Immunoassays have had extensive applications in many fields. Although CIA's with the use of serum (*12–14*) have been described, the mechanism of the cooperativity has not been understood. On the basis of the observations of Weintraub *et al.* (*13*) that the effect is lost when F(ab) fragments are prepared, and on the basis of our findings with monoclonal antibodies (*2, 7*), we assert that formation of a circular complex may frequently be the cause for cooperativity in antisera. Cooperative assays with antisera have had limited application because the range of cooperativity is small and factors needed to develop highly cooperative antisera are unknown. Assays based on monoclonal antibodies should have more utility since appropriate choices of antibodies and their concentrations can be made to optimize the sensitivity and specificity of the assays. Use of multiple monoclonal antibodies also offers the possibility of increasing the specificity of the assay since both antibodies must bind simultaneously. Consequently, as has been shown for a sandwich assay (*4*), the partial cross-reactivity of either antibody with interfering substances may be reduced in a factorial fashion. We have made similar observations for hCG (not shown). Requirements necessary to perform the CIA include antigens that have at least two epitopes, antibodies that bind to each epitope, and the ability of the antibodies and antigen to cooperate in binding. (We use the term "cooperativity" in the sense of forming a circular complex; cooperativity caused by an allosteric transition of the antigen is not required.) Practical application of the CIA with the use of radiolabeled antigen will require that all unknowns be analyzed at two concentrations because of the biphasic nature of the binding curve at low antigen concentration. Use of radiolabeled antibody should reduce this problem since the assays are biphasic only at very high antigen concentrations. As in all radioimmunoassays, the ultimate sensitivity of the assay is limited by the specific activity of the tracers employed. We envision that the CIA will be readily suited for enzyme immunoassays.

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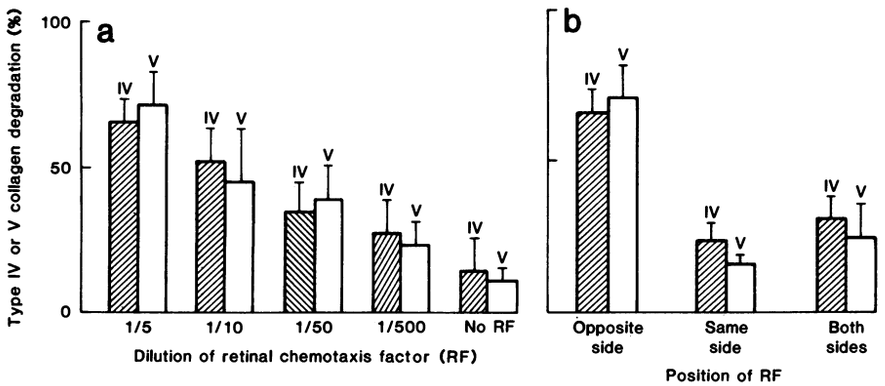
## Basement Membrane Collagen: Degradation by Migrating Endothelial Cells

Abstract. *One of the first steps in neovascularizaton is dissolution of the basement membrane at the point of endothelial outgrowth. An assay was developed to determine whether basement membrane collagens (types IV and V) are degraded by endothelial cells migrating toward a chemotactic stimulus. Fetal bovine endothelial cells were placed on one side of a filter containing the collagen substrate, and a chemoattractant derived from retinal extracts was placed on the opposite side. Degradation of both type IV and type V collagens was observed when the retinal factor was placed on the side of the filter opposite the endothelial cells. Metalloproteinases that cleaved type IV and type V collagens could be extracted from the endothelial cells with detergents. Such endothelial cell–associated (possibly membrane-bound) proteinases may locally disrupt the basement membrane and facilitate the outgrowth of capillary sprouts toward the angiogenic stimulus.*

Dissolution of the vascular basement membrane is associated with migration of endothelial cells out of the vascular channel toward an angiogenic stimulus (*1, 2*). Individual migrating endothelial cells do not resynthesize a basement membrane until they become arranged in tubular capillary loops (*1*). The subendothelial basement membrane, a dense meshwork of collagen, glycoproteins, and proteoglycans (*3–7*), does not contain pores large enough to allow cell passage. Hence, movement of endothelial cells through the basement membrane may involve proteolytic disruption of this structure. Endothelial cells elaborate a collagenase that can degrade interstitial type I collagen (*8*), but this collagenase does not degrade type IV or type V collagens, which are the structural components of the basement membrane (*6, 9, 10*). Metalloproteinases that degrade type IV or type V collagens have been identified in tumor cells (*9, 11*), macrophages (*12*), and bone (*13*) but not in endothelial cells. We therefore developed an assay to identify proteases capable of degrading basement membrane collagens.

A chemotactic stimulus was used to induce endothelial cells to migrate into a nitrocellulose filter containing bound, labeled, native type IV or type V collagen. Collagens labeled biosynthetically with [$^{14}$C]proline were purified as described (*9, 11, 14*), and 10 μg of the collagen dissolved in 0.05*M* tris-HCl, 0.9*M* NaCl, and 0.1 percent bovine thyroglobulin was incubated with nitrocellulose filters (Millipore SCWP; 13 mm; pore size, 8 μm) in a volume of 1 ml at 4°C for 10 hours. Substrate binding efficiency was greater than 90 percent. The filters were washed in phosphate-buffered saline, *p*H 7.4, and clamped in a chemotaxis chamber. The substrates were thus extracted, purified, and incubated with the nitrocellulose under nondenaturing conditions. The substrates were judged to be native because of their tertiary structure observed by rotary-shadowing electron microscopy and their insensitivity to digestion by α-thrombin at 30°C (*14*). The endothelial chemoattractant we used was derived from retina extracts (*15*). Fetal bovine endothelial cells ($0.2 \times 10^5$) suspended in 1 ml of serum-free RPMI 1640 medium were applied to one side of the filter. After a 2-hour wait for the cells to attach, the retinal factor was added to one or both sides of the chamber. The chamber was incubated for 18 hours at 37°C. The filters were then removed, washed, and dissolved in scintillation medium (Biofluor); the amount of $^{14}$C was determined in a liquid scintillation



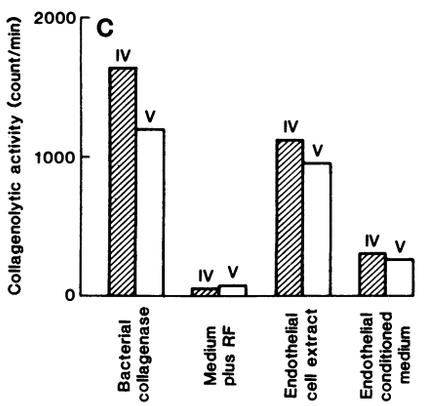




Fig. 1. Degradation of type IV and type V basement membrane collagens by endothelial cells migrating toward the retinal factor chemoattractant. The amount of collagen degraded is presented as a proportion of the maximum radioactivity released by purified bacterial collagenase. Data are the mean and standard deviation of quadruplicate experiments. Retinal factor alone at a dilution of 1/5 showed no degradative activity compared to plain medium. (a) Dose response for retinal factor–induced degradation of type IV or type V collagen. The retinal factor was placed on the side of the filter opposite the endothelium. (b) Effect of position of the retinal factor in the chemotaxis chamber. Retinal factor was diluted 1/5 (10 μg/ml). Degradation was significantly ($P < .05$) less when the retinal factor was placed on the same side as the endothelium or on both sides than when it was placed on the opposite side. A similar finding was observed when retinal factor was diluted 1/10 (not shown). (c) Source of endothelial collagenolytic activity. Endothelial cells were exposed to retinal factor (1/5) for 18 hours as described above. The collagenolytic activity was measured after trypsin activation. Proteases were extracted with 0.1 percent Triton X-100 in 0.05*M* tris-HCl, 0.9*M* NaCl, *p*H 7.6. Bacterial collagenase served as a positive control. Most of the degradative activity was associated with the cells (quadruplicate assays, with standard deviation less than 10 percent of the mean) and was increaed by exposure to retinal factor. The activity expressed reflects that derived from $1 \times 10^6$ cells.

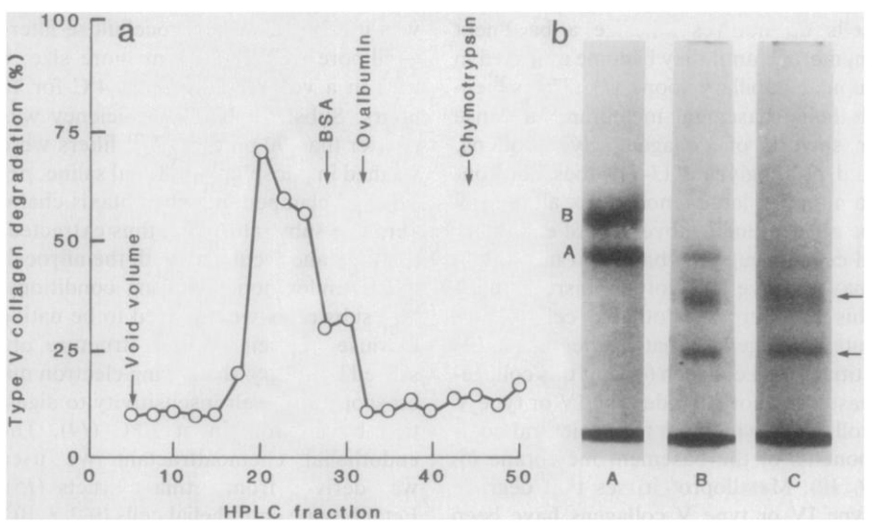



Fig. 2. Isolation of a type V collagenolytic metalloproteinase from migrating endothelial cells. The crude activity was obtained from detergent extracts of endothelial cell membrane fractions by the method of Duran and Cabib (*17*). It was further isolated by chromatography on chelating Sepharose 4B and elution with EDTA (*18*). (a) The active material eluted from the chelating column was subjected to HPLC and the fractions were assayed for type V collagenolytic activity (*11*); a major peak of degradative activity eluted at an apparent molecular size of 80 kilodaltons. Positions of molecular weight standards are shown; *BSA*, bovine serum albumin. (b) Polyacrylamide–acrylamide gel electrophoresis of specific cleavage products of type V collagen: (lane A) substrate (50 μg) alone—bands *A* and *B* are the two different α chains of type V collagen; (lane B) substrate (50 μg) plus endothelial-derived metalloproteinase (1 μg) after incubation for 6 hours at 35°C; (lane C) substrate (50 μg) plus endothelial-derived metalloproteinase (1 μg) after incubation for 18 hours at 35°C.

system (Beckman, LS 235). When the retinal factor was placed on the side of the filter opposite the endothelial cells in the chemotaxis chamber, degradation of both type IV and type V collagen was observed (Fig. 1A). Staining of the endothelial cells that migrated through the filter verified that the number of migrating cells was dependent on the dose of retinal factor (*15*). Cleavage of the substrate released from the nitrocellulose filter was analyzed by high-performance liquid chromatography (HPLC) and gel electrophoresis. Type IV procollagen substrate ($\alpha_1$ IV and $\alpha_2$ IV chains) has a molecular size of 170 to 185 kilodaltons. Degradation fragments released into the medium had a molecular size less than 50 kilodaltons. The amount of degradation was dependent on the concentration of the retinal factor for both types of collagen substrate (Fig. 1a). Degradation was related to directed endothelial migration. When the retinal factor was placed in the endothelial side of the chamber or in both sides, the degradative activity was markedly reduced (Fig. 1b). Results with bovine capillary endothelial cells were similar to those with fetal bovine aortic endothelial cells (*16*). Assay of the endothelial conditioned medium from the chemotaxis chamber did not reveal significant amounts of secreted material with type IV and type V collagen degrading activity (either latent or active) (Fig. 1c). However, when active material was extracted directly from the endothelial cells, collagenolytic activity was recovered in significant amounts (Fig. 1c). The extracted enzymes were inhibited by chelating agents, but not by inhibitors of thiol or serine proteases, and were classified as neutral metalloproteinases.

A neutral metalloproteinase specific for type V collagen was further isolated from the endothelial membrane fraction extract. As shown by HPLC (Sepharogel TSK 3000), the collagenolytic material had a molecular size in the range of 80 kilodaltons, with little or no activity between 20 and 50 kilodaltons (Fig. 2). Specific cleavage of both chains of type V collagen was produced (Fig. 2) at 35°C. The partially isolated protease failed to degrade collagens I, II, III, or IV. The endothelial proteinases that degrade basement membrane collagens may be present on or in the cell membrane. The surface area of the cell in contact with substrate is maximized in the migration assay we used. The chemotactic stimulus may directly induce the exposure of proteases on the cell surface. Alternatively, the chemotactic stimulus may primarily stimulate migration, and indirectly this may induce

membrane changes that expose proteases. Cell-associated—as opposed to secreted—proteases could be subject to tight local control by the cell. Degradation of the basement membrane by such proteases would occur only at the point of contact of the cell surface with the matrix. This hypothesis is consistent with ultrastructural studies of early angiogenesis (*1, 2*). The endothelial metalloproteinases identified for the first time in this study may be of importance in the physiologic turnover of the subendothelial matrix in the established microvasculature. Control of such enzymes may be altered in pathologic processes that exhibit a defective or altered basal membrane such as diabetes and cancer invasion.

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14. The tertiary structure of the native type IV and type V collagen substrates was examined by metal shadow-casting and electron microscopy (*5*). Type IV collagen molecules appeared as ropelike structures and retained the globular region at one end and the 7-S domain at the other end. Type V collagen appeared as intertwined ropelike filaments. α-Thrombin degrades denatured but not native type IV and type V collagen at 30°C [L. A. Liotta, R. H. Goldfarb, V. P. Terranova, *Thromb. Res.* **21**, 663 (1981)]. α-Thrombin degraded less than 5 percent of the native substrates bound to nitrocellulose. In contrast, if the substrates were heat-denatured, α-thrombin degraded 48 and 64 percent of the type IV and type V collagen, respectively.
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28 October 1982; 28 December 1982

## Murine I-Aβ Chain Polymorphism: Nucleotide Sequences of Three Allelic I-Aβ Genes

Abstract. *The polymorphism of immune response genes plays a critical role in determining the immune capabilities of a particular individual. The molecular nature of this polymorphism was studied by examining the structure of the coding portions of three alleles of the I-Aβ chain gene, an immune response gene whose protein product constitutes a subunit of the I-A molecule. Comparison of the I-Aβ chains encoded by these alleles revealed an amino acid sequence divergence of 5 to 8 percent. The differences were found to be a series of short alterations clustered in the amino terminal half of the polypeptide.*

The major histocompatibility complex (MHC)-linked immune response (Ir) genes determine the phenotypic ability of an animal to develop a high level of humoral or cell-mediated immunity to a defined antigen (*1, 2*). Recent studies have revealed that the class II, MHC-encoded, cell-surface glycoproteins (Ia in the mouse and DR or DR-like in man) are the structural products of these Ir genes, whose function is involved in complex regulatory interactions among

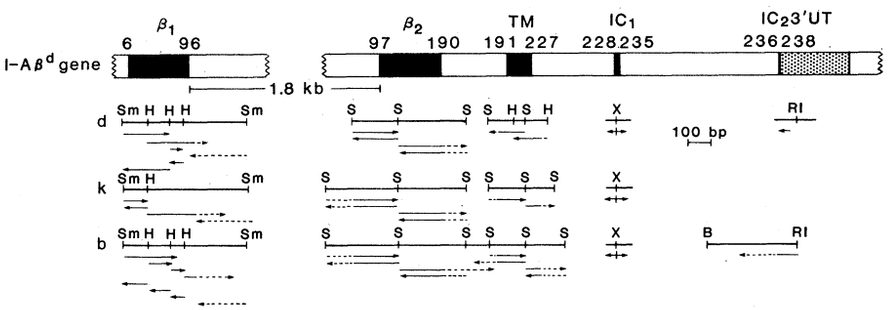


Fig. 1. The organization and sequencing strategy of I-Aβ genes in the mouse. The exons of the I-$A\beta^d$ gene are represented by solid bars in the coding regions and by a stippled bar in the 3′ untranslated (3′ UT) region. The exons encoding the two extracellular domains are labeled $\beta_1$ and $\beta_2$. The distance between the $\beta_1$ and $\beta_2$ exons is 1.8 kb in the I-$A\beta^d$ gene, 2.0 kb in the I-$A\beta^b$ gene, and 2.7 kb in the I-$A\beta^k$ gene. The exon encoding the transmembrane region is labeled TM, and the two exons encoding the intracytoplasmic region are labeled $IC_1$ and $IC_2$. The numbers above the solid bars indicate the amino acid residues encoded by that exon. Where an intron divides a codon between base positions 1 and 2, the amino acid residue for that codon is assigned to the 5′ exon. The 3′ UT region is contiguous with $IC_2$, and the sequence of the first 220 nucleotides of the 3′ UT region does not show an intervening sequence (data not presented). Genomic clones were mapped with restriction enzymes, and the locations of the exons encoding the TM, IC, and 3′ UT regions were determined by hybridization to the mouse I-Aβ cDNA clone pI-Aβ-1 (*7*). The locations of the exons encoding the $\beta_1$ and $\beta_2$ domains were determined by hybridization to a human HLA-DR-like β-chain cDNA clone (*10*). The appropriate restriction fragments from the three genomic clones were subcloned into the plasmid vectors pBR322 or pBR327. The subclones were digested with the restriction enzymes Sau 3AI, Hpa II, or Sma I and further subcloned into M13 mp9 for nucleotide sequencing by the dideoxy chain termination method (*19*). The M13 recombinants were screened with the human HLA-DR-like β-chain cDNA probe. The inserts in these M13 recombinants were sequenced and aligned according to their homology with either the human HLA-DR-like β-chain cDNA sequence or the mouse pI-Aβ-1 cDNA sequence. The sequencing strategies for the d, k, and b haplotypes are presented below the organization of the gene. The restriction sites are designated as follows: *Sm*, Sma I; *H*, Hpa II; *S*, Sau 3AI; *X*, Xho I; *RI*, Eco RI; and *B*, Bam HI. Arrows represent the extent of reading from a restriction site. The sequences from the Xho I site in $IC_1$ in all three haplotypes and from the RI site in the 3′ UT region in the d haplotype were determined by the chemical degradation method of Maxam and Gilbert (*20*). All other arrows represent sequences derived from M13 subclones. The dashed portions of the arrows represent sequences determined but not presented in Fig. 2. For all M13 subclones from the three haplotypes which had inserts starting at analogous restriction sites, the products of the sequencing reactions were subjected to electrophoresis side by side on the sequencing gels in order to facilitate comparisons between the haplotypes.