenase, and the activation of 5-lipoxygenase is Ca^{2+} dependent (37), this is an especially significant parameter of signal transduction. Binding of IgG by FcRs is known to result in a rise in intracytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) (26). We therefore compared the rise in $[Ca^{2+}]_i$ generated by aggregated IgG in control and β_2-deficient PMN. The increase in $[Ca^{2+}]_i$ in β_2-deficient PMN was consistently equal to or higher than control PMN (Fig. 7). Similarly, the rise in intracellular Ca^{2+} induced by FMLP is equivalent in control and β_2-deficient PMN. IB4 had no effect on the rise in $[Ca^{2+}]_i$ in normal PMN in response to either fluid phase aggregated IgG or FMLP (data not shown).

Results were equivalent in either Ca^{2+} or EGTA containing media consistent with previous reports that FcR-dependent increases in $[Ca^{2+}]_i$ result entirely from release of intracellular stores (data not shown) (22). These results confirm the conclusion of the superoxide studies, that some signaling from FcR ligation is normal in LAD cells and not dependent on β_2 integrins. Moreover, the FcR and FMLP receptor-mediated alterations in $[Ca^{2+}]_i$ are normal immediately after ligand binding, at a time when adhesion and PMN morphology are equivalent in control and β_2-deficient PMN. This suggests that the defect in LTB_4 production in LAD cells may result from a subsequent step in signal transduction. This interpretation is consistent with the abnormalities in adhesion to IC, which occur only after several minutes of initially normal cell behavior.

Discussion

Integrins are best known for their involvement in a wide variety of cell adhesive phenomena, involving associations with both the extracellular matrix and with other cells. In addition, there is increasing evidence that integrin receptors are necessary for signal transduction events that occur subsequent to ligand binding (19). However, the mechanism of involvement of integrins in signal transduction remains controversial. In the case of the leukocyte β_2 integrins, studies by Nathan et al. have demonstrated that tumor necrosis factor–induced superoxide production in adherent PMN requires β_2 integrins (31). In addition, cAMP levels in adherent PMN are affected by β_2 integrins (30). Jaconi et al. have demonstrated that the Ca^{2+} oscillations observed in adherent PMN are abolished by anti-β_2 mAbs (20). Recent studies have shown that mAbs directed against the β_2 integrins LFA-1 and p150,95 can stimulate superoxide production (4), and that certain anti-Mac-1 and anti-β_2 mAbs can increase cAMP levels in PMN (17). These observations suggest a link between β_2 integrins and leukocyte signal transduction events.

Our own previous data have hinted at a role for β_2 integrins in signal transduction during phagocytosis, since phagocytosis via several different receptors is deficient in LAD cells or in normal cells treated with anti-β_2 mAb (17). The fact that this abnormality can be clearly separated from adhesion of the opsonized particles to the PMN plasma membrane has suggested a role for the β_2 integrins at a later step in the process of ingestion. Since phagocytosis can be modeled as a series of signal transduction events (39), these data suggest the possibility that β_2 integrins mediate signal transduction at some step during this complex cellular activity.

In an attempt to investigate this hypothesis in more detail and to simplify the system, we have examined the role of β_2 integrins in PMN responses to ICs. We have demonstrated a role for the β_2 integrins in the generation of LTB_4, a potent PMN chemoattractant. LTB_4 production by PMN adherent to IC-coated surfaces or PMN stimulated by fluid phase aggregated IgG is markedly decreased in the absence of the β_2 integrins. In contrast, other signal transduction pathways initiated by FcR ligation are intact in the absence of functioning β_2 integrins, including superoxide production and the rise in $[Ca^{2+}]_i$ in response to fluid phase aggregated IgG. Similarly, LTB_4 production by PMN stimulated with FMLP is decreased by IB4 (an anti-β_2 mAb), yet there is no effect of IB4 on degranulation as measured by lysozyme release in response to FMLP. Together these data suggest a role for the β_2 integrins in LTB_4 synthesis in response to receptor-mediated stimuli. In contrast, LTB_4 synthesis in response to ionomycin is independent of the β_2 integrins.

LTB_4 synthesis requires coordinated phospholipase-mediated release of arachidonic acid from the membrane and metabolism via 5-lipoxygenase (21, 29, 37). To investigate which step in LTB_4 synthesis requires β_2 integrins, arachidonate release in response to PMN adhesion to ICs was measured in control and IB4-treated PMN. Arachidonate release was almost unaffected by IB4. However, there is more than a 100-fold greater release of arachidonate compared with LTB_4 on a molar basis (arachidonic acid = 248 pmol/10^6 PMN; LTB_4 = 1.3 pmol/10^6 PMN), suggesting that only a small pool of arachidonate is utilized for LTB_4 production. In view of the large excess of arachidonic acid, and demonstrated requirements for coordinated arachidonic acid release for subsequent metabolism, it is not yet possible to be certain whether the β_2 integrins are needed for release of a minor but metabolically relevant pool of arachidonic acid, or alternatively are involved in subsequent steps in LTB_4 generation.

5-Lipoxygenase catalyzes the next two steps in LTB_4 synthesis. This enzyme is known to require Ca^{2+} and ATP for maximal activity (36). Since β_2-deficient PMN generate a normal amount of LTB_4 in response to a Ca^{2+} ionophore, this demonstrates that the enzymatic machinery for LTB_4 production is present. In addition, it shows that an extreme rise in intracytoplasmic Ca^{2+} concentration is a sufficient stimulus for LTB_4 production even in β_2-deficient cells. Although we have demonstrated that the initial rise in $[Ca^{2+}]_i$ generated by aggregated IgG or FMLP was equivalent in control and β_2-deficient PMN, the synthesis of LTB_4 is delayed beyond this early time period. It is possible that the role of the β_2 integrins in LTB_4 synthesis may be related to alterations in $[Ca^{2+}]_i$ that occur subsequent to initial ligand binding (23). This hypothesis would be supported by the observations of Jaconi et al. (20) demonstrating that the β_2 integrins are necessary for the generation of sustained Ca^{2+} oscillations in adherent PMN. Alternatively, other steps in 5-lipoxygenase activation, such as translocation to the membrane or association with 5-lipoxygenase activating protein, may be dependent on the β_2 integrins (11, 29, 35).

It has been postulated that Mac-1 may serve as the transmembrane link for IgG-mediated functions of the glycophosphatidyl inositol-linked form of FcR III (45). In this regard it is of interest that PMN adherent to IC-coated surfaces generated LTB_4 by a mechanism that required FcRII. Moreover, superoxide production in response to IC, an FcRIII-dependent event (8), was normal in LAD cells. Thus our data suggest that FcRII- rather than FcRIII-dependent cell activation events require β_2 integrins. The involvement of the β_2 integrins in IC-stimulated LTB_4 production, therefore, suggests a cooperative interaction between the β_2 integrins and FcRII. This is consistent with the previous observations of a role for Mac-1 in IgG-mediated phagocytosis of monocytes that express little if any glycophosphatidyl inositol-linked FcRIII, again suggesting cooperativity of a β_2 integrin with transmembrane FcRs (6).

There is a growing body of evidence for a role for the β_2 integrins in leukocyte signal transduction (4, 17, 20, 30, 31). This would suggest that the host defense defect in LAD is more profound than simple inability of phagocytes to recognize known ligands for β_2 integrins. Our results demonstrate that the β_2 integrins are involved in LTB_4 production by IC- and FMLP-stimulated PMN. Since LTB_4 plays an important role in PMN accumulation at inflammatory sites, it is intriguing to speculate that in addition to abnormal endothelial binding, recruitment of PMN to sites of inflammation may be decreased due to a defect in LTB_4 generation.

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