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11. ELAM-1–mediated binding was measured as previously described [A. Dobrina *et al.*, *Immunology* **67**, 502 (1989)]. MAbs were added to appropriate wells as follows: antibody to ELAM-1 (anti–ELAM-1) [H18/7, immunoglobulin G2a (IgG2a)] (*3*) was used at 2 μg/ml; antibody to α4 (anti-α4) (P4C2, IgG3) (*13*) was used as a 1:100 dilution of ascites fluid. Cell adhesion was performed at 4°C to reduce nonspecific adhesion of LEC11 cells. Each experiment was repeated with at least two independently derived endothelial cell cultures. Data is expressed as the mean percent of input cells bound for triplicate samples and the SEM is given in each case.
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19. ELAM-1 cell adhesion was measured as in Fig. 1, except that washed, ^{51}Cr-labeled HL-60, LEC11, or LEC12 cells were incubated for 20 min at 37°C and chilled for 20 min at 4°C in the presence of MAb containing hybridoma culture supernatants [anti–SLe^x-2; SNH-4, IgG3 (A. K. Singhal, E. Nudelman, S. Hakomori, unpublished results); anti–Le^x-1; SH-1, IgG3 and anti–Le^x-2; FH-2, IgM (A. Singhal *et al.*, *Cancer Res.* **50**, 1375 (1990)], or purified MAb diluted to 10 μg/ml in DMEM containing 5% fetal calf serum (FCS) anti–SLe^x-1; CSLEX-1, IgM) [K. Fukushima *et al.*, *ibid.* **44**, 5279 (1984)] and anti–ELAM-1 (*3*), before binding of the cells to IL-1β activated human umbilical vein endothelial cells (HUVEC).
20. HUVEC, which had been activated for 4 hours with IL-1β, were washed twice with RPMI 1640 containing bovine serum albumin (BSA) at 5 mg/ml and then incubated in the same buffer with liposomes containing various glycosphingolipids at a final concentration of 5 μg/ml (300 μl per well) for 30 min at 37°C and the plates were held an additional 15 min at 4°C. Approximately 10^5 ^{51}Cr-labeled HL-60 or Jurkat cells (*13*) were added to the incubation mixture in 50 μl and cell adhesion was measured as described for Fig. 1. Liposomes were formed as follows: 100 μg glycolipid was added to 500 μg phosphatidylcholine (Sigma, egg yolk) and 300 μg cholesterol (Sigma) in chloroform-methanol (2:1) and the whole solution evaporated to dryness by N_2 in 15-ml screw-cap tubes. Before use, the pellet was dissolved in 100 μl of absolute ethanol and sonicated for 2 min. Phosphate-buffered saline (PBS) (1.9 ml) was added while sonicating for an additional 10 min. The liposome suspension was diluted 1:10 in RPMI 1640 containing BSA (5 mg/ml) before addition to the HUVEC. Glycolipids listed in Table 1 were either purified or biosynthetically produced at the Biomembrane Institute and were characterized by nuclear magnetic resonance (NMR) and mass spectrometry and found to be identical to previously reported structures [S. Hakomori, E. Nudelman, S. B. Levery, R. Kannagi, *J. Biol. Chem.* **259**, 46729 (1984); Y. Fukushi, E. Nudelman, S. B. Levery, S. Hakomori, H. Rauvala, *ibid.*, p. 10511]. S-diLe^x was synthesized enzymatically by adding fucosyl residues with a cell line as enzyme source and SH (Table 1) as substrate. diLe^x was similarly synthesized with nLc6 as substrate [E. H. Holmes, G. K. Ostrander, S. Hakomori, *J. Biol. Chem.* **261**, 3737 (1986)]. SPG and SH (Table 1) were purified from bovine red blood cells, and nLc6 was produced by chemical removal of terminal sialyl residue from SH.
21. We thank N. Kovack for technical advice; J. Harlan and H. Grey for advice and insightful discussions during the course of this work; M. Bevilacqua for his continued interest during this work and for the H18/7 MAb to ELAM-1; P. Terasaki for anti-SLe^x MAb (CSLEX); E. A. Wayner for the P4C2 MAb and the Jurkat cell line; P. Stanley for the LEC11, LEC12, and CHO-K1 cell lines; and M. A. Weiss and the obstetrical nurses of Sharp Memorial Hospital, San Diego, for helping to obtain umbilical cords.

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Recognition by ELAM-1 of the Sialyl-Le^x Determinant on Myeloid and Tumor Cells

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Endothelial leukocyte adhesion molecule-1 (ELAM-1) is an endothelial cell adhesion molecule that allows myeloid cells to attach to the walls of blood vessels adjacent to sites of inflammation. ELAM-1 recognizes the sialyl-Lewis X (sialyl-Le^x) determinant, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-, a granulocyte carbohydrate also found on the surface of some tumor cell lines. Binding of myeloid cells to soluble ELAM-1 is inhibited by a monoclonal antibody recognizing sialyl-Le^x or by proteins bearing sialyl-Le^x, some of which may participate in humoral regulation of myeloid cell adhesion. Stimulated granulocytes also release an inhibitor of ELAM-1 binding that can be selectively adsorbed by monoclonal antibody to sialyl-Le^x.

INTEGRAL MEMBRANE ADHESION PROteins of a nascent family share a common extracellular domain organization consisting of an NH_2-terminal lectin-like segment, an epidermal growth factor (EGF)–related element, and multiple complement receptor motifs (*1, 2*). One member of this family, ELAM-1 (*3*), mediates the adhesion of myeloid cells to endothelial cells activated by interleukin-1 (IL-1), tumor necrosis factor, or substance P (*4*).

To identify the ELAM-1 domains necessary for cell adhesion, we first localized the binding sites for two monoclonal antibodies (MAb) to ELAM-1 (anti–ELAM-1): H18/7, which blocks leukocyte adhesion to activated endothelium, and H4/18, which does not. Progressive deletion from the COOH-terminus showed that the lectin plus EGF-repeat elements were required for expression of H18/7 binding, whereas H4/18 reactivity required the first three complement-related repeats as well (Fig. 1). A restriction fragment exchange between ELAM-1 cDNA and the related Leu-8 (LECAM-1) cDNA (*2*) showed that H18/7 bound to a determinant encoded in the first 75% of the lectin domain, and that the equivalent determinants for MAbs to Leu-8 (anti–Leu-8) and TQ1 were similarly encoded in the first 75% of the LECAM-1 lectin domain (Fig. 1).

To study the possible lectin-carbohydrate interactions suggested by epitope mapping, we prepared a soluble ELAM-1 protein chimera (ELAM-Rg) consisting of the ELAM-1 extracellular domain joined to the hinge and CH2 and CH3 domains of human immunoglobulin G1 (IgG1) (*5–7*) as a disulfide-linked dimer from supernatants of transfected COS-7–m6 cells (Fig. 1).

When plastic dishes coated with goat antibodies to human IgG were incubated with supernatants containing ELAM-Rg, the treated plastic acquired the ability to specifically bind granulocytes and the myeloid cell lines HL-60 and THP-1. Other myeloid cells and some carcinoma cells bound to the ELAM-1–coated plastic, whereas dishes coated with CD8 fusion protein (*5*) showed negligible affinity for all cells tested (*8*). Adhesion to ELAM-1 correlated with the presence of the CD15 [Le^x or lacto-*N*-fucopentaose III (*9, 10*)] determinant, but not with the determinants associated with CD17 [lactosyl ceramide (*11*)], CD65 [VI^{3}NeuAcIII^{3}FucnorLcnOse$_6$Cer (*12*)], or sulfatides (*13*). However, the correlation with CD15 was imperfect, and digestion of

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the cells with neuraminidase abolished binding to ELAM-Rg, but increased binding to CD15 MAb (Fig. 2), consistent with the reported masking of CD15 determinants by sialic acid (*10*).

The association with CD15 and the sensitivity to neuraminidase suggested that the sialylated form of CD15 might represent the physiological ligand. A MAb that recognizes the sialyl-Le^x determinant, CSLEX1 (*14*), gave a good correspondence between the surface density of sialyl-Le^x and the rank order for the number of cells bound per unit area of ELAM-Rg–coated plastic (Table 1). CSLEX1 completely inhibited adhesion of myeloid cells to ELAM-1, whereas CD65 and CD15 MAb had no activity under identical conditions (Fig. 2).

CSLEX1 recognizes sialyl-Le^x joined β (*1, 3*) to galactose (*14*). Neutrophil sialyl-Le^x determinants are predominantly found on tetra-antennary asparagine-linked glycans whose individual strands are built up from poly(*N*-acetyl-lactosamine) chains bearing variable α(1, 3)–linked fucose substitution (*15*). Although CSLEX1 blocks binding, the structure recognized by ELAM-1 might be more complex than the structure recognized by CSLEX1. To establish the minimum structure for ELAM-1 binding, we evaluated chemically characterized glycans bearing sialyl-Le^x determinants.

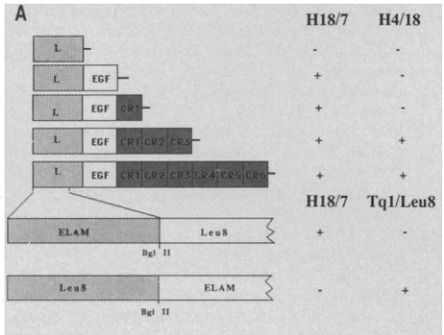

Fig. 1. (**A**) Characterization of the ELAM-1 binding domain, and creation of a soluble chimera. Carboxyl-terminal deletions of ELAM-1 were created by PCR with primers terminating at the domain boundaries indicated. The resulting ELAM cDNA fragments were fused both to the transmembrane and intracellular coding portions of a CD7 cDNA (*28*) as well as to IgG1 Fc segments (*5*), and plasmids bearing the resulting fusions were transfected into COS-7–m6 cells. The reactivity to MAbs was determined by indirect immunofluorescence microscopy of fixed, permeabilized cells. Results shown are representative of transfections of three to six independent isolates of the constructs shown. Leu-8 (LECAM-1)/ELAM-1 chimeras were created by restriction-fragment interchange from a conserved Bgl II site in the lectin domain. A full-length ELAM-1/IgG1 chimera was prepared as described (*6*). (**B**) Electrophoretic analysis of ELAM-Rg purified by adsorption to and elution from protein A–agarose (*5*). Lane 1, reducing; lane 2, nonreducing.

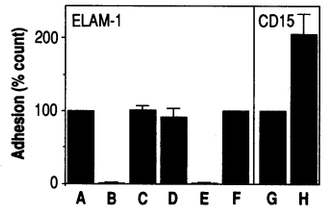

Fig. 2. Effect of neuraminidase and MAb CSLEX1 treatment on binding of HL-60 cells to ELAM-Rg–coated dishes. HL-60 cells (10^6) were incubated in 50 μl of 0.15 M NaCl, 4 mM $CaCl_2$, pH 5.5, for 1 hour at 37°C in the presence or absence of 41.5 mU of neuraminidase [from *Vibrio cholerae*, type II (Sigma)]. Cells were washed three times with PBS, and adherence to ELAM-Rg–or PM-81 (anti-CD15)–coated dishes was determined. The absence of nonspecific (for example, proteolytic or glycolytic) degradation of cell surface structures during the neuraminidase digestion was affirmed by indirect immunofluorescence and flow cytometry with myeloid cell MAbs. Dishes were coated with purified PM-81 (10 μg ml^{-1} in 50 mM tris-HCl, pH 9.5) and subsequently treated as in Table 1. In MAb-inhibition studies, 10^6 HL-60 cells were incubated with MAb (1:50 in PBS) for 30 min on ice, then cross-linked with affinity-purified goat antibody to mouse IgM (Organon Teknika, at 20 μg ml^{-1} in PBS) for 30 min on ice, and fixed with 2% formaldehyde in PBS for 20 min. Cells were washed three times in PBS–1% glycine, and incubated with ELAM-coated dishes as described in Table 1. Data (as percent of control) represent mean ±SD of triplicate determinations in three independent experiments. A, no MAb; B, CSLEX-1; C, CD15 PM-81; D, CD65 VIM-2; E, neuraminidase; F, no enzyme; G, no enzyme; and H, neuraminidase.

One source of well-defined sialyl-Le^x is amniotic fluid (*16*). The sialyl-Le^x group of amniotic fluid mucins is joined β (*1, 6*) to a 3-substituted *N*-acetylgalactosamine, which in turn is attached directly to the polypeptide backbone through O-linkage to serine or threonine (*16*). Although neither the adjacent residue nor the linkage is the same as for granulocyte glycans, both amniotic fluid and purified amniotic fluid mucins efficiently block binding of myeloid cells to immobilized ELAM-1 (Fig. 3A).

Another source of sialyl-Le^x is fucosylated α_1-acid glycoprotein (α_1-AGP) (*17*). Fucose is present on a minor fraction of N-linked α_1-AGP glycans (*18*), and our results indicate that a modest (35 ± 9%) reduction in binding of HL-60 cells to ELAM-Rg adsorbed to plastic can be achieved with 200 μg ml^{-1} of the protein (*8*). Similarly high protein concentrations, ~1 mg ml^{-1}, are required to effect partial blockade of antibody to CD15 (anti-CD15) binding (*10*), in keeping with the relative scarcity of fucosylated glycans on the native protein (*18*). To extend these results, we prepared enzymatically fucosylated α_1-AGP in vitro.

α_1-Acid glycoprotein is a good substrate for the α(1,3)fucosyltransferase of amniotic fluid, an enzyme that forms sialyl-Le^x and Le^x from sialylated and nonsialylated precursors, respectively (*19*). We isolated amniotic fluid fucosyltransferase by affinity chromatography (*20*) and evaluated its ability to convert α_1-AGP into an ELAM-1 ligand. α_1-AGP incubated with fucosyltransferase in the presence of GDP-fucose bound significantly more ELAM-Rg than did α_1-AGP

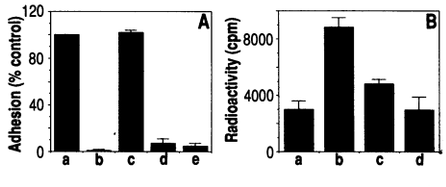

Fig. 3. (**A**) Human amniotic fluid (HAF) and purified amniotic fluid mucins inhibit myeloid cell adhesion to ELAM-1. HAF was either used without purification, fractionated by centrifugal ultrafiltration (100-kD nominal cutoff; Centricon 100, Amicon), or fractionated by phenol extraction and size-exclusion chromatography (Sephacryl S-300 HR) in 4 M guanidinium chloride (*16*) to yield purified mucins, which were used at a protein concentration of approximately 150 μg ml^{-1}. Binding to ELAM-Rg–coated plastic was performed as described in Table 1. Results (expressed as percent of control) are the average of triplicate determinations in two independent experiments. Similar results were obtained with mucins purified in a related manner from amniotic fluid proteins soluble in 4% dichloracetic acid (not shown). a, Control; b, HAF; c, HAF <100 kD; d, HAF >100 kD; e, mucin. (**B**) Fucosylation mediates binding of ELAM-Rg to α_1-AGP. α(1,3)Fucosyltransferase was isolated from concentrated amniotic fluid by fetuin-agarose chromatography as previously described (*19*). Guanosine diphosphate (GDP)-[^{14}C]fucose (0.8 μCi; 225 Ci/mol) and bovine α_1-AGP (100 μg, Sigma) were added to a reaction mix containing 25 mM tris-HCl, pH 7.0, 35 mM $MgCl_2$, and 1 mM adenosine triphosphate (ATP) in a final volume of 120 μl, and incubated at 37°C for 24 hours, after which approximately 10% of the labeled fucose had been incorporated into trichloroacetic acid–insoluble material. The unincorporated label was removed by centrifugal ultrafiltration (Centricon 10, Amicon), and 20 μl of a 1:5 dilution of the resulting material, or of a similarly constituted reaction mixture without GDP-fucose, was adsorbed to plastic dishes or to 96-well microtiter plates (Falcon 3911, Becton Dickinson). Binding of soluble ELAM- or CD8-Rg was measured with a radioiodinated goat antibody to human IgG (Du Pont Biotechnology Systems) after incubating the wells at 22°C with Rg at 1 μg ml^{-1} for 1 hour, washing with PBS, incubating with labeled second antibody for an additional hour, washing, and measuring in a gamma counter. Results are expressed as mean ±SD of quadruplicate determinations and are representative of two independent experiments. a, α_1-AGP; b, fucosylated α_1-AGP; c, no GDP-fucose; d, CD8-Rg–fucosylated α_1-AGP.

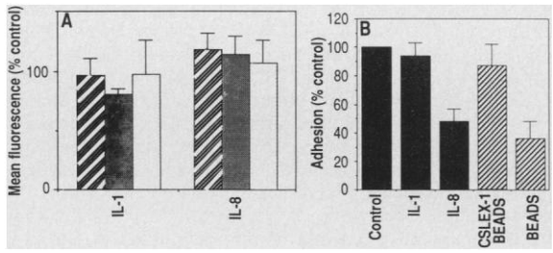

Fig. 4. (**A**) Effect of IL-1 and IL-8 on CD15, CD65, and sialyl-Le^x surface expression on human granulocytes. Granulocytes were incubated with IL-1β (10 ng ml^{-1}, Pepro Tech, Rock Hill, NJ) or IL-8 (25 ng ml^{-1}, Pepro Tech) for 20 min at 37°C, washed three times, and incubated with the indicated MAbs on ice. Results are given as the relative mean fluorescence intensity (MFI) determined by flow cytometry, as a percent of the MFI of granulocytes incubated in parallel without cytokines, and are representative of four experiments. Hatched bars, CD15–PM-81; black bars, CSLEX-1; white bars, CD65–VIM-2. (**B**) Supernatants harvested from granulocytes exposed to IL-8 block binding to ELAM-1. Granulocytes (5×10^7 per ml) were incubated with IL-1 (10 ng ml^{-1}) or IL-8 (25 ng ml^{-1}) for 1 hour at 37°C. Supernatants were collected after centrifugation, and incubated with ELAM-coated dishes. Cells were added after 30 min and binding was determined as in Table 1. Immunoadsorption was performed with 40 μl of protein A–agarose (Sigma) to which 10 μg of affinity-purified rabbit antibody to mouse IgM were adsorbed, followed by 5 μl of CSLEX1 ascites. Control beads were similarly prepared, but not incubated with CSLEX1 or incubated with IgM anti-CD14 (not shown). The beads were washed with PBS and incubated with the supernatants for 1 hour at 4°C. Results are expressed as the percent of cells bound, relative to the number bound in the presence of supernatants of granulocytes incubated without cytokine under the same conditions. Data shown are mean ± SD of triplicate determinations in three independent experiments.

Table 1. Carbohydrate antigen densities and ELAM-1 adhesion. Human granulocytes, the myeloid cell lines HL-60, THP-1, and U-937, the T cell line HSB-2, and the colon carcinoma line WiDr were simultaneously analyzed for the antigens shown and for binding to ELAM-Rg–coated plastic. Human granulocytes were isolated from freshly drawn, heparinized whole blood by centrifugation through Ficoll–sodium diatrizoate (Mono-Poly Resolving Medium, Flow Laboratories, McLean, VA). The U-937 line maintained in our laboratory lacks sialyl-Le^x, unlike the line used by Terasaki and coworkers (*14*). MAb reactivity was determined by indirect immunofluorescence with MAbs PM-81 (*29*) (anti-CD15; Medarex, W. Lebanon, NH), CSLEX-1 (*14*), or VIM2 (*12*) (anti-CD65). Surface antigen scores represent the percent of cells positive by flow cytometry as follows: −, < 10%, +, 10 to 25%, ++, 25 to 75%, and +++, > 75%. Adhesion to ELAM-Rg was measured in bacterial culture dishes (Falcon 1008) to which affinity purified goat anti-human IgG antibody (Organon Teknika/Cappel) had been adsorbed at 10 μg ml^{-1} in 50 mM tris-HCl, pH 9.5, for at least 1 hour. Remaining protein binding sites were then blocked by overnight incubation with 1 mg ml^{-1} bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The dishes were incubated with ELAM-Rg (~1 μg ml^{-1}) for 30 min at 22°C, washed with PBS, overlaid with cells (10^6 ml^{-1}) for 10 min at 22°C, and washed three times with PBS. The adherent cells per unit area of dish were enumerated with the aid of an ocular reticle and scored as follows: +++, > 75 cells; ++, 75 to 25 cells; +, 25 to 10 cells; and −, < 10 cells (average of triplicate determinations).

Cells	CSLEX-1	CD15 PM-81	CD65 VIM2	Adhesion
Granulocytes	+++	+++	+++	+++
HL-60	+++	+++	+++	+++
THP-1	+++	+++	+++	+++
WiDr	++	+++	+	++
U-937	−	++	++	−
HSB-2	−	++	+	−

alone or α_i-AGP incubated with enzyme in the absence of GDP-fucose (Fig. 3) (*21*).

The fucosylated glycans of asialo–α_1-AGP bear the Le^x determinant linked β(1,4) to mannose, whereas the nonfucosylated termini bear *N*-acetyl-lactosamine joined either β(1,4), β(1,2), or α(1,6) to mannose (*18*). Hence, neither the α_1-AGP sialyl-Le^x determinants nor the potential fucosyl adducts to *N*-acetylglucosamine can be joined to galactose. These results, and those showing inhibition of ELAM-1 binding by mucin O-linked glycans, demonstrate that the sialyl-Le^x grouping by itself has appreciable affinity for ELAM-1.

Release of IL-8 from IL-1–treated endothelial cells causes granulocytes to lose the ability to bind to IL-1–induced endothelium (*22*). Neither IL-1 nor IL-8 caused a substantial reduction in the expression of cell surface sialyl-Le^x (Fig. 4A). However, supernatants harvested from cultures of granulocytes treated with IL-8, but not IL-1, blocked the adhesion of HL-60 cells to immobilized ELAM-Rg, and the binding inhibition could be specifically reversed by adsorption of the supernatants with solid-phase CSLEX1, but not with the immunoadsorption matrix alone or with matrix preloaded with anti-CD14 (Fig. 4B) (*8*).

Although the distribution of sialyl-Le^x on granulocytes has not been studied in detail, the related CD15 determinants are associated with the adhesion proteins of the CD11-CD18 complex, the CR1 complement receptor CD35, and soluble proteins contained in intracellular granules (*23*). MAbs to CD15 increase neutrophil adhesion to endothelium by a CD11a-CD18 (LFA-1)–dependent mechanism (*24*), making plausible the idea that binding of granulocytes to ELAM may deliver a signal to the granulocyte that predisposes it to diapedesis.

In addition to monocytes and granulocytes in adult blood, the sialyl-Le^x determinant is expressed by fetal tissues and tumor cells of diverse origins (*14*). Previous studies have shown that colon carcinoma cells possess the ability to bind ELAM-1 (*25, 26*), and that increased fucose content in cell-surface carbohydrates correlates with tumor invasiveness (*27*).

Fucosylation of the acute-phase reactant protein α_1-AGP is elevated threefold in cirrhosis, and the fucose groups are located in the sialyl-Le^x grouping (*17*). The demonstration here that in vitro fucosylation of α_1-AGP substantially increases its affinity for ELAM-1 suggests that increased liver fucosyltransferase activity may provide a humoral mechanism for feedback inhibition of granulocyte extravasation in chronic inflammation.

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6. ELAM-1 cDNA was genetically fused to human IgG1 genomic sequences with the aid of a synthetic splice donor appended to the ELAM-1 coding sequence at a location immediately before the portion encoding the membrane-spanning domain. Synthetic oligonucleotides having the sequence CGGAATTCCAGTACTACTCACCTGGAATGT-TGGACTCAGTGG and CCAGATATACGCGT-TGACATTGATTATTGACTAGTTATT, corresponding to the splice donor/COOH-terminus of ELAM-1 and to a location in the vector upstream of the inserted cDNA, respectively, were prepared. Polymerase chain reaction (PCR) with these oligonucleotides and an ELAM-1 cDNA expression plasmid as template, yielded a fragment that was subcloned into expression vector πH3M (*7*). The subcloned fragment was released by restriction endonuclease digestion and ligated to IgG1 expression plasmid. The resulting construct was transfected into COS-7–m6 cells and the desired fusion protein was recovered from the supernatant as described (*5*). The initial construct and a subsequent version in which the majority of the PCR-amplified segment was replaced by restriction fragment interchange to avoid potential mutations introduced during amplification, showed identical binding activity.
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The Enzymic Nature of Antibody Catalysis: Development of Multistep Kinetic Processing

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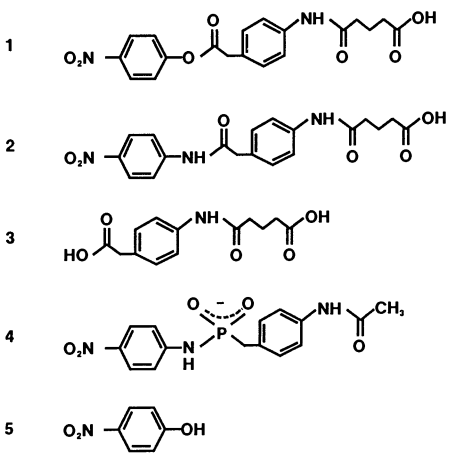
Detailed kinetic investigations of a catalytic antibody that promotes the hydrolyses of an anilide and phenyl ester show that this catalyst uses a multistep kinetic sequence resembling that found in serine proteases to hydrolyze its substrates, although antibody was elicited to a single transition-state analog. Like the serine proteases the antibody catalyzes the hydrolysis reactions through a putative covalent intermediate, but unlike the enzymes it may use hydroxide ion to cleave the intermediates. Nevertheless, the antibody is a potent catalyst with turnover at higher pH values rivaling that of chymotrypsin. This analysis also reveals that turnover by the antibody is ultimately limited by product desorption, suggesting that improvements in catalytic efficiency may be achieved by judicious changes in the structure of the substrate, so that it is not superimposable on that of the eliciting hapten.

A VARIETY OF REACTIONS HAVE NOW been catalyzed by antibodies elicited to transition-state analogs and include a number of important reaction types (*1, 2*). These catalysts resemble enzymes in their mode of action, since substrate transformation is achieved through a preliminary binding event that generates a reactive antibody-substrate complex that in turn dissociates to products. Thus comparisons between appropriate enzyme-antibody pairs have been made, but these are based primarily on steady-state Michaelis-Menten parameters. We report the detailed description of the kinetic sequence of an antibody, NPN43C9, that catalyzes the hydrolysis of an aromatic amide as well as the corresponding aromatic ester (*3*). The resemblance of the number and mechanistic nature of the steps in this pathway to those of the serine proteases informs an analysis of the catalytic characteristics of this antibody relative to chymotrypsin. The appearance of a multistep kinetic sequence limited for the ester by-product desorption emphasizes an adaptive advantage of the esterase enzyme over the abzyme and identifies structural features of the eliciting transition-state analog that may be modified for catalytic advantage.

The antibody-catalyzed hydrolysis of the *p*-nitrophenyl ester **1** and *p*-nitroanilide **2** have been examined by both pre- and steady-state kinetic techniques. The steady-state Michaelis-Menten parameters, k_{cat}/K_M and k_{cat}, as a function of pH are displayed in Fig. 1. The data can be fit either to a reaction mechanism involving the titration of a group at the antibody binding site whose dissociation promotes substrate hydrolysis or to one that features a change in the rate-limiting step around an intermediate antibody-bound species due to changes in pH (*4*). An examination of the rates of product release from the antibody (Table 1), measured by stopped-flow competition experiments monitoring the increase in antibody fluorescence (340 nm) upon dissociation of the product molecule, revealed that the rate constants for product dissociation are the parent acid >*p*-nitroaniline (PNA) >*p*-nitrophenol (PNP), suggesting that the nitro residue is a major determinant of antigen binding. Moreover, the rate constant for PNP release (45 ± 10 s^{-1}) sets the maximum value of k_{cat} (40 ± 6 s^{-1}) for ester hydrolysis (the pH-independent region, pH > 9 in Fig. 1). (Error limits are ± SE throughout.) Thus, the antibody-catalyzed hydrolysis of **1** proceeds through at least two steps whose relative importance in determining the rate of a reaction cycle change with pH. The identification of k_{cat} (pH > 9) with the rate constant for *p*-nitrophenolate release and the 10^4 difference in k_{cat} values (pH > 9) for **1** and **2** moreover eliminate a mechanism involving productive



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