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A new procedure for establishing functional monoclonal antibodies capable of inhibiting E- or P-selectin-dependent cell adhesion

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Employing a new procedure, we established many monoclonal antibodies (mAbs) which inhibit E- or P-selectin-dependent cell adhesion. One of these mAbs is capable of staining selectin in paraffin-embedded histological sections. The procedure is based on immunization of BALB/c mice with irradiated mouse myeloma NS-1 cells (syngeneic HAT-sensitive fusion partner cells) transfected with cDNA encoding human E- or P-selectin. Resulting NS-1 transfectant cells permanently express human E- or P-selectin as immunogen. The mAbs are useful for detecting selectins by flow cytometric and immunohistological methods, and for inhibiting selectin-dependent adhesion in experimental models. In contrast, the majority of anti-selectin mAbs previously established do not have these capabilities.

***Keywords*: selectin, NS-1, transfection, immunohistology, cell binding**

***Abbreviations*: Ig, immunoglobulin; mAb, monoclonal antibody**

Introduction

E-selectin (expressed on endothelial cells) and P-selectin (expressed on endothelial cells and platelets) are part of an important defence mechanism for recruitment of neutrophils into tissues, for tissue repair or destruction of foreign microorganisms (for review see [1]). They may also be involved in tumour cell invasion and metastasis (for review see [2], and Discussion). Following the discovery of these selectins in 1989 [3, 4], there have been many investigations of their carbohydrate ligands [5–10]; for review see [11]. Some anti-E- or P-selectin mAbs so far established are capable of staining selectin expressed on histologic sections from frozen tissues ('frozen sections'), but no mAb has been reported to stain paraffin-embedded sections. Activated endothelial cells in culture are stained by anti-E- but not by anti-P-selectin mAbs, whereas native endothelial cells in frozen sections are stained by anti-P-selectin mAbs. Activated platelets are strongly stained by and reactive with anti P-selectin mAbs. Since E- and P-selectin expressed on activated endothelial cells and platelets are involved in mediation of inflammatory processes (i.e. recruitment of neutrophils to site of inflammation), anti-E- and -P-selectin mAbs are expected to inhibit this process. In fact, murine anti-P-selectin mAbs significantly reduce reperfusion injury of rabbit ear. The effectiveness is similar to that of anti-CD18 mAb [12].

We developed a new method allowing establishment of many mAbs directed to E- or P-selectin that strongly inhibit E- or P-selectin-dependent cell adhesion. One of the mAbs is capable of staining selectins expressed on endothelial cells in paraffin-embedded sections.

Materials and methods

Cell lines used

Human promyelocytic leukemia HL60 and mouse myeloma NS-1 and Sp2/0 cell lines were purchased from ATCC. Establishment of NS-1E and NS-1P is described below. Chinese Hamster Ovary cell line CHODG44 and its E- and P-selectin-expressing transfectants (CHODG44-E and -P) were established as described previously [13].

Expression of E- or P-selectin in NS-1 cells through transfection of selectin genes

NS-1 is a HAT-sensitive mouse myeloma cell line which has been used extensively as a cell fusion partner for hybridoma production [14]. In this experiment, NS-1 was transfected

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with: (i) commercially available E-selectin cDNA carried by vector pCDM8 (R&D Systems, Minneapolis, MN) plus pSV2/neo (ATCC); or (ii) P-selectin cDNA cloned in this laboratory from HEL cells and carried by pRC/CMV as described previously [13]. The transfection was performed by electroporation. Transfectants were selected by culturing in medium containing 500 $\mu g\,ml^{-1}$ of G418 (Gibco, Grand Island, NY), and were screened by mAb against P-selectin (AC1.2) (Becton-Dickinson) or against E-selectin (3B7) [15] (donated by Dr Walter Newman, Otsuka Pharmaceutical, Rockville, MD), or by binding activity to HL60 cells. Resulting E- and P-selectin-expressing cell lines were termed NS-1E and NS-1P respectively. Expression of E- and P-selectin by these cell lines (as determined by adhesion to HL60 cells and by reactivity with mAbs) was permanent, and was not affected by freeze/thaw or passage of many generations.

Immunization of mice with NS-1E and NS-1P, and establishment of hybridoma

NS-1E or NS-1P cells (2×10^7 in 100 µl) were irradiated under 7000 rad and mixed with an equal volume (100 µl) of adjuvant MPL/TDM/CWS emulsion (Ribi Immunochem Inc., Hamilton, MT). The mixtures were used for i.p. immunization of BALB/c mice once per week for 3 weeks. After about 3 weeks the mice were boosted, and splenocytes were harvested and fused with actively-growing NS-1 cells in the presence of polyethylene glycol according to established procedures. Antibody-producing hybridomas were screened by ability of supernatant to stain NS-1E or NS-1P cells, but not control NS-1 cells. Screening of hybridoma was also made by direct binding assay of antibody to plates coated with E- or P-selectin fusion protein (see below). Mouse IgG isotypes were determined with a mouse mAb isotyping kit (Isostrip, Boehringer Mannheim, Indianapolis, IN).

Table 1. mAbs directed to P- or E-selectin, established by the novel procedure.

mAb	*Isotype*	*Inhibits HL60 binding to P-selectin*	*Inhibits HL60 binding to E-selectin*
Anti-human P-selectin			
P1A	$mIgG_1$	No	
P1C	$mIgG_1$	No	
P1D	$mIgG_1$	No	
P5A	$mIgG_{2b}$	Yes	
P5B	$mIgG_1$	Yes	
P5C	$mIgG_1$	Yes	
Anti-human E-selectin			
E1A	$mIgG_1$		No
E1B	$mIgG_{2b}$		No
E1C	$mIgG_1$		Yes

Binding of mAbs to E- or P-selectin, or selectins expressed on the surface of cells or tissues

Culture supernatants of various hybridomas (established as shown in Table 1) were adjusted in their IgG concentration (to 5–10 µg per ml of RPMI medium) and tested for cell binding by flow cytometry. Briefly, cells (2×10^5) were incubated with mAb on ice for 1 h, washed twice with PBS containing 1% BSA and 0.1% sodium azide ('washing buffer'), incubated with 1:40 diluted FITC-conjugated goat anti-mouse IgG on ice for 1 h, washed with washing buffer, and analysed on EPICS flow cytometer (Coulter Corp., Hialeah, FL). Mouse IgG (mIgG) (5–10 µg per ml of RPMI medium) was used as control. Positivity (%) and intensity index (mean fluorescence intensity [MFI] with mAb divided by MFI with control mIgG) were calculated.

Inhibition of selectin-dependent cell adhesion by mAbs

HL60 cells show both E- and P-selectin-dependent cell adhesion. Soluble fusion proteins of human E- or P-selectin with Fc domain of human Ig were prepared as described previously [13]. Flat-bottom 96-well ELISA plates (Falcon, Bectin-Dickinson, Lincoln Part, NJ) were coated with goat anti-human IgG (Fc fragment-specific) antibody (Jackson Immunoresearch Lab, West Grove, PA) in PBS (5 $\mu g\,ml^{-1}$, 50 µl per well), and incubated overnight at 4 °C. Each well was then washed with PBS, blocked with 3% BSA in PBS, incubated with 100 µl of culture supernatant containing $\approx 0.2\ \mu g\,ml^{-1}$ of fusion protein for 2 h at room temp, and washed three times with PBS. Various concentrations of anti-E- or anti-P-selectin mAb were added to the plates, which were then incubated for 60 min at room temperature and washed with PBS. HL60 cells labeled with [^{3}H]thymidine in culture were washed and resuspended in binding buffer (HBSS–1% FCS–10 mM HEPES (pH 7.4)–0.05% azide). 1×10^5 HL60 cells suspended in 50 µl binding buffer solution were added to wells coated with selectin fusion protein. For control wells, 5 mM EDTA was added. After 20 min at room temperature, unbound cells were washed out with binding buffer, and bound cells were detached with trypsin/EDTA and detected using a scintillation counter.

Preparation of histological sections and in situ staining of selectins by mAbs

Colonic tumors and surrounding normal colon tissue, surgically resected at the National Cancer Center Hospital, Tokyo, Japan, were fixed in acetone, treated with methylbenzoate and xylene, and embedded in paraffin as previously described ('AMeX method') [16]. Frozen human tonsil sections (fixed in acetone at–20 °C for 1 min and stored at −80 °C) were purchased from Histologic Services, Seattle, WA. Thawed sections, and deparaffinized and hydrated sections, were incubated in 0.3% hydrogen peroxide in methanol at room temperature for 30 min to eliminate

endogenous peroxidase activity, washed three times in PBS, incubated in 2% normal pig serum in PBS at room temperature for 30 min, washed again, overlaid with 150 µl of culture supernatant of mAb (e.g. P5A), and incubated at 4 °C overnight in a moist chamber. mIgG was used as negative control. Sections were washed again and stained with biotinylated secondary mAb, peroxidase-conjugated avidin and 3′,3′-diaminobenzidine. These reagents were included in a Vectastain ABC kit (Vector, Burlingame, CA). Sections were counterstained with Hematoxylin, dehydrated, and mounted.

Results

Antibodies established

We transfected mouse myeloma NS-1 and Sp2/0 cells by electroporation with plasmids containing E- or P-selectin. We established permanent transfectants (NS-1E and NS-1P) from NS-1 cells, but were unable to establish stable transfectants from Sp2/0 cells. We established six hybridomas secreting anti-human P-selectin mAbs and three hybridomas secreting anti-human E-selectin mAbs after fusion with NS-1 cells according to the method described originally by Köhler and Milstein [17]. Their Ig isotypes and ability to inhibit selectin-dependent adhesion of HL60 cells are summarized in Table 1.

Reactivities of mAbs

Results of flow cytometry analysis of the six anti-P-selectin mAbs listed in Table 1 are shown in Table 2. Each of the mAbs reacted strongly with P-selectin-expressing NS-1P cells (but not with parent NS-1 cells), P-selectin-expressing CHODG44-P cells (but not with parent CHODG44 cells), and thrombin-activated platelets (but only weakly with nonactivated platelets). Reactivities of the three anti-E-selectin mAbs listed in Table 1 are shown in Table3. Each of the mAbs reacted strongly with E-selectin-expressing NS-1E cells (but not with parent NS-1 cells), and E-selectin-expressing CHODG44-E cells (but not with parent CHODG44 cells). As expected, anti-P-selectin mAbs did not react with E-selectin-transfected cells, and anti-E-selectin mAbs did not react with P-selectin-transfected cells (data not shown).

Table 3. Reactivity of anti-E-selectin mAbs analyzed by flow cytometry.

	mIgG		E1A		E1B		E1C	
	%*	Index*	%	Index	%	Index	%	Index
Exp. 1								
NS-1	2.8	1.0	1.5	0.8	5.5	1.1	14.7	1.3
NS-1E	1.6	1.0	95.7	27.2	92.7	23.3	97.4	42.0
Exp. 2								
CHODG44	2.5	1.0	2.1	1.0	2.8	1.0	8.7	1.2
CHODG44-E	2.0	1.0	85.8	18.8	79.6	12.4	88.3	21.5

* %, percentage of positive cells. Index, MFI with mAb/MFI with control mIgG.

mAbs that inhibit P- and E-selectin-dependent cell adhesion

Among the mAbs listed in Table 1, three anti-P-selectin mAbs (P5A, P5B, P5C) strongly inhibited P-selectin-dependent HL60 cell adhesion but had no effect on E-selectin-dependent adhesion (Table 4). Among the three anti-E-selectin mAbs, E1C strongly inhibited E-selectin-dependent adhesion, while the other two showed weaker inhibition. None of the three mAbs had any effect on P-selectin-dependent adhesion (Table 4).

The dose-dependent inhibitory effects of P5A and E1C on P- and E-selectin-dependent adhesion of HL60 cells are

Table 2. Reactivity of anti-P-selectin mAbs analyzed by flow cytometry.

	mIgG		P1A		P1C		P1D		P5A		P5B		P5C	
	%*	Index*	%	Index	%	Index	%	Index	%	Index	%	Index	%	Index
Exp. 1														
NS-1	1.9	1.0	31.3	2.7	2.0	1.0	1.1	1.0	1.7	1.0	1.7	1.0	1.9	1.0
NS-1P	1.5	1.0	89.0	20.4	93.6	19.5	24.7	22.6	89.9	16.1	89.4	15.7	93.2	19.8
Exp. 2														
Non-activated platelet	3.0	1.0	18.4	6.1	10.2	3.4	9.9	3.0	NT		NT		NT	
Thrombin-activated platelet	3.1	1.0	92.5	187.5	89.8	107.9	85.3	88.3	NT		NT		NT	
Exp. 3														
CHODG44	2.2	1.0	2.9	1.0	1.7	1.0	1.9	1.0	2.3	1.0	2.1	1.0	2.7	1.0
CHODG44-P	2.5	1.0	83.8	14.7	87.2	16.9	99.3	59.3	97.6	38.3	97.0	37.4	97.6	37.1

* %, percentage of positive cells. Index, MFI with mAb/MFI with control mIgG.

Table 4. Inhibitory effect on HL60 cell adhesion to P- and E-selectin-coated plates by anti-P- and anti-E-selectin mAbs.

	P-selectin	E-selectin
mIgG	0	0
P1C	8.2 ± 17.4*	NT
P5A	96.1 ± 0.7	1.6 ± 13.2
P5B	91.2 ± 0.5	− 6.3 ± 6.6
P5C	87.0 ± 1.2	− 10.7 ± 0.6
E1A	− 5.3 ± 15.4	28.4 ± 5.9
E1B	− 0.8 ± 6.4	36.3 ± 2.8
E1C	4.8 ± 1.1	91.3 ± 0.2
EDTA	88.9 ± 0.3	76.2 ± 1.5

*mean inhibition (%) ± half of range (from duplicate experiments).

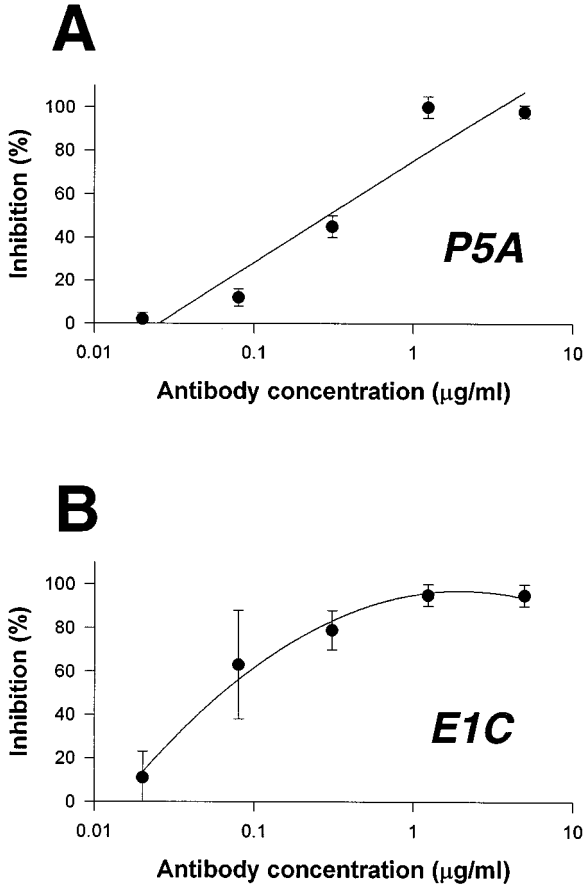

Figure 1. Dose-dependent inhibition by mAbs P5A (Panel A) and E1C (Panel B) of P- and E-selectin-dependent adhesion of HL60 cells. mAb concentrations used in the experiments were 0.02, 0.08, 0.31, 1.25, and 5.0 μg ml^{-1}. Values shown are mean ± SD of triplicate experiments.

shown in Fig. 1A and B respectively. 100% inhibition of P-selectin-dependent adhesion was achieved with ≈ 1 μg ml^{-1} of P5A. Near-complete inhibition of E-selectin-dependent adhesion was achieved with ≈ 1 μg ml^{-1} of E1C.

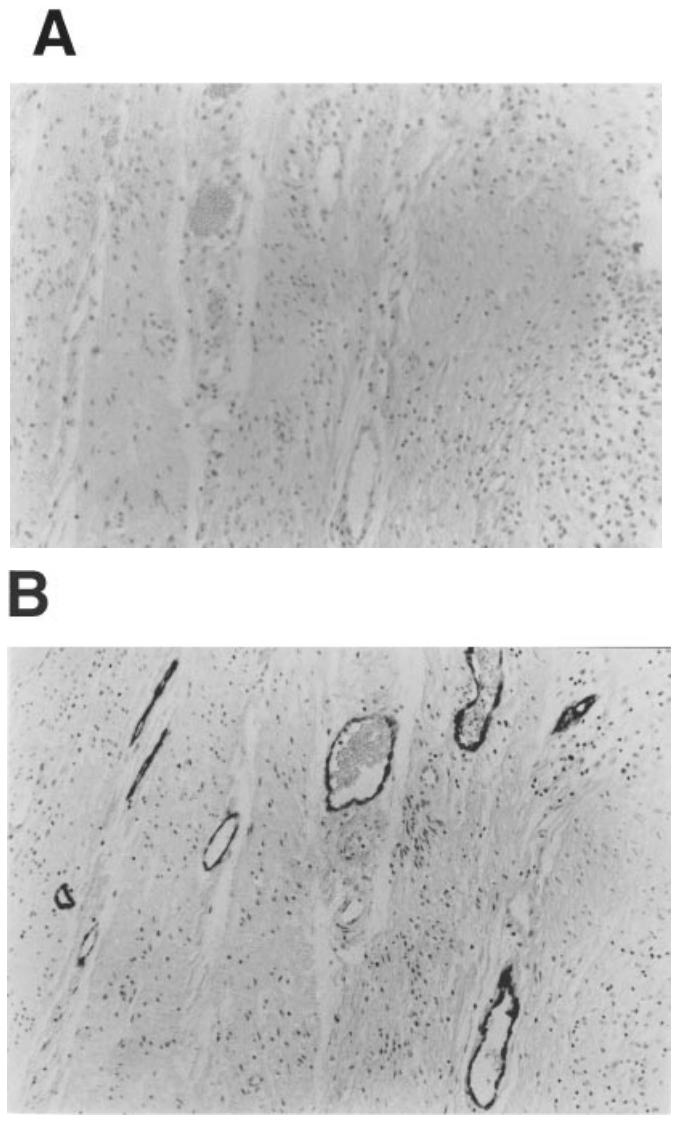

Figure 2. Immunostaining of P-selectin expressed on microvascular endothelial cell layers in paraffin-embedded sections of muscular tissue adjacent to colonic tumors. Panel A, control mIgG. Panel B, mAb P5A.

mAbs capable of detecting selectin expression in situ on immunohistology

mAb P5A showed strong immunohistological staining of P-selectin expressed on endothelial cells in paraffin-embedded sections (prepared by AMeX method) of tissues adjacent to colonic tumors (Fig. 2), in addition to frozen human tonsil sections (data not shown).

Discussion

The new procedure described here provides an efficient means of establishing mAbs directed to human E- or P-selectin. The method is based on immunization of BALB/c mice with irradiated NS-1 cells (syngeneic fusion partner cells) which are transfected with cDNA of these selectin genes. Plasmids containing E- or P-selectin cDNA

as described in Materials and methods were used, and permanently transfected cell lines were established. The efficiency of mAb production by this procedure is attributable to the fact that NS-1 cells syngeneic to BALB/c mice were used not only as immunogens after transfection but also as partner cells for hybridoma production. The transfected NS-1 cells were also used for screening. Our previous trials using thrombin-activated human platelets and megakaryocytic cell lines were unsuccessful.

This paper describes establishment of mAbs directed to selectins. However, the same procedure can be applied to various other cell surface antigens whose cDNA sequences are known. Thanks to recent advances in recombinant DNA technology, it has become much easier to isolate cDNA and establish transfectants. Previous methods involved purification of cell surface proteins (which are minor components in many cases) by time-consuming multi-step purification from a large quantity of starting material. In some cases, sufficient quantity of starting material was not available. With the new procedure, transfected fusion partner cells may also be useful for screening.

Selectin expression at the surface of endothelial cells and platelets is an important step for: (i) initiation of the inflammatory process; and (ii) tumor invasion and metastasis. Many tumor cells express sialosyl-Le^x (SLe^x) or SLe^a [18], which are now considered to be tumor-associated ligands of E- and P-selectin involved in process (ii) above [2, 19]. Our recent studies suggest that the ligand involved in process (i) is not entirely SLe^x or SLe^a, but rather 'myeloglycan' or its analogues for E-selectin [20, 21], and a still-unknown structure assembled on 'PSGL-1' glycoprotein for P-selectin [13, 22, 23]. Tumor cells are capable of activating platelets and endothelial cells to express selectins [24]. Systematic studies on expression patterns of E- and P-selectin associated with tumor cell growth *in vivo* are essential for understanding the mechanism of tumor cell invasion and metastasis. Immunohistological staining data (e.g. Fig. 2) are the first step in this direction. Figure 2 shows strong staining of P-selectin in microvascular endothelial cell layers adjacent to colonic tumor masses. Further investigation of this interesting phenomenon is in progress.

Successful application of the new procedure for the production of functional mAbs is hereby reported. By fusing genes encoding variable regions of such functional mouse mAbs with genes encoding other regions or domains of human IgG, we can potentially create 'humanized' functional mAbs capable of inhibiting human E- and P-selectin activity, inflammatory processes, or tumor metastasis. The mAbs are also useful for histopathological studies. Our preliminary experiments show that two of the anti-P-selectin mAbs (P1A and P5A) cross-react with rabbit P-selectin, as detected by staining of rabbit platelets. These mAbs may be useful for detection of P-selectin expression in a rabbit ear inflammation model, widely used for *in vivo* evaluation of anti-inflammatory drugs.

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