A MONOCLONAL ANTIBODY TO SIALOPHORIN (CD43) INDUCES HOMOTYPIC ADHESION AND ACTIVATION OF HUMAN MONOCYTES

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Sialophorin, or CD43 (1), also known as leukosialin (2, 3) and large sialoglycoprotein (4), is an abundant, heavily glycosylated, sialic acid–bearing surface protein on human lymphocytes (5). The molecule is defective on lymphocytes of patients with the inherited immune deficiency Wiskott-Aldrich syndrome (5). Anti-CD43 mAb was shown to be a potent inducer of lymphocyte proliferation (6), suggesting that CD43 is the surface receptor of a lymphocyte stimulation pathway.

Sialophorin (CD43) is also an abundant surface molecule on blood monocytes (7), cells that play critical roles in immune defense mechanisms. Monocytes can be activated to enhanced defense functions by a number of agents, including cytokines such as IFN-γ (8) and bacterial cell wall products (9). Activated monocytes are characterized by the ability to kill certain microorganisms and tumor cells (10) and to produce reactive oxygen intermediates, including hydrogen peroxide (11).

In this study we tested the antisialophorin mAb L10 (5) for its ability to trigger activation of human monocytes. We show that the anti-CD43 mAb induces activation of monocytes as judged by increased hydrogen peroxide–producing capacity. Activation of monocytes by anti-CD43 mAb is compared with activation of monocytes by rIFN-γ. The involvement of homotypic adhesion in the pathway of monocyte activation by anti-CD43 mAb is demonstrated.

Materials and Methods

Antibodies. Mouse mAbs were used as follows: L10, an IgG1, against human sialophorin (CD43) (5); TA-1, an IgG2a, against human LFA-1 (CD11a/CD18) (12); mAb 44 (a gift of Dr. Robert Todd III, University of Michigan Medical School, Ann Arbor, MI), an IgG2a against human Mo1 α subunit (CD11b) (13, 14); mAb 60.3 (a gift of Dr. Patrick Beatty, Fred Hutchinson Cancer Research Center, Seattle, WA), an IgG1 against CD18, the common β subunit of human LFA-1, Mo1, and p150,95 (15); and the irrelevant control mAb M12, an IgG1 against guinea pig CD11b (16). TA-1 precipitated M_r 170,000 and 95,000 polypeptides from lysates of ^{125}I-labeled monocytes, and the anti-CD11b mAb OKM1 (17) (Ortho Diagnostic Systems, Inc., Westwood, MA), tested in parallel, precipitated M_r 155,000 and 95,000

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polypeptides (T. W. LeBien, unpublished findings). The failure of TA-1 to precipitate CD11b indicates that TA-1 recognizes the α, rather than the β, subunit of LFA-1. The IgG content of ascites was quantified by radial immunodiffusion (18) in agar gels containing 0.01 vol of rabbit anti-mouse IgG antiserum (Miles Laboratories, Inc., Naperville, IL). Homogeneous antibodies were prepared from ascites by adherence to protein A-agarose at pH 8.0 and elution at low pH (19).

Reagents. rIFN-γ (10^6 U/ml) was from Amgen Biologicals, Thousand Oaks, CA. Medium 199, RPMI 1640, and HBSS were from Gibco Laboratories, Grand Island, NY; gentamycin was from M. A. Bioproducts, Walkerville, MD; and FCS was from HyClone Laboratories, Logan, UT. Culture solutions and supernatants were assayed with a chromogenic Limulus amoebocyte lysate assay (M. A. Bioproducts) to insure that LPS content was $\leq$0.01 ng/ml.

Cells. Human peripheral blood from healthy volunteers was drawn into plastic vials containing acid-citrate-dextrose (NIH formula A; Fenwal Laboratories, Deerfield, IL), and mononuclear cells were purified by Ficoll-Hypaque centrifugation. The mononuclear cells (2×10^5 in 200 μl RPMI 1640 medium with 10% FCS) were incubated in 96-well tissue culture plates (Becton Dickinson & Co., Oxnard, CA) for 1 h at 37°C. Nonadherent material was decanted and the wells were washed with RPMI 1640 at 37°C. The adherent cells ($\sim 0.5 \times 10^5$/well), ~96% monocytes as judged by esterase staining (20), were cultured in RPMI 1640 with 10% FCS and 50 μg/ml gentamycin for 24 h and were washed with RPMI 1640. Platelet contamination, quantified at this point by microscopic examination, was $\leq$8 platelets/100 monocytes.

Monocyte Activation. mAb (ascites or purified IgG) or IFN-γ in RPMI 1640 containing 10% FCS were added to washed 1-d-old adherent monocytes, which were then cultured for 0.5–48 h.

Hydrogen Peroxide Assay. Secretion of H_2O_2 in response to PMA was measured by the modified scopoletin fluorescent assay (21). The monolayers were incubated with 7 μM scopoletin, 20 μg/ml horseradish peroxidase, 65 ng/ml PMA, and 1 mM NaN_3 in a volume of 200 μl. DNA content, determined in parallel wells, as described (22), indicated that none of the experimental treatments altered the number of cells per well. Hydrogen peroxide production was expressed as ng H_2O_2/μg DNA/h.

Assay for Monocyte Aggregation. All plates were assigned coded identification by an individual not otherwise involved in the study. Wells were scored for aggregates, defined as the association of $\geq$10 cells, using a Nikon phase contrast microscope.

Results

Anti-CD43 mAb L10 Induces Hydrogen Peroxide Production in Human Monocytes. Isolated human blood monocytes were allowed to mature in culture for 1 d, then treated with the anti-CD43 mAb L10 for 48 h. Hydrogen peroxide release measured in the presence of the triggering agent PMA was increased five- to sevenfold in L10-treated monocytes in comparison with monocytes treated without antibody or with subclass-matched irrelevant antibody (M12 mAb) (Fig. 1). Optimal hydrogen peroxide-producing capacity was induced by 15 μg/ml L10, and higher levels had no additional effect. L10 used in these experiments was pure IgG, indicating that its action is not attributable to other components. The level of peroxide-producing capacity induced by optimal L10 mAb concentrations (1.6 ± 0.3 nmol H_2O_2/μg DNA/h) was comparable with that induced by optimal levels of rIFN-γ (1.3 ± 0.4 nmol H_2O_2/μg DNA/h) (mean ± SE of six experiments; e.g., Fig. 1). As previously shown for IFN-γ-activated monocytes, L10-treated monocytes require the triggering agent PMA to be present in the hydrogen peroxide assay (not shown).

Time Course of Enhancement of Hydrogen Peroxide Capacity Induced by L10 mAb. L10 induction of increased hydrogen peroxide-producing capacity of monocytes is a time-dependent reaction with maximum capacity reached after 24 h with 15 μg/ml L10

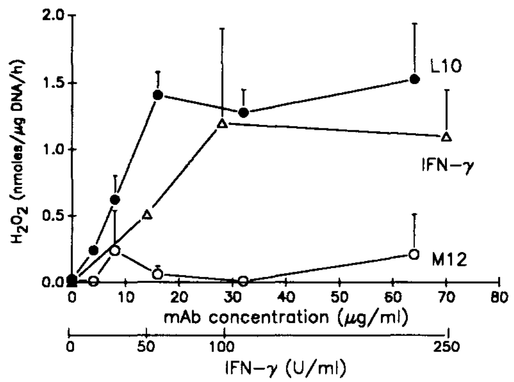

FIGURE 1. Hydrogen peroxide-producing capacity induced in monocytes by varying concentrations of L10 mAb. Isolated adherent monocytes, which had matured in culture for 1 d, were treated with the indicated concentration of purified L10 IgG (●), the IgG fraction of the irrelevant mAb M12 (○), or rIFN-γ (Δ). The hydrogen peroxide-producing capacities measured after 48 h of treatment are means ± SE of triplicate determinations.

(Fig. 2). In contrast, in IFN-γ-treated monocytes, maximum peroxide production was reached after 48 h with 200 U/ml (Fig. 2). Further increase of the IFN-γ concentration did not shorten the time (not shown). Thus, maximum hydrogen peroxide-producing capacity is reached after 24 h of treatment of monocytes with optimal L10 concentration or 48 h with optimal IFN-γ.

Anti-CD43 mAb Induces Homotypic Adhesion of Monocytes. The adherence properties of L10-treated monocytes were also examined, since IFN-γ-induced monocyte activation is known to proceed via a stage involving homotypic cell adhesion (23). When 1-d-old adherent monocytes were treated with L10 for 24 h, increased numbers of aggregates were found relative to monocytes treated with subclass-matched irrelevant antibody (Fig. 3) or monocytes incubated without antibody (not shown). The increase of monocyte aggregation was dependent on L10 concentration with maximal aggregation at 5–10 µg/ml; 20 and 40 µg/ml L10 were partially inhibitory (Fig. 3). The level of monocyte aggregation induced by L10 mAb was comparable with that induced by IFN-γ (shown below).

Time Course of Monocyte Aggregation Induced by the Anti-CD43 Antibody L10. Monocyte aggregation induced by L10 is a time-dependent process, with significant aggregation detected after 1 and 4 h, and maximal aggregation reached at 8 h of L10 treatment (Fig. 4). The requirement for 1–8-h incubation strongly suggests that L10 does not function solely as a crosslinking agent. L10-induced monocyte aggregation

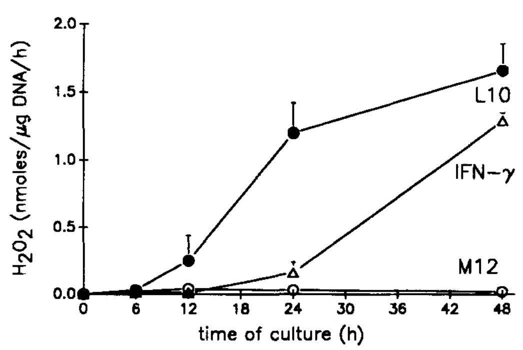

FIGURE 2. Time course of enhancement of hydrogen peroxide-producing capacity of monocytes treated with L10 mAb or IFN-γ. 1-d-old adherent monocytes were cultured for the indicated time with L10 ascites at 15 µg IgG/ml (●), 15 µg IgG/ml of the irrelevant M12 ascites (○), or rIFN-γ at 200 U/ml (Δ). The hydrogen peroxide production values are means ± SE of triplicate determinations.

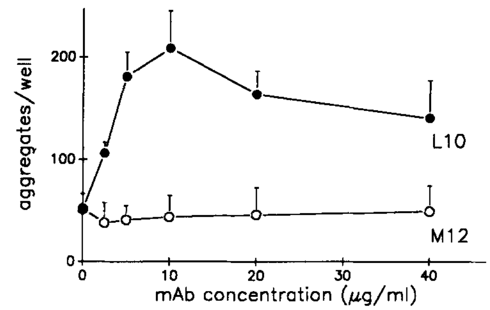

FIGURE 3. Homotypic adhesion of monocytes induced by varying concentrations of L10 mAb. Beginning 1 d after isolation, adherent monocytes were treated for 24 h with the indicated concentration of purified L10 IgG (●), or the irrelevant mAb M12 (○). The number of aggregates/well are means ± SE of quintuplicate determinations.

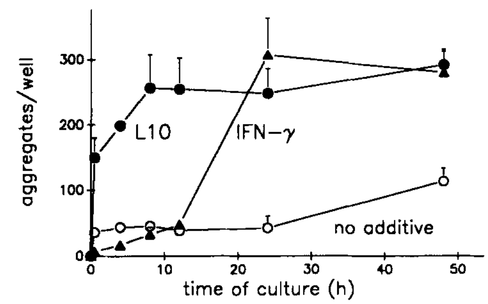

FIGURE 4. Time course of homotypic adhesion of monocytes induced by L10 mAb or IFN-γ. Beginning 1 d after isolation, adherent monocytes were treated with 10 μg/ml purified L10 IgG (●), 200 U/ml of rIFN-γ (▲), or medium alone (○). The number of aggregates/well are means ± SE of quintuplicate determinations.

remained maximal at 12, 24, and 48 h (Fig. 4). In contrast, monocyte aggregation induced by IFN-γ is nondetectable at 12 h and maximal at 24 h (Fig. 4).

Role of Cations in Monocyte Aggregation Induced by Anti-CD43. When monocytes were depleted of Mg^{2+} and Ca^{2+}, induction of aggregation by L10 was almost completely abrogated (Table I). The capacity of the cells to undergo L10-induced homotypic adhesion was restored by addition of 1 mM Mg^{2+}; addition of Ca^{2+} had no effect. Thus, Mg^{2+} is required to support L10-induced monocyte aggregation, a finding inconsistent with mechanisms in which L10 acts solely as a crosslinking agent.

The Anti-LFA-1 Antibody TA-1 Inhibits Monocyte Aggregation Induced by the Anti-CD43 Antibody L10. IFN-γ-induced homotypic adhesion of monocytes was shown to in-

TABLE I

Aggregation of Monocytes Induced by L10 mAb is Mg^{2+} Dependent

	Aggregates per well	
Medium	Without mAb	With L10 mAb
Complete	99 ± 24	354 ± 42
Depleted	45 ± 11	107 ± 17
Depleted, + Mg^{2+}	174 ± 33	469 ± 54
Depleted, + Ca^{2+}	15 ± 5	148 ± 15
Depleted, + Mg^{2+} and Ca^{2+}	147 ± 30	408 ± 19

Adherent monocytes that had been cultured for 1 d in complete medium, were incubated for 2 h in HBSS + FCS (complete medium) or Mg^{2+}/Ca^{2+}-free HBSS + dialyzed FCS (depleted medium). L10 ascites diluted 1:100 in complete or depleted medium were added, and cell aggregation was evaluated after a further 4-h incubation. Where indicated, 1 mM Mg^{2+} and/or Ca^{2+} was added to depleted cell cultures simultaneously with L10 addition. The data are the means ± SE of quadruplicate determinations.

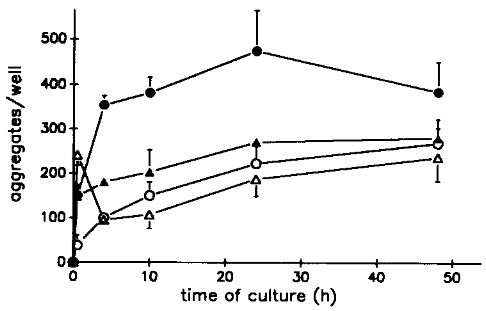

FIGURE 5. Homotypic adhesion of monocytes induced by L10 mAb is inhibited by TA-1 mAb. 1-d-old adherent monocytes were cultured for the indicated time with medium alone (○), TA-1 mAb ascites at 1:10 dilution (△), 10 μg/ml of L10 IgG (●), and L10 + TA-1 (▲). The number of aggregates/well are means ± SE of triplicate determinations. Similar results were obtained with L10 mAb at 20 μg/ml (not shown).

volve the heterodimeric surface molecule LFA-1 (CD11a/CD18) (23). To determine whether LFA-1 is involved in L10-induced homotypic adhesion, the monocytes were treated with L10 mAb in the presence of the anti-LFA-1α (anti-CD11a) mAb TA-1. TA-1 mAb completely abrogated the induction of monocyte aggregation by 10 or 20 μg/ml L10 mAb (Fig. 5). The mAb 44, an anti-Mol-α (anti-CD11b) antibody, at the same concentration, had no effect on L10-induced monocyte aggregation (not shown). The mAb 60.3 (anti-CD18) directed against the common β subunit of Mol and LFA-1 caused slight inhibition of L10-induced monocyte aggregation (not shown). These findings indicate that L10-induced monocyte aggregation is a Mg^{2+}-requiring process, that like IFN-γ-induced monocyte aggregation, involves LFA-1 surface molecules.

Discussion

The present study demonstrates that L10, an mAb that binds to sialophorin (CD43), induces increased hydrogen peroxide-producing capacity and homotypic adhesion of human monocytes, two characteristics associated with the activated state of these cells. The requirement for Mg^{2+} and 1–8-h incubation strongly indicates that L10-induced monocyte aggregation is not a simple case of antibody crosslinking of cells. In direct comparisons, the extent of L10-induced enhancement of hydrogen peroxide-producing capacity and homotypic adhesion were found to be comparable with the effects induced by the prototypic monocyte-activating agent IFN-γ.

The finding that Mg^{2+} depletion abrogates, and Mg^{2+} reconstitution restores, L10-induced monocyte aggregation suggested the involvement of adhesion molecules of the Leu-CAM family. These are heterodimer surface glycoproteins consisting of CD18 and either CD11a (LFA-1), CD11b (Mol, Mac-1), or CD11c (p150,95) (reviewed in reference 24). LFA-1 has been shown to mediate heterotypic and homotypic interactions of leukocytes, including IFN-γ-induced aggregation of monocytes (23).

TA-1, a LFA-1α mAb, reduced L10-induced aggregation to baseline levels, indicating that LFA-1 is instrumental in L10-induced monocyte aggregation. An anti-CD18 mAb, 60.3 (anti-β chain), had a slight inhibitory effect on L10-induced monocyte aggregation. A previous study showed that IFN-γ-induced monocyte aggregation, like L10-induced monocyte aggregation, was inhibited completely by anti-LFA-1α mAb, but only partially by an anti-CD18 mAb that was completely inhibitory in a lymphocyte system (23). The lesser sensitivity of IFN-γ-induced monocyte aggregation to inhibition by anti-CD18 mAb was attributed to the presence on monocytes of multiple CD18 heterodimers (23).

We do not know whether there is any relatedness of the findings reported here, i.e., activation of monocytes by anti-CD43 mAb, and an earlier study in the mouse system demonstrating activation of macrophages by mAb against both subunits of CD11b/CD18 (25). Treatment of human monocytes with the anti-CD11b mAb 44 failed to stimulate hydrogen peroxide-generating capacity (H. G. Remold, unpublished results) or homotypic adhesion; additional anti-CD11b/CD18 mAbs were not examined.

Human sialophorin (CD43), the surface molecule recognized by L10 mAb, is found on thymocytes, T lymphocytes, some B lymphocytes, neutrophils, monocytes, and platelets (6, 7). It is a highly glycosylated acidic molecule (∼60% carbohydrate), consisting of a COOH-terminal intracellular region subject to phosphorylation by protein kinase C (26), a single transmembrane region, and an extracellular region (27). Sialophorin has one *N*-linked carbohydrate unit and 80–90 sialic acid-bearing *O*-linked units, distributed, more or less uniformly, throughout the ∼235-amino acid extracellular region (27, 28).

The present study is the first examination of sialophorin function in monocytes, but the role of sialophorin in lymphocyte function has received some attention. Surface CD43 is deficient and/or defective in lymphocytes of patients with the inherited immune deficiency Wiskott-Aldrich syndrome (5). Sialophorin is thought to be a component of a pathway of T cell activation, since L10 mAb triggers proliferation of human T lymphocytes (6). L10-induced lymphocyte proliferation is quantitatively comparable with lymphocyte proliferation induced by mAbs directed against CD3/TCR, and both processes are monocyte dependent and lead to increased surface expression of HLA-DR and IL-2-R (6). The putative natural ligand for CD43 is unknown. Two other anti-CD43 mAbs, B1B6 and E11B, induce IL-2 secretion by lymphocytes, synergize with PMA in inducing lymphocyte proliferation, and induce lymphocyte aggregation (4). Lymphocyte aggregation induced by these anti-CD43 mAbs, like the anti-CD43-induced monocyte aggregation documented in this study, requires the presence of Mg^{2+} and is abrogated by anti-LFA-1 mAb (4).

Brief incubation of lymphocytes with L10 mAb was shown to generate increased levels of diacyl glycerol, inositol phosphates, and intracellular Ca^{2+}, as well as conversion of protein kinase C from a soluble to a membrane-associated form (29). All of these L10-induced events are known to be associated with protein kinase C activation in other systems (30). Moreover, two of these early changes, increased levels of inositol phosphates and intracellular Ca^{2+}, were also demonstrated in monocytes treated with L10 (29).

Since monocyte activation induced by L10 and IFN-γ have multiple common characteristics, the possibility was considered that L10 acts via the IFN-γ pathway. L10-induced release of IFN-γ from lymphocytes is a highly unlikely explanation, first of all, because the cell populations were ≥95% monocytes. It is certain that IFN-γ and L10 bind to different molecules, since the receptors for both have been sequenced (27, 31). Most importantly, the more rapid appearance of activation-associated changes in L10-treated monocytes relative to IFN-γ-treated monocytes provides strong evidence that L10 action does not depend on the IFN-γ-R pathway.

Indeed, the documentation of parallel changes in anti-CD43-treated lymphocytes and anti-CD43-treated monocytes, i.e., early signaling involving inositol phosphates, Ca^{2+}, and protein kinase C, and the appearance at later times of homotypic adhesion, suggest that the pathway of CD43 activation is independent of molecules, like the IFN-γ-R and the TCR/CD3 complex, that are specific to only one of these cells. Cumulatively, these findings indicate that CD43 is the surface receptor of an independent pathway of activation of lymphocytes and macrophages; the pathway has early events common to lymphocytes and monocytes and later events specific to each cell.

Summary

Treatment of human monocytes for 24–48 h with the anti-CD43 mAb L10 caused five- to sevenfold stimulation of hydrogen peroxide-producing capacity, an established characteristic of activated monocytes. Peroxide-producing capacity induced by L10 antibody (1.6 $\pm$ 0.3 nmol $H_2O_2/\mu g$ DNA/h) was comparable with that induced by IFN-γ (1.3 $\pm$ 0.4 nmol $H_2O_2/\mu g$ DNA/h), but appeared more rapidly (maximal at 24 h) than in the IFN-γ-treated monocytes (maximal at 48 h).

Treatment of monocytes with L10 mAb also caused dramatic increase in aggregation (homotypic adhesion). Induction of monocyte aggregation by L10 mAb required incubation for 1–8 h in the presence of Mg^{2+} and was abrogated by TA-1, an anti-LFA-1-α mAb. Thus, L10-induced monocyte activation proceeds via a Mg^{2+}-requiring aggregation stage involving LFA-1. Whereas the extent of monocyte aggregation induced by L10 mAb and by IFN-γ were comparable, the L10-induced aggregation occurred more rapidly (maximal at 8 h) than the IFN-γ-induced aggregation (maximal at 24 h). The more rapid appearance of aggregation and increased hydrogen peroxide capacity in L10-treated monocytes suggests that the L10-induced activation pathway is independent of IFN-γ- and IFN-γ-R dependent events. These findings suggest that the surface molecule CD43 is the receptor of an independent activation pathway that leads in lymphocytes to proliferation and in monocytes to activation.

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