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A BLOCKING MONOCLONAL ANTIBODY TO ENDOTHELIAL-LEUKOCYTE ADHESION MOLECULE-1 (ELAM1)

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ELAM1 is a leukocyte adhesion molecule induced on human umbilical vein endothelial cells (HUVECs) by inflammatory cytokines. Balb/C mice were immunized with COS cells transiently expressing cell-surface ELAM1 after transfection with ELAM1 cDNA. After fusion, ELAM1-specific monoclonal antibodies (Mabs) were identified by selective adhesion to ELAM1-expressing, but not control, CHO cells, and to cytokine-treated but not untreated HUVECs. One Mab, designated BB11, binds to and immunoprecipitates ELAM1 expressed on HUVECs, COS and CHO cells. BB11 blocks the interaction of ELAM1 with human PMN, the human myelomonocytic cell line HL60, and the human colon carcinoma line HT29.

ELAM1 is an induced endothelial cell protein that contributes to the adhesion of PMN to cytokine-treated HUVECs *in vitro* (1,2). It is one of a family of adhesion molecules related to mammalian lectins, epidermal growth factor, and complement regulatory proteins (2). ELAM1 is present at sites of inflammation *in vivo*, but is not expressed in the normal vascular bed, and likely plays an important role in PMN extravasation at inflammatory sites *in vivo* (1-3).

We have recently described the direct expression cloning of ELAM1, using adhesion to HL60 cells to select functional clones (4). We have already exploited this powerful method to clone a novel lymphocyte adhesion molecule expressed on cytokine-treated HUVECs, which we have called VCAM1 (5). However, the lack of specific Mabs to characterize the expressed proteins is problematic. In this report we describe a blocking Mab to ELAM1, generated after immunization with COS cells transfected with ELAM1 cDNA, and transiently expressing ELAM1 protein on the cell surface. This method should be applicable to other cell surface molecules where cDNAs are available but Mabs are lacking.

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MATERIALS AND METHODS

Cells. HUVECs were isolated and subcultured as described (4) and used for assays at passages 3-5. HL60, COS7, CHO and HT29 cells were maintained, and human PMN isolated, as described (4,6). The generation of stable ELAM1-expressing CHO cell lines will be detailed elsewhere. Briefly, the ELAM1 cDNA (4) was inserted into vector pBG341JOD, which contains an expression casette for the dihydrofolate reductase cDNA gene. CHO.DHFR$^-$ cells were transfected with pBG341JOD.ELAM by electroporation (6), methotrexate selection performed, and clones expressing sufficient ELAM1 to bind HL60 cells detected directly by adhesion assay. Two cell lines, CHO338 and CHO660, were used in these studies.

Immunoprecipitation. All cells were labelled with [35S]-cysteine and extracted with Triton X100 as described for HUVECs (4). Cell supernatants were precleared for 2-3 hr at 4°C with protein A-Sepharose IgG, incubated overnight at 4°C with protein A-sepharose-BB11, the beads washed 5 times with PBS pH 7.2, 0.5% Tween 20, 0.05% SDS, 0.1% BSA, and 0.02% sodium azide, boiled in 2% sample buffer, and the eluate run on a 4-20% SDS gradient gel. N-glycanase treatment was performed as described (2).

Adhesion Assays. HUVECs were grown in 48 well cluster plates as described (4). Control CHO cells, or CHO338 or CHO660 cells, were grown in 48 well or 96 well plates, and used within 2 days of reaching confluence. HL60 and HT29 cells were labelled, and adhesion assays performed, as described (4). For inhibition assays, the appropriate target cells were preincubated for 30 min at room temperature with Moabs at 10 µg/ml, cells added to washed monolayers, and the assays performed as above. Moabs 60.3 and 4B9, to inhibitory epitopes on CD18 and VCAM1, respectively, were the gift of Dr. John Harlan.

Hybridoma Generation. Eight to ten week old female Balb/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were immunized i.p. (0.5 ml) every two weeks for eight weeks with 2-5 million COS cells, and bled from the tail vein 7-10 days after each boost. COS cells were transfected with ELAM1 DNA by electroporation as described (4), and 72 hr after transfection, were harvested with 5 mM EDTA in Dulbecco's PBS (GIBCO Laboratories, Grand Island, NY) 1% BSA, by incubation for 30 min. at 37°C. The cells were collected and washed (200 x g) once with DPBS, 5 mM EDTA, and then twice with DPBS without EDTA. The cells were resuspended in a small volume of DPBS, counted in trypan blue and scored for viability. Cells used for immunization were always greater than 95% viable. Sera were analyzed for antibodies which blocked HL60 adhesion to 4 hour IL-1 activated HUVECs. One mouse was selected, boosted with COS ELAM-1 cells, and spleen cells were fused to P3X63Ag8.653 cells as described (7). Hybrid cells were selected in HAT supplemented medium and grown initially in the presence of a commercial cloning factor (IGEN, Rockville, MD). Culture supernatants were screened for differential binding to a stable transfectant of ELAM1 in CHO cells vs untransfected CHO cells, using radiolabeled goat anti-mouse immunoglobulin (New England Nuclear, Boston, MA). Specificity was corroborated with an ELISA using IL-1 activated HUVECs, and selected cultures examined for inhibition of adhesion. Positive cultures were subcloned at limiting dilution. Monoclonality was confirmed by subclass analysis using goat anti-mouse immunoglobulin coated plates and a kit from Hyclone Laboratories (Logan, Utah). BB11 ascites was raised in pristane primed Balb/c mice. The monoclonal antibody was purified on protein A coupled to sepharose 4B (Zymed, CA). The ascites was diluted 1:3 into 1.5M glycine, 3M NaCl, pH 8.9 and passed over a 10 ml column of protein A pre-equilibrated in the same buffer. Bound antibody was eluted with (0.05M) acetate saline buffer, pH 3.7 and dialyzed against PBS pH 7.2.

RESULTS AND DISCUSSION

Generation of an ELAM1-selective Mab. Balb/c mice were immunized at biweekly intervals with COS cells transiently expressing ELAM1. Anti-ELAM1-secreting hybridomas were selected using as a primary screen binding to CHO338 but not CHO control cells. In parallel assays, positives were also readily detected by binding to cytokine-treated but not control HUVECs, using a standard ELISA assay. One well, BB11, consistently scored in each binding assay and also inhibited in the adhesion assay. Originally a mixed clone containing IgG2b and IgM, BB11 was subcloned at limiting dilution yielding 7 subclones. ELAM1 specificity and adhesion inhibition resided with the IgG2b subclones. Specificity for ELAM1 was confirmed by reactivity with CHO660 but not control CHO.JOD cells (Fig. 1A), with cytokine-treated but not control HUVECs (Fig. 1A), and with COS cells transiently expressing ELAM1 but not control COS cells (not shown). All of the subclones behave similarly and are referred to hereafter as BB11. Mab BB11 immunoprecipitates proteins of about 110 Kd and 120 Kd from ELAM1-expressing HUVECs (Fig. 1B). N-glycanase treatment generates a protein core of about 80 Kd (Fig. 1B). BB11 immunoprecipitates a protein of about 96 Kd from ELAM1-expressing but not control COS cells (Fig. 1B). These data are in agreement with published results (2). BB11 also immunoprecipitates proteins of about 130 Kd and 100 Kd from CHO660 but not control CHO cells (Fig. 1B).

A number of cell surface molecules have now been cloned by direct expression and functional assay without using specific Mabs, including the adhesion molecules ELAM1 (4), VCAM1 (5), and ICAM2 (8), and the cytokine receptors for IL6 (9), and GMCSF (10). Our results show that

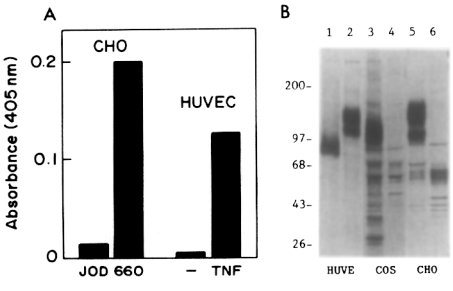

Figure 1. A) BB11 binds ELAM1: control CHO cells (JOD) or ELAM1- expressing CHO cells (660), or HUVECs with or without TNF treatment (10 ng/ml,4h), were incubated with Mab BB11 (10 µg/ml, 60',23°C), washed, incubated with alkaline phosphatase-coupled anti-mouse antibody (1:5000,60',23°C), washed and developed using standard protocols. B) BB11 immunoprecipitates ELAM1: HUVECs expressing ELAM1 (TNF, 10 ng/ml,4h), either N-glycanase treated (lane 1) or untreated (lane 2); COS cells expressing ELAM1 (lane 3) or control COS cells (lane 4); CHO cells expressing ELAM1 (lane 5) or control CHO cells (lane 6).

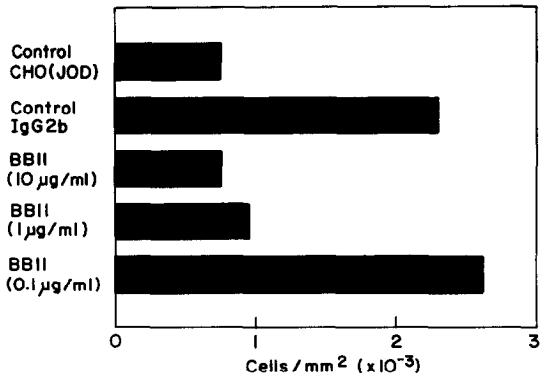

Figure 2. Mab BB11 inhibits HL60 adhesion to ELAM1: CHO cells expressing ELAM1 were preincubated with Mab BB11 (10 µg/ml,30',23°C) and the ability of HL60 cells to adhere as compared to control CHO cells (JOD) was assessed (see methods).

immunization of mice with COS cells expressing genes cloned into the CDM8 expression vector is adequate for the subsequent generation of specific Mabs, even though expression is transient (11).

Inhibition of HL60 Adhesion. The promyelocytic cell line HL60 has proved particularly useful for the characterization of the ELAM1 adhesion pathway (1,2). We have used it to clone ELAM1 by direct expression, and to examine its adhesion function (4). As shown in Figure 2, Mab BB11 at a concentration of 10ug/ml blocks completely HL60 adhesion to CHO660 cells, while a control IgG2b Mab has no effect on HL60 adhesion. However, BB11, even at optimal doses, only partially inhibits HL60 binding to TNF-treated HUVECs (Fig. 3), suggesting the existence of a second adhesion pathway. We have found that HL60s also bind to VCAM1, another adhesion molecule expressed on cytokine-treated HUVECs (5).

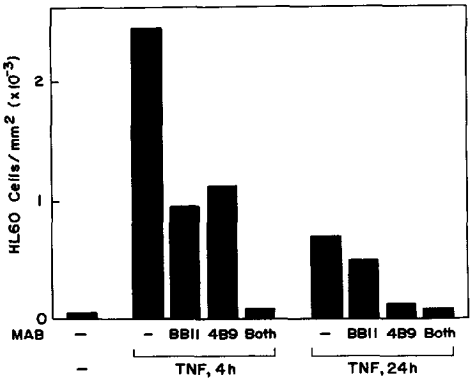

Figure 3. HL60 adhesion to HUVECs: HUVECs, either treated with TNF (10 ng/ml,4h or 24h) or untreated, were preincubated with BB11 and/or anti-VCAM1 Mab 4B9 as indicated (each 10 µg/ml,30',23°C) and HL60 adhesion assessed.

Adhesion to VCAM1 is fully blocked by the anti-VCAM1 Mab 4B9 (12). BB11 in conjunction with 4B9 blocks HL60 binding completely to cytokine-treated HUVECs, both at 4h post cytokine treatment, when the ELAM1 pathway predominates (60-90% inhibition by BB11, n=4), and at 24h when the VCAM1 pathway predominates (Fig. 3).

PMN binding to HUVECs. Mab 60.3, which recognizes a blocking epitope on CD18, partially inhibits the binding of PMN to cytokine-treated HUVECs in our assays (4). As shown in Figure 4, BB11 also partially inhibits, and in conjunction with 60.3 abolishes completely, PMN binding to cytokine-treated HUVECs. These data are in agreement with recently published information using different anti-ELAM1 and anti-CD18 antibodies (13).

HT29 binding to HUVECs. Recent studies have shown that a number of human cell lines established from solid tumors show a selective increase in adhesion to cytokine-treated HUVECs (14,15). In particular, a series of colon carcinoma cell lines, including HT29, bound to HUVECs with a time course consistent with ELAM1-mediated adhesion (14). We have reported that HT29 cells adhere selectively to ELAM1 expressed in COS cells (4). Figure 4 shows that BB11 abolishes completely the increased binding of HT29 cells to HUVECs treated with TNF for 4h, showing that increased HT29 adhesion is all ELAM1-mediated, and confirming recently published information (16).

In summary, immunization with COS cells transiently expressing cloned cDNAs should be a general method for the generation of Mabs to new cell surface proteins. In particular, we have generated by this means a potent blocking Mab to the cytokine-induced human endothelial cell adhesion protein ELAM1. This Mab should greatly help in evaluating the role of ELAM1 in leukocyte/endothelial cell adhesion and transendothelial migration, both in vitro and in vivo.

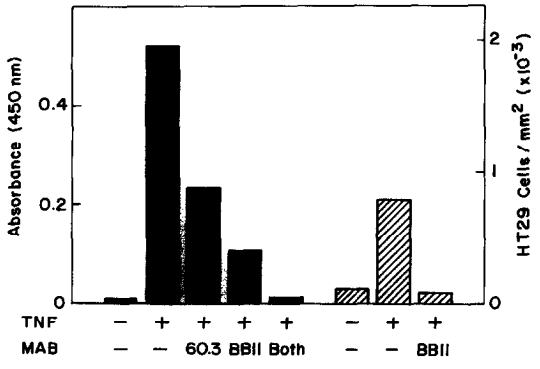

Figure 4. PMN and HT29 adhesion to HUVECs: HUVECs, either treated with TNF (10 ng/ml, 4h) or untreated, were preincubated with BB11 (10 µg/ml,30',23°C) where indicated, and adhesion of PMN (either untreated or treated with Mab 60.3,50 µg/ml,20',23°C) or HT29 cells was assessed.

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