

Short communication

Integrin VLA-6 ($\alpha 6\beta 1$) mediates adhesion of mouse bone marrow-derived mast cells to laminin

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The development of mast cells from bone marrow precursors and their function as the mucosal- or connective-tissue-type mast cell are critically dependent on microenvironmental factors. Extracellular matrix proteins, such as collagen, fibronectin, and laminin, may represent insoluble components of the microenvironment. Recent studies have described multiple isoforms of laminin isolated from different tissues. In the present study, adhesion of mouse bone marrow-derived mast cells (BMMC) and long-term mast cell lines to Engelbreth-Holm-Swarm (EHS) tumor laminin, rat laminin, human merosin, and human placental laminin was compared. The greatest level of adhesion was found with human laminin as the substrate. By use of a newly prepared mouse VLA- $\alpha 6$ integrin-specific mAb (MA6) together with the previously described mAb GoH3, VLA-6 ($\alpha 6\beta 1$) integrin was found to be expressed and utilized by BMMC and long-term mast cell lines. VLA-6 has been described as a major laminin receptor with roles in diverse cell functions including cell growth and differentiation. BMMC have been shown to express a 32/67-kDa laminin receptor. Therefore, in addition to the 32/67-kDa laminin receptor described in early studies, BMMC also express VLA-6 integrin, which may have roles in the regulation of their development.

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Mast cells are frequently found in close proximity to blood vessels, skin, and nerves (1). Therefore, in addition to functioning as effector cells in allergic reactions, they may have roles in providing the first line of defense of the immune system. This notion is supported by the demonstration of a wide spectrum of inflammatory cytokine production by mast cells in recent years (2, 3). In the murine system, bone marrow-derived mast cells (BMMC) are readily prepared by culturing bone marrow precursors in the presence of interleukin (IL)-3. Both *in vivo* and *in vitro* studies have demonstrated that BMMC can be induced to undergo further differentiation into either the connective-tissue-type or mucosal-type mast cells depending on their anatomic locations, indicating that microenvironmental factors have pivotal roles in regulating mast cell development (4, 5). Thus, diverse

cytokines such as IL-3, IL-4, stem cell factor (or *c-kit* ligand), or nerve growth factor, alone or in combinations, have been shown to induce differentiation of BMMC (6–8).

The extracellular matrix (ECM) proteins may represent the insoluble compartment of the microenvironment. Laminin constitutes one of the major ECM components of the basement membrane and is known to have effects on diverse cell functions, such as cell migration, growth, and differentiation (9–11). Various members of the integrin family, VLA-1 ($\alpha 1\beta 1$), VLA-2 ($\alpha 2\beta 1$), VLA-3 ($\alpha 3\beta 1$), VLA-6 ($\alpha 6\beta 1$), $\alpha 6\beta 4$, and $\alpha 7\beta 1$, have been shown to mediate cell interaction with laminin substrate (12, 13). VLA-6 ($\alpha 6\beta 1$) serves as the major receptor for laminin (12). A 32/67-kDa laminin receptor has been described for mediating adhesion of BMMC to laminin (14). While the role of the 32/

67-kDa laminin receptor in cell function is still unclear, VLA-6 has been extensively documented to have effects on cell morphogenesis and development (15, 16). In the present study, the utilization of VLA-6 for interaction with laminin by BMMC and long-term cultured mast cell lines was assessed with the mAbs GoH3 and MA6. Previous studies have shown that GoH3 is specific for $\alpha 6$ integrins, binding both human and mouse VLA-6 ($\alpha 6\beta 1$) and $\alpha 6\beta 4$ integrins (17). MA6 is a newly prepared $\alpha 6$ -specific mAb described here.

Material and methods

Cell culture

The mouse melanoma cell line B16F1 was provided by Dr B. Zetter (Harvard Medical School, Boston, MA, USA). Mouse factor-dependent mast cell line MC/9 (18), the myelomonocytic cell line WEHI-3, the myeloma cell line P3X63Ag8 (P3), and the antimouse κ -light-chain-specific hybridoma 187.1 were obtained from American Type Culture Collection (Rockville, MD). The hybridoma secreting DNP-specific mouse IgE was kindly provided by Dr D. H. Katz (Medical Biology Institute, La Jolla, CA, USA). The mouse squamous carcinoma cell line SSC-VII has been described in previous studies (19). The mouse factor-dependent mast cell line CFTL-15 was kindly provided by Dr J. H. Pierce (NCI, Bethesda, MD, USA [20]). Mouse BMMC were prepared by culturing bone marrow cells from Balb/c mice in the presence of IL-3, as previously described (21). After 3–4 weeks of culture, greater than 95% of BMMC were stained by toluidine blue, as assessed by averaging the percentages of cells stained in five fields, each containing approximately 100 cells. BMMC (3–4 weeks) thus prepared were used in this study.

Antibodies

MA6 is a hybridoma prepared by fusion of spleen cells from a Fischer rat immunized with 1×10^8 SSC-VII cells (i.p.) with the mouse myeloma P3X63Ag8.653, according to established method (22). Hybridomas were selected for production of binding antibodies which immunoprecipitated typical $\alpha\beta$ integrin heterodimers from VLA-6-expressing B16F1 cells. MA6 (IgG2a/ κ) was selected for further characterization after repeated limiting dilution. mAb GoH3 against VLA- $\alpha 6$ integrins was kindly provided by Dr A. Sonnenberg (The Netherlands Cancer Institute, The Netherlands) (17). mAb MB1.2 specific for mouse $\beta 1$ integrin subunit was prepared in this laboratory and is described elsewhere (23).

Flow cytometry and immunoprecipitation

Immunofluorescence staining was carried out as described previously (24). All antibodies were used at predetermined saturating concentrations. Immunoprecipitation from cell lysate of ^{125}I -surface-labeled cells by the lactoperoxidase catalyzed reaction was performed according to established procedures (24). For immunodepletion experiments, the cell lysate was immunoprecipitated with GoH3 in combination with antirat agarose (ARA) three times and then subjected to another three rounds of ARA to remove the remaining VLA-6/GoH3 complexes. The VLA-6-depleted cell lysate was then used for immunoprecipitation by MA6. In the reciprocal experiment, cell lysate was first depleted of the antigen of MA6 before being used for immunoprecipitation by GoH3. The isolated material was eluted with SDS-PAGE elution buffer, analyzed by SDS-PAGE (6%), and then visualized by autoradiography.

Adhesion assays

Cell adhesion to laminin matrix protein (Gibco BRL, MD, USA) with fluorogenic compound BCECF (Sigma, MO, USA)-labeled cells was carried out according to established procedures (24). A final volume of 100 μl containing 5×10^4 cells was added to a 96-well microtiter plate (ICN, Horsham, PA, USA) precoated with laminin or bovine serum albumin as control protein. Cells were allowed to bind for 45 min at 37°C. Results are shown as percentage of cell adhesion based on fluorescence of bound and the total 5×10^4 cells. Where indicated, the cells were stimulated with 50 nM phorbol ester (PMA) for 15 min at 37°C before carrying out the adhesion experiment. For inhibition of cell adhesion, the appropriate mAbs were added to the microtiter wells at 10 $\mu\text{g/ml}$ final concentration.

Results

Two mAbs were used for the characterization of the expression of VLA- $\alpha 6$ integrins. GoH3 is known to bind both human and mouse VLA-6 ($\alpha 6\beta 1$) and $\alpha 6\beta 4$ integrins (17, 25). MA6 is a newly prepared $\alpha 6$ -specific mAb. As shown in Fig. 1a, removal of VLA-6 from B16F1 cell lysate by a combination of GoH3 and antirat agarose (ARA) resulted in the loss of antigen for subsequent isolation by MA6. Similarly, immunoprecipitation of antigen for MA6 resulted in a major reduction in VLA-6 integrin to be isolated by GoH3 (Fig. 1b). Faint bands detectable by GoH3 may be attributable to incomplete antigen depletion.

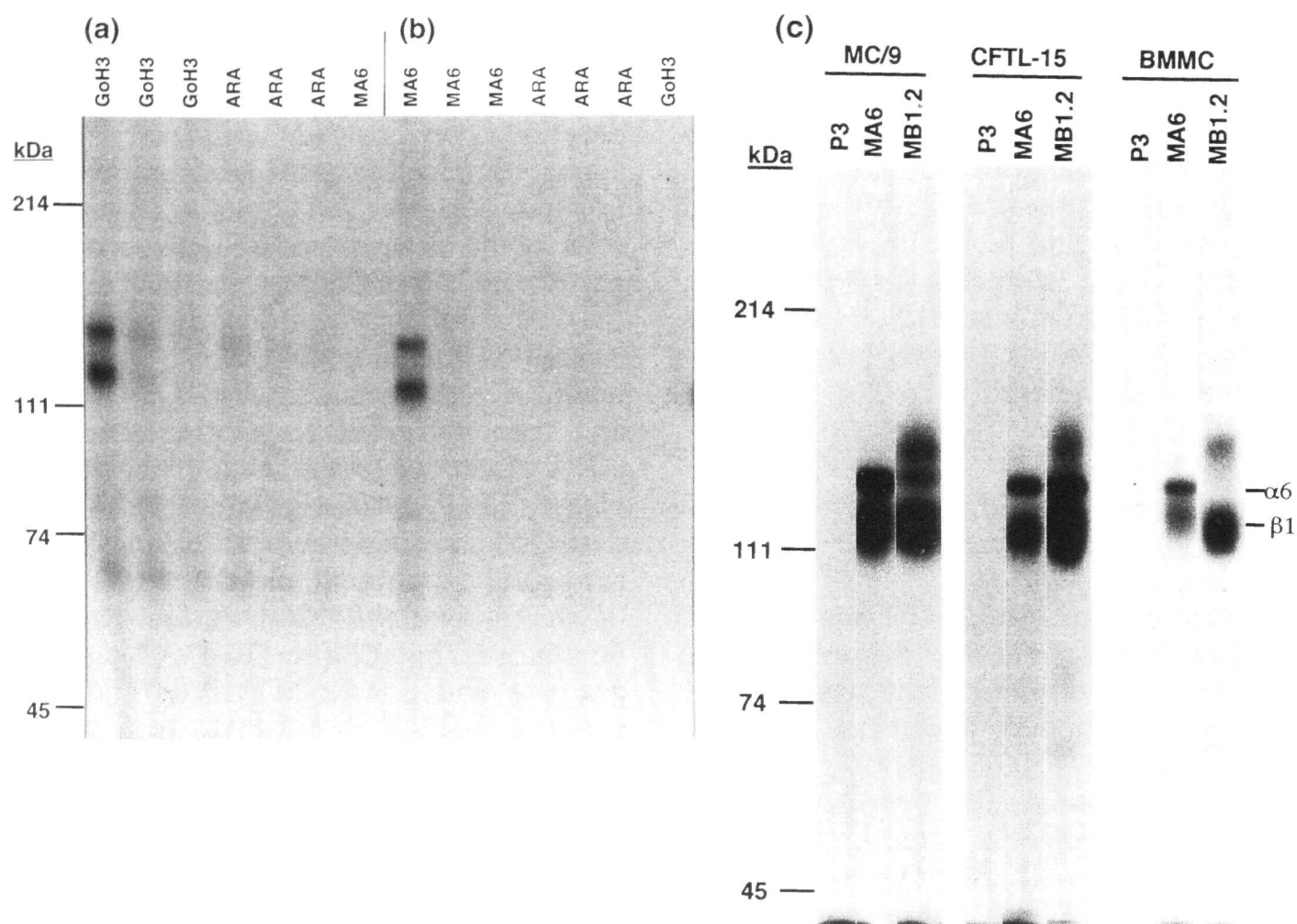


Fig. 1. Immunoprecipitation of VLA-6 ($\alpha 6\beta 1$) integrin by mAbs GoH3, and MA6 and VLA ($\beta 1$) integrins by mAb MB1.2. For determination of specificity of MA6 for mouse VLA-6 integrin, ^{125}I -labeled B16F1 cell lysate was immunoprecipitated three times with GoH3 in combination with ARA (GoH3), and remaining immune complexes were removed by another three rounds of ARA alone (ARA) before using MA6 for immunoprecipitation (a). Similarly, MA6 and GoH3 were used in reverse order (b). Results are from single experiment that has been duplicated. Similar results were also obtained with SSC-VII cells (data not shown). For detection of VLA-6 expression on mast cell lines MC/9, CFTL-15, and BMMC (3–4 weeks), ^{125}I -labeled cell lysates were immunoprecipitated with MA6 and MB1.2 (c). Results are from single experiment that has been repeated three times.

Results of reciprocal immunodepletion between GoH3 and MA6 were also obtained with mouse squamous carcinoma SSC-VII cells (data not shown). In addition, both MA6 and GoH3 immunoprecipitated a 200-kDa band typical of $\beta 4$ integrin subunit from SSC-VII cells, indicating that both mAbs are $\alpha 6$ -integrin specific with binding activities for both VLA-6 ($\alpha 6\beta 1$) and $\alpha 6\beta 4$ (data not shown). The specificity of MA6 for $\alpha 6$ integrins was confirmed by competitive binding studies. Thus, binding of MA6 was inhibited by preincubation of B16F1 cells with GoH3 (data not shown). Therefore, our results show that MA6 and GoH3 share the same specificity for $\alpha 6$ integrins. However, the epitopes involved are distinct because VLA-6-mediated cell adhesion was inhibited by GoH3 while MA6 had no effect (data not shown). Therefore, both mAbs were used for the detection of $\alpha 6$ integrin expression on BMMC.

For determination of the expression of VLA-6 on BMMC and long-term cultured mast cell lines, immunoprecipitation experiments were carried out with MA6 (Fig. 1c). ^{125}I -labeled $\alpha 6$ and $\beta 1$ integrin

subunits were isolated by MA6 from BMMC, as well as the long-term cultured mast cell lines, MC/9 and CFTL-15. Similar results were also obtained when GoH3 was used (data not shown). In comparison, mAb MB1.2 specific for the $\beta 1$ subunit isolated all associated α subunits including the cleaved form of $\alpha 4$, seen as 70- and 80-kDa bands, from BMMC and CFTL-15 cells. The expression of VLA-6 was further confirmed by flow cytometry using MA6 and GoH3 for detection of $\alpha 6$, and MB1.2 for detection of the $\beta 1$ subunit (Fig. 2).

Studies in recent years have described multiple isoforms of laminin isolated from different tissues (26–28). In addition, mouse thymocytes have been shown to bind human laminin to greater extents than mouse EHS tumor laminin (29). To characterize the involvement of VLA-6 in BMMC adhesion to laminin, we first compared cell adhesion to human merosin, mouse EHS tumor laminin, rat L2 tumor laminin, and anti-A chain purified human laminin (Fig. 3a). Human laminin appeared to be the most effective substrate for adhesion of BMMC (3–4 weeks), MC/9, and CFTL-15 cells. In

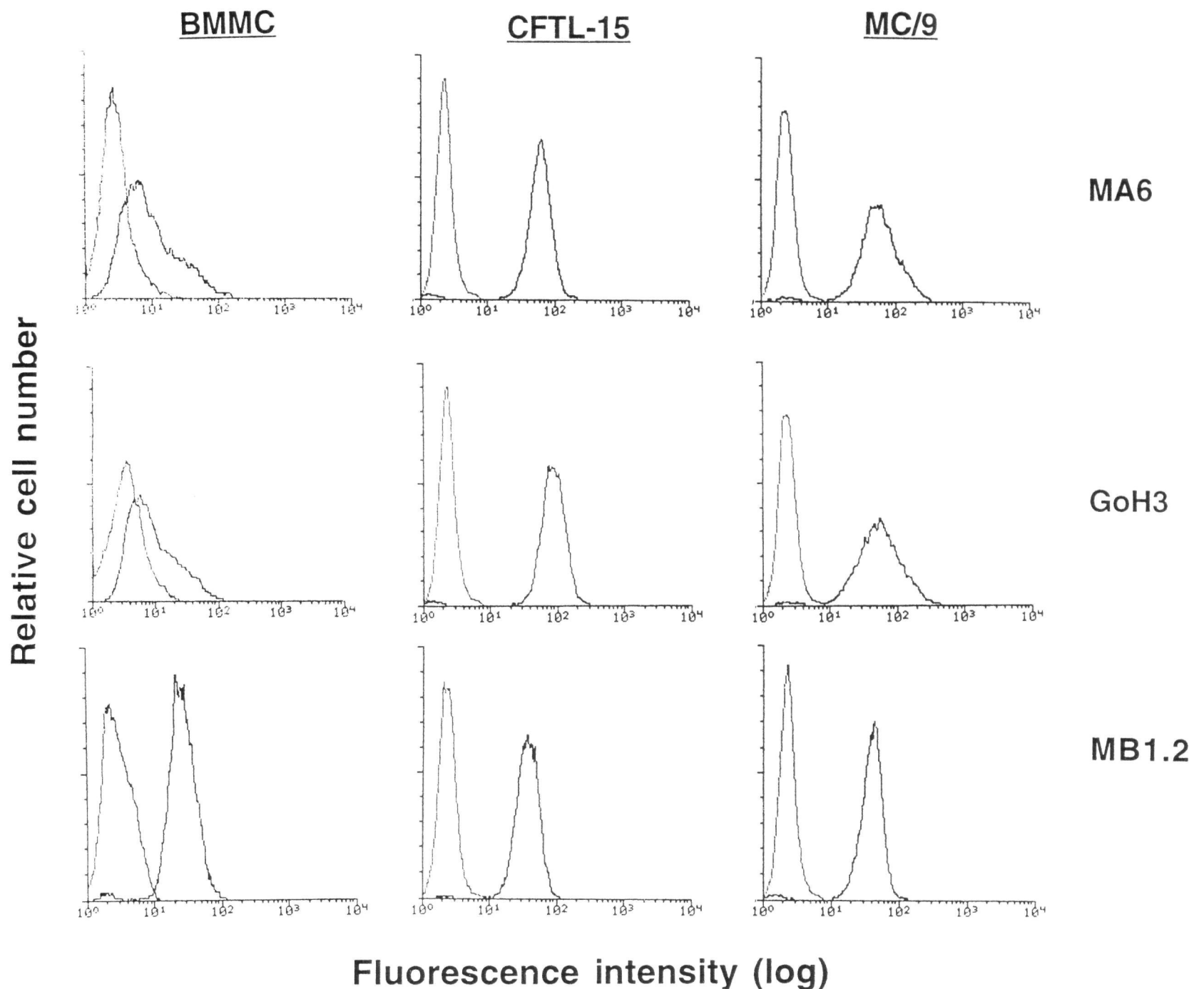


Fig. 2. Flow cytometry analysis of VLA- α 6 and - β 1-integrin subunit expression on mast cell lines MC/9, CFTL-15, and BMMC (3–4 weeks). Immunostaining with MA6 or GoH3 for α 6 detection, or MB1.2 for β 1 detection (dark lines) is shown relative to that of nonspecific mAb P3 (light lines). Results are from single experiment that has been repeated three times.

agreement with previous studies (14), adhesion of BMMC required activation. Thus, adhesion of BMMC and CFTL-15 cells to human laminin was significantly increased upon stimulation with PMA ($P < 0.05$). However, adhesion of MC/9 cells to human laminin was not significantly different after PMA stimulation. Human laminin was used in the subsequent studies to facilitate the detection of VLA-6 mediated adhesion. Adhesion of BMMC, MC/9, and CFTL-15 cells was carried out in the presence of blocking mAb GoH3 (Fig. 3b). In comparison with mAb P3 and nonblocking MA6, adhesion of MC/9 and CFTL-15 cells was inhibited by GoH3 ($P < 0.005$). Significant inhibition by GoH3 was detected for BMMC, MC/9, and CFTL-15 after stimulation by PMA ($P < 0.005$). Therefore, the results show that BMMC and the long-term

cultured mast cells utilized VLA-6 integrin for interaction with laminin substrate.

Discussion

The development of mast cells is critically regulated by microenvironmental factors (4, 5). Studies in recent years have focused on the role of cytokines in mast cell growth and differentiation (6–8). However, ECM proteins may represent the insoluble component of the microenvironment. Laminin constitutes one of the major ECM components of the basement membrane where mast cells are frequently found (1). Early studies have described a nonintegrin 32/67-kDa receptor for mediating BMMC adhesion to EHS tumor laminin (14). However, members of the integrin family

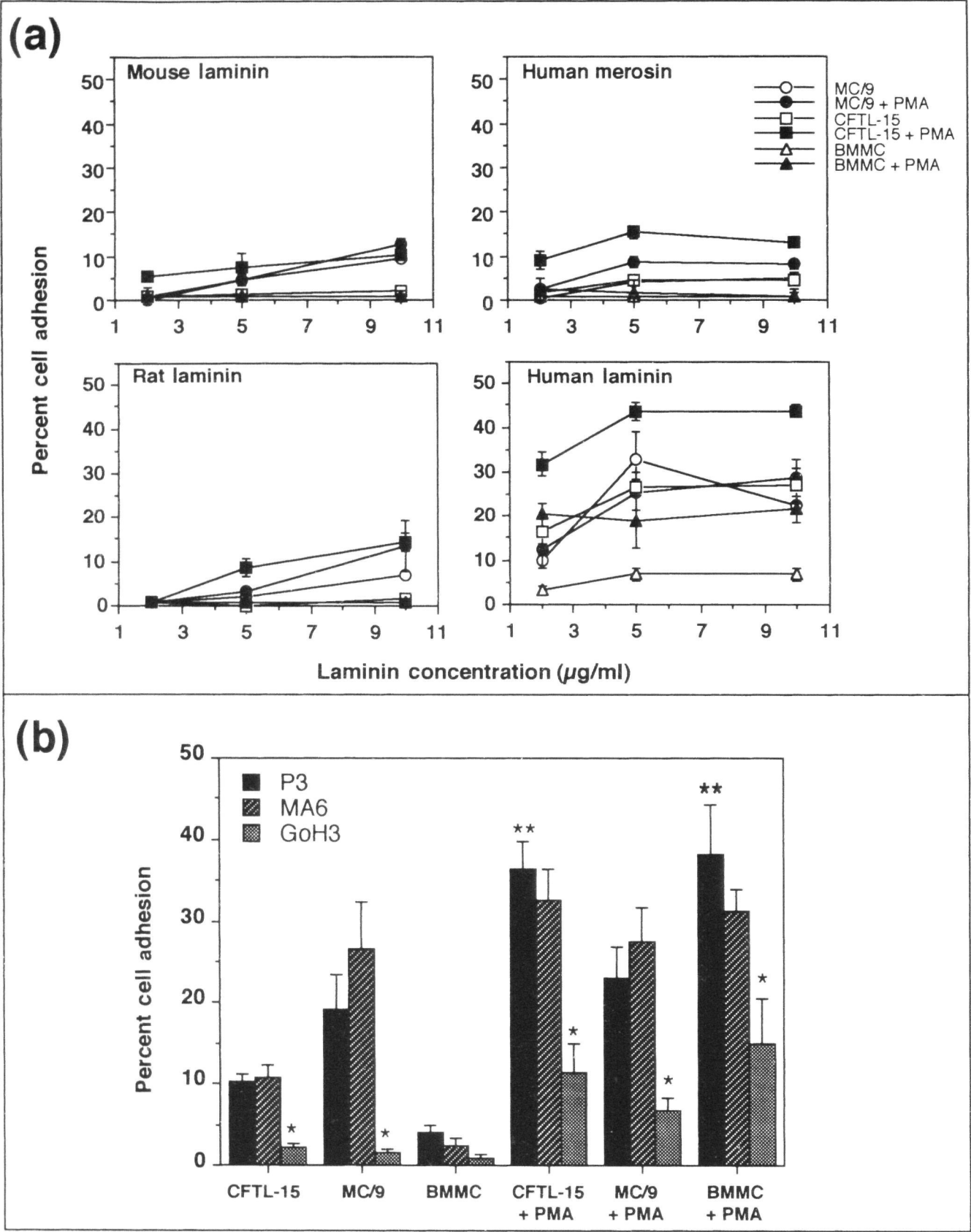


Fig. 3. Involvement of VLA-6 integrin in adhesion of BMMC and mast cell lines to laminin. Adhesion of BMMC (3–4 weeks), MC/9, and CFTL-15 cells to mouse EHS tumor laminin, rat L2 tumor laminin, human merosin, and human laminin was carried out in single experiment to facilitate comparison of relative extents of adhesion (a). Where indicated, cells were stimulated with PMA (50 nM) for 15 min at 37°C before adhesion assays. Inhibition of VLA-6 function was assessed by carrying out adhesion assays on human laminin (4 $\mu\text{g/ml}$) in presence of mAb GoH3, MA6, or control P3 (b). Results are expressed as percentages of total cells bound. Each value represents mean $\pm$ SD of triplicates from experiment that has been repeated three times. Asterisks (*) indicate significant difference in level of adhesion as result of inhibition by GoH3 ($P < 0.005$), and (**) indicate significant difference in level of adhesion as result of PMA stimulation ($P < 0.005$).

have been shown to serve as receptors for laminin and to regulate diverse cell functions including cell growth and differentiation (12).

The VLA- $\alpha 6$ subunit can be expressed in association with the $\beta 1$ subunit or the alternate $\beta 4$ subunit (30, 31). In the present study, both the

newly prepared MA6 and the prototypic mAb for $\alpha 6$ integrins GoH3 (17) demonstrated the expression of $\alpha 6$ integrins on BMMC and long-term cultured mast cell lines. In addition, only labeled bands of $\alpha 6$ and $\beta 1$ subunits, with M_r of about 140 and 120 kDa, respectively, were

immunoprecipitated; no labeled 200-kDa band typical of the $\beta 4$ integrin subunit was detected. Therefore, it is apparent that BMMC, MC/9, and CFTL-15 cells expressed VLA-6 ($\alpha 6\beta 1$), but not $\alpha 6\beta 4$ integrin.

Our results indicate preferential binding of VLA-6 on mast cells to human laminin in comparison to human merosin, mouse EHS, and rat L2 tumor laminin. The human laminin used in this study was purified from placenta, using mAb specific for the human laminin A chain. Therefore, it is likely to consist of both A/B1/B2 and A/B1s/B2 isoforms. However, VLA-6 has been shown to bind different laminin isoforms including EHS tumor laminin (A/B1/B2 isoform); bovine heart laminin consisting of the A/B1/B2, Am/B1/B2, and Am/B1s/B2 isoforms; human UMSCC-22B cell-derived kalinin; and human laminin (32). Whether binding to specific laminin isoforms is regulated by the cell type on which VLA-6 is expressed is not clear. Recent studies have shown that VLA-6 on mouse thymocytes bound human placental laminin better than EHS tumor laminin (29). In comparison, VLA-6 expressed on the T-cell clone Ar-5 and choriocarcinoma JAR cells readily bound EHS tumor laminin (25, 33). As suggested by Chang et al. (29), the different adhesion properties of mouse VLA-6 integrin for laminin from different sources (mouse, rat, and human) may be due to subtle differences in the binding sites involved, the requirement of other laminin receptor(s) for effective binding, regulation by factors such as cytokines, and/or changes in the conformation that favor ligand binding. In addition, both the mouse and rat laminin used in this study are tumor cell-derived, and it has been suggested that the mouse EHS tumor-derived laminin may not be representative of laminin matrix proteins found *in vivo* (28).

In earlier studies, BMMC were shown to bind EHS tumor laminin upon stimulation and a 32/67-kDa receptor was involved (14). Results from the present study also show that adhesion of BMMC required activation, and human laminin appeared to be the most effective substrate among the different laminins compared. Adhesion was VLA-6 mediated since binding was inhibited by blocking mAb GoH3. It was observed that inhibition of PMA-stimulated adhesion by GoH3 was incomplete; to determine whether the remaining binding activity was attributable to the 32/67-kDa receptor will require confirmatory studies. VLA-6 has been described as a major laminin receptor with roles in cell morphogenesis and development (15, 16). Therefore, results from this study demonstrating VLA-6 being expressed and utilized by BMMC for interaction with laminin may have relevance to

studies of ECM proteins as microenvironmental factors involved in mast cell development and function.

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