Immune Complex-stimulated Neutrophil LTB₄ Production Is Dependent on β₂ Integrins

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Abstract. The β₂ 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 β₂ integrins, phagocytosis of IgG-opsonized particles by activated neutrophils (PMN) requires these integrins. This observation suggests a role for β₂ integrins in phagocytosis subsequent to particle binding. To investigate the mechanism of involvement of β₂ integrins in IgG-mediated functions, we examined the role of β₂ integrins in adhesion to immune complex (IC)-coated surfaces. Initial adhesion and spreading on IC-coated surfaces were equivalent in control and β₂-deficient phagocytes. However, both genetically β₂-deficient PMN and PMN treated with the anti-β₂ mAb IB4 subsequently detached from the IC-coated surfaces. To determine whether biochemical consequences of IgG activation were also affected by β₂ deficiency, LTB₄ production in response to Fc receptor ligation was assessed. LTB₄ production by β₂-deficient PMN adherent to IC-coated surfaces was markedly decreased in comparison with control PMN. Importantly, LTB₄ production by PMN stimulated with fluid phase heat-aggregated IgG also required the β₂ 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 β₂-deficient PMN. The initial rises in intracytoplasmic [Ca²⁺], in response to aggregated IgG also were unaffected by inhibition of β₂ integrins. These data show that lack of β₂ integrins does not inhibit all FcR-dependent signal transduction. Finally, LTB₄ 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 β₂ integrins in a signal transduction pathway leading to sustained adhesion and LTB₄ production in response to IC. Since both β₂ 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 β₂ 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 (β₂), 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 β₂ 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 β₂-deficient polymorphonuclear leukocytes (PMN), yet in most instances the β₂-deficient PMN remain unable to migrate to sites of inflammation (12, 40). In addition, β₂-deficient PMN demonstrate abnormal adhesion and spreading on many surfaces in vitro (5). The involvement of β₂ integrins in homotypic PMN aggregation and in phagocytosis of IgG- and C3b-opsonized particles are other examples of the widespread importance of β₂ integrins for leukocyte adhesion-dependent functions (7). The involvement of the β₂ 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 β₂ 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

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

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Materials and Methods

Adhesion Assay

as described (14). 3G8 F(ab’h (anti-FcRIII) and IV.3 Fab (anti-FcRII) were
ascites using protein A-Sepharose. F(ab’h fragments of IB4 were prepared
produced W632 (anti-HLA-I, IgG2a) was purchased from the American

gests the intriguing hypothesis that a deficiency in LTB,
the ~2 integrins in receptor-mediated LTB4 generation sug-

Required 132 Integrins

involves the ~2 integrins. This demonstration of a role for
the ~2 integrins in receptor-mediated LTB4 generation
suggests the intriguing hypothesis that a deficiency in LTB4
generation may contribute to the chemotactic defect in ~2-
deficient PMN. Moreover, these data establish a role for ~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 ~2 expression were obtained as described (17).

Monoclonal Antibodies

IB4 and 60.3 (both anti-~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’h)2 fragments of IB4 were pre-
tered as described (14). 3G8 F(ab’h)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 ~g/ml BSA and followed by anti-BSA IgG (1.2-20 ~g;
Sigma Chemical Co., St. Louis, MO) to generate IC-coated surfaces. PMN
(3 x 105) in 300 ~l RPM buffer were preincubated ±30 ~g/ml of IB4
(anti-~2 mAb) at 37°C for 15 min, followed by adhesion to IC-coated cov-
erslips at 37°C in a 5% CO2 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 phago-
cytes adherent per 40x 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
MgCl2, 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 MgSO4, 5 mM EGTA, and 3% paraformaldehyde) for 20 min at room
temperature. Cells were stained with 160 ~g/ml rhodamine phalloidin (Molec-
ular 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 epifluores-
cence photomicroscope.

LTB4 Assay

Samples of 3 x 105 PMN were prepared in 300 ~l of RPMI supplemented
to 1 mM Ca2+ and Mg2+. 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 ~g/ml of the
various mAbs, 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 concen-
tration of azide was 0.0001%. Heat-aggregated IgG was prepared using hu-
man IgG as described (14). Aggregated IgG was added at 300 ~g/ml to PMN
in suspension at 1 x 104/ml immediately before incubation for 25 min at
37°C. The studies of the time course for LTB4 production in response to
aggregated IgG or formyl-met-leu-phe (FMLP) were done by adding 300
~g/ml aggregated IgG or 10 -6 M FMLP to 3 x 105 PMN in the fluid phase
in 24-well plates and incubating for the indicated times. LTB4 in the super-
natants was quantitated in duplicate by RIA as described (24).

Lysozyme Assay

Samples of 2 x 106 PMN were prepared in 400 ~l RPMI supplemented
to 1 mM Ca2+ and Mg2+. After preincubation for 1 h at room temperature
with 5 ~g/ml of mAbs, 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 ~g/ml of IB4 (anti-
~2). Reaction mixtures (0.5 ml of HBSS with 1.5 mM Ca2+ and 1.5 mM
Mg2+ with 1% human serum albumin) were prepared on ice containing 3
x 105 PMN and 80 ~g/ml cytochrome c in 24-well plates coated with ICs
described above. Samples were prepared in duplicate. Additional duplic-
cates containing 125 ~g/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 x 105 PMN/20 min (32).

Intracytoplasmic Ca2+ Concentration

PMN were loaded with 2 ~M fura-2 (Molecular Probes, Inc.) as described
(33). Intracytoplasmic Ca2+ concentration was determined in samples of 2
x 106 PMN/ml with a spectrofluorimeter (model P2000; 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 ident-
cific to those used for evaluation of LTB4 production. The buffer was the
same except for the addition of 1 mM of fatty acid-free BSA to serve as a
trap for released arachidonate. After adhesion, supernatants from 3 x 105
PMN were pooled for each point. Arachidonate was extracted and as-
sayed 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 ~2 Integrins

To begin to investigate the mechanism of involvement of the
~2 integrins in IgG-mediated functions we examined the
adhesion of PMN to IC-coated surfaces. Initially we mea-
sured the number of cells adherent to the IC-coated surfaces at sequential time points, comparing control PMN and PMN

treated with F(ab’h)2 of the anti-~2 mAb IB4 (Fig. 1 A). At
the 5- and 12-min time points the same number of control
and F(ab’h)2-treated PMN were adherent. Control PMN con-

continued to maintain adhesion to the IC-coated surface through-
out a 40-min assay. In contrast, the F(ab’h)2-treated cells

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 in-
volvement of the ~2 integrins in PMN interactions with
IgG-containing immune complexes (ICs). We have found
that, although the initial adhesion and spreading of ~2-
deficient PMN on IC-coated surfaces is normal, the ~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 LTB4 is
generated by IC-stimulated PMN, we investigated the role
of the ~2 integrins in IC-stimulated LTB4 production. We
have found that LTB4 generation by PMN adherent to IC-
coated surfaces, or stimulated by fluid phase aggregated IgG
involves the ~2 integrins. This demonstration of a role for
the ~2 integrins in receptor-mediated LTB4 generation
suggests the intriguing hypothesis that a deficiency in LTB4
generation may contribute to the chemotactic defect in ~2-
deficient PMN. Moreover, these data establish a role for ~2
integrins in signal transduction leading to PMN activation
by ICs.

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showed a progressive loss of adhesion, and few cells remained attached by 40 min. Genetically \( \beta_2 \)-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 \( \beta_2 \) integrin on an IC-coated surface, sustained adhesion to this surface requires \( \beta_2 \) integrins.

**Actin Distribution in PMN Adherent to IC-coated Surfaces**

To investigate why \( \beta_2 \) integrins are necessary for sustained adhesion to IC-coated surfaces, we examined the actin distribution of control PMN and genetically \( \beta_2 \)-deficient PMN adherent to IC. After adhesion for 5 min to ICs both the control and \( \beta_2 \)-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 \( \beta_2 \)-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 \( \beta_2 \)-deficient PMN had contracted and detached from the plate (Fig. 2). In addition, PMN treated with the \( \beta_2 \) 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 \( \beta_2 \) integrins are required for the second stage of actin redistribution associated with morphologic polarization, and for sustained adhesion to IC-coated surfaces.

**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 chemotactractant, which can activate PMN with a resultant morphologic polarization (46). In adherent cells polarization is
Fluid Phase–Aggregated IgG-stimulated LTB₄ Production Is Also Dependent on β₂ Integrins

Adhesion and spreading of β₂-deficient PMN is abnormal on many different surfaces. This abnormal adhesion might provide an explanation for the lack of LTB₄ production in response to adherence to IC in LAD- or IB4-treated cells. We therefore tested whether the requirement for the β₂ integrins in LTB₄ production was unique to an IC-coated surface, or whether the β₂ integrins were also required for LTB₄ production initiated by a fluid phase IgG FcR ligand. Control and β₂-deficient PMN were stimulated with heat-aggregated IgG, and LTB₄ production was assayed by RIA. β₂-deficient PMN produced <5% of the amount of LTB₄ produced by aggregated IgG-stimulated control PMN (Fig. 4 A). Similarly, PMN pretreated with IB4 (anti-β₂ mAb) produced only 25% of the LTB₄ generated by control PMN (Fig. 4 A). The time course of LTB₄ production was examined for fluid phase aggregated IgG (Fig. 4 B). This demonstrates that LTB₄ is first measurable at the 15-min time point, and that IB4 inhibits LTB₄ production at both the 15- and 25-min time points.

To determine whether other stimuli for LTB₄ synthesis also required β₂ integrins, adherent and suspension PMN were incubated with 1 μM FMLP. PMN in suspension produced no LTB₄ in response to this high agonist concentration. However, adherent PMN produced 26 pg/million PMN associated with both a ruffling border at the advancing edge and posterior retraction fibers. Since the β₂-deficient PMN were unable to progress to this morphologically polarized stage when adherent to ICs, LTB₄ production was assessed. Control and β₂-deficient PMN were allowed to adhere to IC-coated surfaces for the indicated times and the LTB₄ produced was measured (Fig. 3 A). LTB₄ production by the β₂-deficient PMN was markedly decreased at all time points. At 15 min, when adhesion was similar between LAD and controls, LTB₄ production by β₂-deficient PMN was <20% of the amount produced by control PMN (Fig. 3 B). Similarly, treatment of control PMN with IB4 (an anti-β₂ mAb) resulted in a 40–50% reduction in LTB₄ production (Fig. 3 B). Treatment of control PMN with 60.3, an alternative β₂ mAb, also decreased LTB₄ production by 40 ± 10% (SEM, n = 3). IB4 consistently inhibited LTB₄ production by PMN adherent to IC-coated surfaces over a range of IC densities (48–62%, Fig. 3 C). These data demonstrate that β₂ integrins are involved in LTB₄ production by PMN adherent to IC-coated surfaces. The addition of exogenous LTB₄ (10⁻²–10⁻⁹ M) to the β₂-deficient PMN failed to correct the adhesion abnormality in the β₂-deficient PMN, suggesting that abnormalities in addition to LTB₄ synthesis may prevent normal adhesion and polarization in LAD cells.

Figure 3. (A) LTB₄ production by control and genetically β₂-deficient PMN (LAD) adherent to IC-coated surfaces. LTB₄ was quantitated in the supernatant by RIA. LTB₄ 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) LTB₄ production by PMN adherent to IC-coated surfaces is compared at the 15-min time point. The LTB₄ production by the genetically β₂-deficient PMN (LAD) is <20% of the amount produced by control PMN (presented as the mean ± SEM; n = 3). β₂ mAb (IB4, IgG2a)-treated PMN are compared with control PMN treated with an isotype-matched mAb (W632, anti-HLA I, IgG2a). LTB₄ production by the IB4-treated PMN is 52% of the amount produced by control PMN (presented as the mean ± SEM, n = 6). (C) LTB₄ production by PMN adherent to ICs (BSA/anti-BSA) made with increasing concentrations of anti-BSA ab. A representative experiment is shown. IB4 inhibits LTB₄ production at all concentrations tested that generate LTB₄ (% 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.
Figure 4. (A) LTB₄ production by PMN stimulated with fluid phase aggregated IgG. Genetically β₁-deficient PMN (LAD) produce only 4% as much LTB₄ as control PMN. (Mean LTB₄ production by control PMN was 151 pg/million PMN in 25 min; n = 3.) IB₄ (anti-β₂-)treated PMN produce 25% as much LTB₄ as control PMN stimulated with aggregated IgG. A similar result was obtained using IB₄ F(ab')₂ fragments (n = 4). (B) Time course of LTB₄ produced by PMN stimulated with fluid phase aggregated IgG. There is no detectable LTB₄ produced by 7.5 min after stimulation. IB₄ inhibits LTB₄ production at 15 min (60 ± 7%) and 25 min (55 ± 5%). Data are % inhibition ± SEM; n = 4. (C) LTB₄ produced by PMN stimulated with FMLP. IB₄ inhibits LTB₄ production by 60%; n = 4. (D) LTB₄ production by PMN stimulated with ionomycin. Control or β₁-deficient PMN were treated with 10 µM ionomycin for 10 min at 37°C. An equivalent amount of LTB₄ was produced by the control and the β₁-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 β₂ integrins, may prime PMN for FMLP-induced synthesis of LTB₄. This may be analogous to the priming effect of β₂-dependent PMN adhesion on tumor necrosis factor-α-stimulated superoxide production (31).

Finally, the role of the β₂ integrins in ionomycin-stimulated LTB₄ production was examined. β₂-deficient PMN generated an equivalent amount of LTB₄ as ionomycin-stimulated control PMN (Fig. 4 D). Similarly, IB₄ did not inhibit ionomycin-stimulated LTB₄ production. This shows that LTB₄ production stimulated by a Ca²⁺ ionophore is independent of the β₂ integrins, but IgG- and FMLP-stimulated LTB₄ production requires β₂ integrins, even when the stimulus is in suspension.

We also investigated prostaglandin E₂ production and arachidonic acid release in IC-adherent PMN. We could not detect prostaglandin E₂ synthesis, even in normal PMN. IB₄-treated PMN released 89% as much arachidonate in response to IC as control, W632-treated PMN (72 ng/10⁶ IB₄ treated cells; 81 ng/10⁶ W632 treated cells; n = 2 for each group). Thus, there is ~200-fold more arachidonate released as LTB₄ produced.
Figure 5. Role of FcRs in LTB₄ production by PMN adherent to IC-coated surfaces. (A) LTB₄ 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 LTB₄ production, while adhesion was decreased 9%. This suggests that FcRII is involved in LTB₄ production, since LTB₄ production in the absence of a significant effect on adhesion suggests that FcRII is involved in LTB₄ production by PMN adherent to IC-coated surfaces. The requirement for both FcRII and β₂ integrins in IC-stimulated LTB₄ production suggests a cooperative interaction between the β₂ integrins and FcRII. In contrast, 3G8 (anti-FcRIII)-treated PMN showed a 13% reduction in LTB₄ 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 LTB₄ production and adhesion suggest that FcRIII is not involved in LTB₄ production in PMN adherent to IC-coated surfaces. FcR(s) are involved in LTB₄ production after adhesion to IC-coated surfaces. For these studies PMN were pretreated with Fab of mAb IV.3 (anti-FcRIII) 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 LTB₄ production (Fig. 5 A), while PMN adhesion was decreased less than 10% (Fig. 5 B). The marked reduction in LTB₄ production in the absence of a significant effect on PMN adhesion suggests that FcRII is involved in LTB₄ production by PMN adherent to IC-coated surfaces. The requirement for both FcRII and β₂ integrins in IC-stimulated LTB₄ production suggests a cooperative interaction between the β₂ integrins and FcRII. In contrast, 3G8 (anti-FcRIII)-treated PMN showed a 13% reduction in LTB₄ 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 LTB₄ production and adhesion suggest that FcRIII is not involved in LTB₄ production in PMN adherent to IC-coated surfaces.

IgG FcRII Is Involved in LTB₄ Production by PMN Adherent to IC-coated Surfaces

PMN express both IgG FcRII, a transmembrane receptor family, and the glycosphatidyl inositol–linked form of IgG FcRIII (34). Both receptors have been reported to be involved in LTB₄ 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-β₂)-treated PMN produce equivalent amounts of superoxide. (Presented as the mean nanomoles of superoxide per 3 x 10⁶ PMN in 20 min ± SEM. Assay was performed in duplicate; n = 3).

Figure 7. Aggregated IgG-induced rise in [Ca²⁺]. PMN were loaded with Flura-2 and the [Ca²⁺], was monitored continuously using a spectrofluorimeter. The addition of aggregated IgG (300 μg/ml) resulted in a 200-nM rise in [Ca²⁺], in control PMN. Aggregated IgG induced a 300-nM rise in [Ca²⁺], in the β₂-deficient PMN in the representative experiment shown. The aggregated IgG-induced rise in [Ca²⁺], in β₂-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 [Ca²⁺], 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 LTB4 production by 79% (data not shown). The reduction in adhesion in conjunction with LTB4 production makes it difficult to exclude definitively a supplemental role for FcRIII in LTB4 production.

Some IgG- and FMLP-stimulated Effects on PMN Do Not Require β2 Integrins

To determine whether all signal transduction via FcRs was abnormal in β2-deficient PMN adherent to ICs, superoxide production was measured. Control and IB4 (anti-β2 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 β2 integrins.

Finally, we measured [Ca2+]i in response to aggregated IgG and FMLP. Since the generation of LTB4 requires release both of arachidonic acid and activation of 5-lipoxygenase, and the activation of 5-lipoxygenase is Ca2+ 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 Ca2+ ([Ca2+]i) (26). We therefore compared the rise in [Ca2+]i generated by aggregated IgG in control and β2-deficient PMN. The increase in [Ca2+]i in β2-deficient PMN was consistently equal to or higher than control PMN (Fig. 7). Similarly, the rise in intracellular Ca2+ induced by FMLP is equivalent in control and β2-deficient PMN. IB4 had no effect on the rise in [Ca2+]i in normal PMN in response to either fluid phase aggregated IgG or FMLP (data not shown).

Results were equivalent in either Ca2+ or EGTA-containing media consistent with previous reports that FcR-dependent increases in [Ca2+]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 β2 integrins. Moreover, the FcR and FMLP receptor-mediated alterations in [Ca2+]i are normal immediately after ligand binding, at a time when adhesion and PMN morphology are equivalent in control and β2-deficient PMN. This suggests that the defect in LTB4 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 β2 integrins, studies by Nathan et al. have demonstrated that tumor necrosis factor-induced superoxide production in adherent PMN requires β2 integrins (31). In addition, cAMP levels in adherent PMN are affected by β2 integrins (30). Jaco et al. have demonstrated that the Ca2+ oscillations observed in adherent PMN are abolished by anti-β2 mAbs (20). Recent studies have shown that mAbs directed against the β2 integrins LFA-1 and p150,95 can stimulate superoxide production (4), and that certain anti-Mac-1 and anti-β2 mAbs can increase cAMP levels in PMN (17). These observations suggest a link between β2 integrins and leukocyte signal transduction events.

Our own previous data have hinted at a role for β2 integrins in signal transduction during phagocytosis, since phagocytosis via several different receptors is deficient in LAD cells or in normal cells treated with anti-β2 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 β2 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 β2 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 β2 integrins in PMN responses to ICs. We have demonstrated a role for the β2 integrins in the generation of LTB4, a potent PMN chemoattractant. LTB4 production by PMN adherent to IC-coated surfaces or PMN stimulated by fluid phase aggregated IgG is markedly decreased in the absence of the β2 integrins. In contrast, other signal transduction pathways initiated by FcR ligation are intact in the absence of functioning β2 integrins, including superoxide production and the rise in [Ca2+]i in response to fluid phase aggregated IgG. Similarly, LTB4 production by PMN stimulated with FMLP is decreased by IB4 (an anti-β2 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 β2 integrins in LTB4 synthesis in response to receptor-mediated stimuli. In contrast, LTB4 synthesis in response to ionomycin is independent of the β2 integrins.

LTB4 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 LTB4 synthesis requires β2 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 LTB4 on a molar basis (arachidonic acid = 248 pmol/106 PMN; LTB4 = 1.3 pmol/106 PMN), suggesting that only a small pool of arachidonate is utilized for LTB4 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 β2 integrins are needed for release of a minor but metabolically relevant pool of arachidonic acid, or alternatively are involved in subsequent steps in LTB4 generation.
5-Lipoxygenase catalyzes the next two steps in LTB₄ synthesis. This enzyme is known to require Ca²⁺ and ATP for maximal activity (36). Since β₂-deficient PMN generate a normal amount of LTB₄ in response to a Ca²⁺ ionophore, this demonstrates that the enzymatic machinery for LTB₄ production is present. In addition, it shows that an extreme rise in intracytoplasmic Ca²⁺ concentration is a sufficient stimulus for LTB₄ production even in β₂-deficient cells. Although we have demonstrated that the initial rise in [Ca²⁺]ᵢ generated by aggregated IgG or FMLP was equivalent in control and β₂-deficient PMN, the synthesis of LTB₄ is delayed beyond this early time period. It is possible that the role of the β₂ integrins in LTB₄ synthesis may be related to alterations in [Ca²⁺]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 β₂ integrins are necessary for the generation of sustained Ca²⁺ 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 β₂ integrins (11, 29, 35).

It has been postulated that Mac-1 may serve as the transmembrane link for IgG-mediated functions of the glycosphatidyl inositol-linked form of FcRIII (45). In this regard it is of interest that PMN adhere to IC-coated surfaces generated LTB₄ 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 β₂ integrins. The involvement of the β₂ integrins in IC-stimulated LTB₄ production, therefore, suggests a cooperative interaction between the β₂ 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 glycosphatidyl inositol-linked FcRIII, again suggesting cooperativity of a β₂ integrin with transmembrane FcRs (6).

There is a growing body of evidence for a role for the β₂ 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 β₂ integrins. Our results demonstrate that the β₂ integrins are involved in LTB₄ production by IC- and FMLP-stimulated PMN. Since LTB₄ 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 LTB₄ generation.

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