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11. The expression vector pCMV-Neo-Bam was derived from plasmid BCMGNeo-mIL2 [H. Karasuyama, N. Tohyama, T. Tada, *J. Exp. Med.* **169**, 13 (1989)] by excision of the human beta globin sequences and bovine papilloma virus sequences with Bam HI and Not I. Next, the interleukin 2 (IL-2) sequences present at the unique Xho I site were removed, and the Xho I site was changed to a Bam HI site by linker addition. The vector included CMV promoter/enhancer sequences, which could drive expression of the insert at the Bam HI site, and splicing and polyadenylation sites derived from the rabbit beta globin gene, which ensured proper processing of the transcribed insert in the cells. A pBR322 origin of replication and beta-lactamase gene facilitated growth of the plasmid in *Escherichia coli*. The plasmid conferred geneticin resistance through expression of the neomycin resistance gene under separate control of an HSV thymidine kinase promoter.
12. A 1.8-kb Xba I fragment, extending from nucleotide −130 to 1671 relative to the translation initiation site, was isolated from wild-type or CX3 cDNA clones (*3*). The fragment was blunt-ended with the Klenow fragment of DNA polymerase, ligated to Bam HI linkers, and cloned into the unique Bam HI site in the expression vector pCMV-Neo-Bam.
13. SW480 and SW837 were obtained from American Type Culture Collection (ATCC). RKO cells were obtained through the generosity of M. Brattain. VACO 235 cells are described by J. K. V. Willson *et al.* [*Cancer Res.* **47**, 2704 (1987)]. For transfection, carcinoma cells at 30 to 60% confluence were incubated in a 75-cm^2 flask in 6 ml of Optimem (Gibco) with 5 μg of plasmid DNA and 30 μg of lipofectin [P. L. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987)]. After 5 to 16 hours, the Optimem was replaced with Dulbecco's or McCoy's 5A medium containing 10% fetal calf serum. Selection in geneticin (0.8 mg/ml) began 36 to 48 hours after transfection for colony formation assays. Electroporation was used to transfect VACO 235 cells essentially as described by H. Potter, L. Wier, and P. Leder [*Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984)].
14. Previous studies have shown that, in contrast to rodent cells, primate cells are able to integrate only a small amount of foreign DNA (approximately 6 kb), so that only 10 to 30% of clones selected for the expression of one transcription unit also contain the second unit in an intact form [F. Collabere-Garapin, M. Rhyiner, I. Stephany, P. Kourisky, A. Garapin, *Gene* **50**, 279 (1986); J. H. J. Hoeijmakers, H. Odijk, A. Westerveld, *Exp. Cell Res.* **169**, 111 (1987); L. Mayne *et al.*, *Gene* **66**, 65 (1988); S. W. Dean, L. Kincla, H. R. Sykes, A. R. Lehmann, I. A. Wise, *Exp. Cell Res.* **183**, 473 (1989)].
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16. The p53 gene sequences in exons 5, 6, 7, 8, and 9 were examined essentially as described in (*4*). All previously noted point mutations in p53 genes have involved one of these exons (see *3–5*).
17. Approximately 5 × 10^4 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against human p53 protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein [L. Banks, G. Matlashewski, L. Crawford, *Eur. J. Biochem.* **159**, 529 (1986)]. Ten to 20 randomly selected microscopic fields were analyzed per slide.
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19. Cells were grown for 2 hours in McCoy's 5A medium with 10% fetal calf serum and [^{3}H]thymidine (10 μCi/ml of 50 Ci/mmol, New England Nuclear). After immunocytochemical staining (*17*), slides were dehydrated in ethanol, dipped in NTB-2 emulsion (Kodak), and exposed for 2 weeks at 4°C. Autoradiographs were developed for 2 min in D-19 and stabilized in Rapid Fix (Kodak).
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23. Total cellular RNA was isolated by the acid guanidium extraction method [P. Chomczynski and N. Sacchi, *Ann. Biochem.* **162**, 156 (1987)].
24. RNA (15 μg) from each sample was used in RNase protection experiments. A ^{32}P-labeled RNA probe comprising nucleotides 1450 to 1788 relative to the p53 translation initiation site was generated in vitro from a p53 cDNA subclone in Bluescript with T7 polymerase. Ribonuclease protection was performed as previously described [E. Winter, F. Yamamoto, C. Almoguera, M. Perucho, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7575 (1985); R. M. Myers, Z. Larin, T. Maniatis, *Science* **230**, 1242 (1985)]. Autoradiographs were exposed for 16 to 20 hours.
25. DNA purification, restriction endonuclease digestion, electrophoresis, transfer, and hybridization were performed as described (*1, 3*). The hybridization probe was a 1.8-kb Xba I fragment of p53 cDNA (*12*).
26. We thank A. Preisinger, K. Molkentin, and J. Jackson for technical assistance. This work was supported by grants GM 07184, GM 07309, CA 43703, CA 45967, CA 43460, CA 51504, and CA 35494 from the NIH.

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A β_3 Integrin Mutation Abolishes Ligand Binding and Alters Divalent Cation–Dependent Conformation

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The ligand-binding function of integrin adhesion receptors depends on divalent cations. A mutant $\alpha_{IIb}\beta_3$ integrin (platelet gpIIb/IIIa) that lacks ligand recognition shows immunologic evidence of a perturbed interaction with divalent cations. This was found to be caused by a G → T mutation that resulted in an Asp^{119} → Tyr^{119} substitution in the β_3 subunit. This residue is proximal to bound ligand and is in a conserved region among integrins that are enriched in oxygenated residues. The spacing of these residues aligns with the calcium-binding residues in EF hand proteins, suggesting interaction with receptor-bound divalent cation as a mechanism of ligand binding common to all integrins.

CELL-CELL AND CELL-MATRIX ADHESIVE interactions are essential to development, inflammation, hemostasis, and immune recognition. The integrins are a broadly distributed family of structurally related receptors that contribute to these adhesive reactions by recognition of a multiplicity of extracellular matrix protein ligands including laminin, collagens, fibrinogen, and bone sialoprotein (*1*). In addition, integrins participate in cell-cell interactions by recognition of integral membrane protein ligands including the intercellular adhesion molecules ICAM-1 and ICAM-2 and vascular cell adhesion molecule–1 (VCAM-1) (*2*). Although the integrins differ in ligand recognition specificity, a requirement for millimolar concentrations of physiologic divalent cations is common to the primary recognition function of all integrins (*3*). This dependence of function on divalent cations can be attributed to a low-affinity divalent cation–binding site within the integrins, because millimolar Ca^{2+} or Mg^{2+} can modulate the conformation of a prototype integrin, platelet membrane glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$ also known as gpIIb/IIIa), which is detectable by a monoclonal antibody (MAb) PMI-1 (*4*). Loss of the epitope recognized by this MAb directly correlates with the capacity of $\alpha_{IIb}\beta_3$ to bind fibrinogen. The Cam variant of Glanzmann's thrombasthenia (*4*) is an autosomal recessive hereditary disorder of $\alpha_{IIb}\beta_3$ that is associated with the inability of this integrin to recognize macromolecular (*4*) or synthetic peptide (*5*) ligands. In addition, divalent cations do not regulate the expression of the PMI-1 epitope in Cam platelets (*4*). These characteristics indicate that the presumptive mutation in the Cam receptor leads to defects in binding of both divalent cations and primary ligands. To elucidate the structural basis of integrin function, we identified the point mutation in $\alpha_{IIb}\beta_3$ that causes the Cam variant of Glanzmann's thrombasthenia.

Total RNA was isolated from platelets of normal donors and two affected siblings with Cam variant. For initial sequencing, we

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amplified a 365-bp fragment of β_3 that encodes residues Ser^{97} to Ala^{218} (*6*) and a 681-bp fragment of α_{IIb} that encodes Ser^{226} to Val^{454} and contains the four putative divalent cation–binding sites (*7*). The β_3 region was selected because chemical cross-linking has indicated its proximity to the primary ligand-binding site (*8, 9*). First-strand cDNA was synthesized from 1 μg of total RNA by avian myeloblastosis virus reverse transcriptase and an oligo$(dT)_{12-18}$ primer. The resulting cDNAs were subjected to two consecutive rounds of polymerase chain reaction (PCR) (*10*) with two sets of internally nested primers. Electrophoretic analysis of the amplified material revealed that PCR products of the appropriate size were obtained from both affected and normal donors. Amplified cDNAs were subcloned into the plasmid vector Bluescript for sequence analysis. For each subunit, a single sequence was found from a pool of 50 individual clones. The sequence of the region of α_{IIb} encoding the four putative divalent cation–binding sites obtained from the affected individuals was identical to that present in the normals. In contrast, sequence analysis of the 365-bp fragment of β_3 revealed a single G → T base change resulting in substitution of Asp^{119} by Tyr in mature β_3 (Fig. 1). The base change also introduced an Rsa I restriction site such that the amplified fragments of normal and affected individuals could be differentiated by restriction analysis. Only the abnormal restriction pattern was present in both affected individuals, consistent with homozygosity for an autosomal recessive disease. The authenticity of the observed sequence change was supported by its presence in both affected siblings and in several samples from each individual. No other point mutations or new polymorphisms were detected by sequence analysis in the normal or affected individuals.

Table 1. Reactivity of the monoclonal antibody PMI-1 with transfected CHO cells. Flow cytometric analysis of stable transfectants was performed as described in Fig. 2. PMI-1 was incubated with cells in the presence of 2 mM calcium or 5 mM EDTA. Results are expressed as mean linear fluorescence intensity in arbitrary fluorescence units. Results shown are representative of five separate experiments.

Cells	Anti-gpIIb/IIIa	PMI-1 with	
		Ca^{2+}	EDTA
	Untransfected		
CHO	5.3	30.3	23.2
	Transfectants		
$\alpha_{IIb}\beta_3$	146.2	48.6	96.5
$\alpha_{IIb}\beta_{3Cam}$	201.7	133.6	107.1

To determine whether the identified mutation is associated with the Cam phenotype, the identified single base change was introduced into the wild-type β_3 sequence by site-directed mutagenesis (*11*) and examined for its effect on the expression and function of $\alpha_{IIb}\beta_3$ after transfection into Chinese hamster ovary (CHO) cells. In addition, the effect of the mutation on the ligand-binding function of the vitronectin receptor ($\alpha_v\beta_3$) was also examined since it shares the same β subunit with α_{IIb}.

Full-length cDNAs for α_{IIb}, α_v, β_3, and the mutant β_3 (β_{3Cam}) were subcloned into the vector CDM8 for expression studies. Stably transfected cell lines were established in CHO cells by cotransfection (*12*) with the appropriate α-subunit construct and either wild-type β_3 or β_{3Cam}. The cells were also transfected with a separate CDM8 construct containing the neomycin resistance gene to allow coselection. The expressed receptors were detected and clonal cell lines established with the use of fluorescence-activated cell sorting with specific MAbs.

The single base change did not affect the surface expression of $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (Fig. 2). Furthermore, immunoprecipitates with a polyclonal antibody to β_3 of all transfectants contained a corresponding α subunit, indicating that both subunits were complexed on the cell surface (Fig. 3). The $\alpha_{IIb}\beta_{3Cam}$ transfectant had a divalent cation–binding defect similar to that of the platelets of affected individuals. On normal platelets, the binding of PMI-1 MAb is inhibited by Ca^{2+} and enhanced by EDTA, whereas PMI-1 will bind to Cam platelets in the presence or absence of divalent cations (*4*). This pattern is repeated for the wild-type and the Cam transfectants (Table 1).

The capacity of the expressed receptors to bind representative peptide ligands that contained ArgGlyAsp [RGD (*13*)] was examined by flow cytometry with the MAb LIBS1 that recognizes an epitope on β_3 integrins that is dependent on occupancy of the receptor by ligand (*14*) and is not modulated by millimolar concentrations of divalent cations. RGD peptide ligands up-regulated the binding of MAb LIBS1 to wild-type $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ but did not up-regulate the binding of this MAb to both receptors containing β_{3Cam} (Fig. 2).

The direct interaction of the expressed receptors with RGD ligands was further assessed by affinity chromatography on the hexapeptide KYGRGDS coupled to Sepharose-4B (*15*). Detergent extracts of surface radioiodinated transfected cells were applied to the affinity matrix. After incubation overnight at 4°C, the column was washed with the inactive peptide GRGESP and then eluted with the active peptide GRGDSP. Recombinant wild-type $\alpha_{IIb}\beta_3$ and wild-type $\alpha_v\beta_3$ were bound and specifically eluted from the affinity matrix (Fig. 3). In contrast, both receptors containing the β_{3Cam} subunit did not bind and elute from the affinity matrix. These results indicate that the single

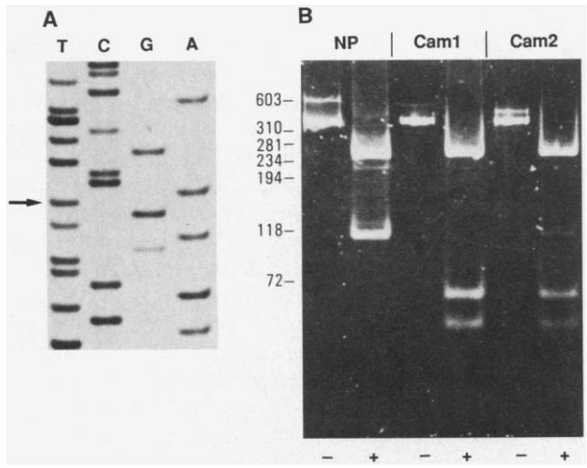

Fig. 1. First-strand cDNA synthesis was performed with a kit according to the manufacturer's recommendations (Invitrogen). One-fifth of the reverse transcriptase reaction (50-μl total volume) was subjected to two consecutive rounds of PCR with two sets of internally nested primers. Sequences of oligonucleotide primers for PCR amplification are stated 5′ to 3′; (+) and (−) denote sense and antisense primers, respectively, for each primer pair. For gpIIb: first primer pair: 2PCR3 (+), CTCGAGTTACCGCCCAGGCATCCTT, and 2PCR4 (−), CACAGCTCTTCACAGCAGGATTCAG; second pair: 2PCR1 (+), AGCAACCCAGAGTACTTCGACGGCT, and 2PCR2 (−), CTGGCTGAGCTCTGTACACAGCCAC. For gpIIIa: first primer pair: 3PCR3 (+), AATCCATCGAGTTCCCAGTGAGTGA, and 3PCR4 (−), TGGCATCAGTGGTAAACACCAGCAA; second pair: 3A5ECO (+), TCGAAGAATTCCTCCATCCAA, and 3A3BAM (−), GGCATCTCGGATCCGTGACAC. Underlined bases denotes non-gpIIIa sequence substitutions to facilitate directional subcloning. (**A**) DNA sequence analysis of the amplified gpIIIa RGD peptide cross-linking region from Cam variant. A portion of the autoradiogram of the sequence from a pool of clones corresponding to bases 443 to 464 [numbering based on (*6*)] is shown. The single base substitution of a T for a G at base 453 is indicated with an arrow. (**B**) Identification of Cam mutation by restriction analysis. The second-round PCR product corresponding to bases 387 to 752 (4 μl) was fractionated on a 5% polyacrylamide gel before (−) or after (+) digestion with endonuclease Rsa I. In control samples, which lacked input cDNA, no product was observed after two rounds of PCR. NP, pooled normal platelet donors; Cam1 and Cam2, two affected siblings with Cam variant. Position of size markers is shown on left.

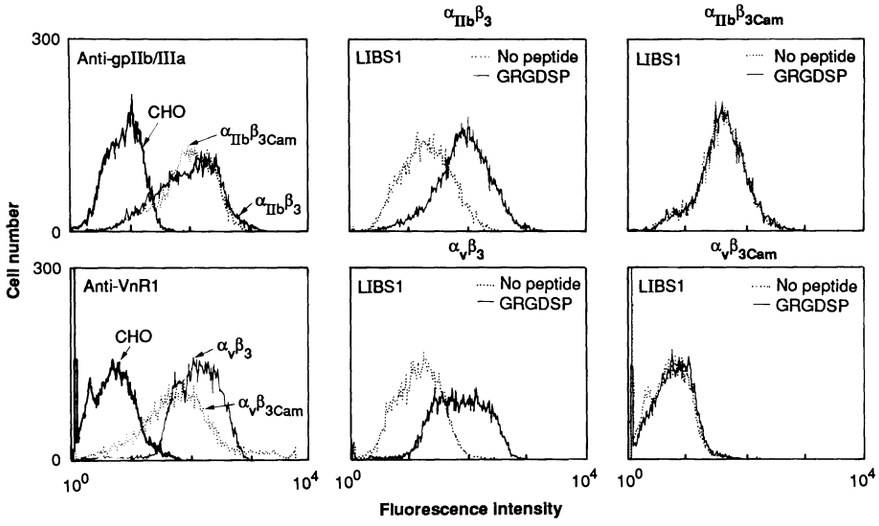

Fig. 2. Flow cytometric analysis of capacity of transfected cells to bind RGD ligands. Fluorescence-activated cell sorting (FACS) was performed as described (*12*). Briefly, an aliquot of transfected cells (5×10^5) in RPMI 1640 was incubated with the first antibody for 20 min on ice. Cells were pelleted, washed, resuspended in RPMI 1640, and incubated with fluorescein isothiocyanate–conjugated goat antibody to mouse immunoglobulin (Tago). After 20 min on ice, the cells were pelleted, resuspended in 0.5 ml of RPMI 1640, and analyzed on a FACS IV analyzer (Becton-Dickinson). For LIBS1 binding analysis, the GRGDSP peptide, at a final concentration of 1 mM, was added together with LIBS1 MAb (0.1 μM) in the first incubation. Results are expressed as histograms of cell number (linear scale) on the ordinate versus fluorescence intensity (log scale) on the abscissa. The reporting antibody is listed in the upper left of each panel. Antibody to vitronectin receptor 1 (anti-VnR1) is a MAb specific for $\alpha_v\beta_3$.

base change alone established the Cam phenotype in both of these β_3 integrins. Since the two affected individuals have no evident manifestation other than bleeding (*4*), other integrins [for example, $\alpha_v\beta_5$ (*16*)] may compensate for the lack of functional $\alpha_v\beta_3$.

Asp^{119} is close to Lys^{125}, which is proximal to bound RGD peptide (*8*). This Asp is absolutely conserved among integrin β subunits as are Ser^{121}, Ser^{123}, Asp^{126}, and Asp^{127}. An oxygenated residue at the position corresponding to Ser^{130} in β_3 is also present in all β subunits with the exception of the drosophila β subunit (*17*). This clustering of oxygenated residues could provide coordination sites for divalent cations as their linear spacing approximates that of the residues in the calcium-binding loop of EF hand proteins (*18*). The occurrence of Met^{124} in place of the invariant Gly in the EF loop (*18*) suggests the structure of this region will differ from the EF loop. Thus, further analysis will be required to assign function to the other residues. The proximity of the ligand-binding and hypothesized cation-binding site suggests the possibility of direct interactions between the bound cation and ligand as has been suggested for thrombospondin binding to $\alpha_v\beta_3$ (19) and for β_2 integrin function (*20*). Ligand binding to $\alpha_{IIb}\beta_3$ increases the binding of MAb PMI-1 to its divalent cation–sensitive epitope (*21*), the function of all integrins is divalent cation–dependent (*3, 22, 23*), and the region surrounding Asp^{119} is conserved indicating that this may be a general feature of ligand recognition by integrins. Furthermore, small peptide ligands that interact with certain integrins [RGDX (*23*), LGGAKQAGDV (*24*), and LHGHPEILDVPST (*25*)] contain oxygenated residues and in two cases, the D is critical for function (*26, 27*). This hypothesis predicts that β-subunit mutations affecting the Asp residue corresponding to Asp^{119} in β_3 should inhibit ligand binding function in other integrins as would mutations affecting four other oxygenated residues. In addition to this proposed common mechanism of ligand binding, integrins differ in peptide recognition specificity (*28*). The structural basis of this specificity should be elucidated by a further analysis of the topography of the binding interactions (*29*).

Fig. 4. Alignment of the region of β_3 containing the Cam mutation with the deduced sequences (*6, 16, 17, 30*) of other integrin β subunits. The aligned sequences are from human, avian, and frog (*Xenopus laevis*) β_1, human β_2, β_3, β_4, β_5, and β_6, and *Drosophila melanogaster* l(1)mys (lethal myospheroid gene product). Asp^{119} of β_3, the residue substituted by Tyr in affected Cam individuals is circled. Lys^{125} of β_3, the proposed residue to which receptor-bound RGD peptide becomes cross-linked by bifunctional reagents, is boxed. Oxygenated residues that align with those in the EF loop consensus sequence are shaded. X, Y, Z, −X, and −X refer to vertices of the calcium coordination octahedron (*18*).

β_1	D	L	S	Y	S	M	K	D	D	L	E	N
β_2	D	L	S	Y	S	M	I	D	D	L	R	N
β_3	D	L	S	Y	S	M	K	D	D	L	W	S
β_4	D	F	S	N	S	M	S	D	D	L	D	N
β_5	D	L	S	L	S	M	K	D	D	L	D	N
β_6	D	L	S	A	S	M	D	D	D	L	N	T
Avian (β_1)	D	L	S	Y	S	M	K	D	D	L	E	N
Frog (β_1)	D	L	S	F	S	M	K	D	D	L	E	N
l(1)mys	D	L	S	K	S	M	E	D	D	K	A	K
EF loop	X		Y		Z	G		-X			-Z	

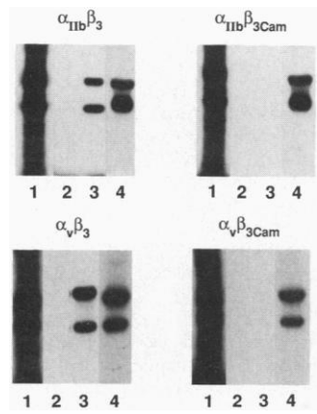

Fig. 3. RGD affinity chromatography and immunoprecipitation of extracts of stably transfected cell lines. CHO cells stably expressing the wild-type or mutant receptors were radioiodinated by the lactoperoxidase-H_2O_2 method then solubilized in buffer containing 10 mM Hepes (pH 7.5), 0.15 M NaCl, 50 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM leupeptin, and 10 mM *N*-ethylmaleimide. The extract was applied to a KYGRGDS–Sepharose 4B column (1-ml column volume). Unbound proteins were eluted with 30 ml of column buffer, identical to lysis buffer except the octylglucoside was lowered to 25 mM. The column was washed sequentially with 3 ml of buffer containing GRGESP peptide (1 mg/ml), followed by 3 ml of buffer containing GRGESP peptide (1 mg/ml), followed by 3 ml of buffer, and finally 3 ml of buffer containing GRGDSP (1 mg/ml). Fractions were analyzed by electrophoresis on 6.5% SDS-polyacrylamide gels under nonreducing conditions followed by autoradiography. Lanes 1, starting material; lanes 2, GRGESP eluted material; lanes 3, GRGDSP eluted material; and lanes 4, immunoprecipitate of starting material obtained with a polyclonal anti-β_3 antibody. Immunoprecipitation was performed as described (*12*).

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Presentation of Exogenous Antigen with Class I Major Histocompatibility Complex Molecules

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Soluble antigens (Ags) in the extracellular fluids are excluded from the class I major histocompatibility complex (MHC)–restricted pathway of Ag presentation in most cells. However, an exogenous Ag can be internalized, processed, and presented in association with class I MHC molecules on specialized Ag-presenting cells (APCs). These APCs express class II molecules and can simultaneously present exogenous Ags to both class I and class II MHC–restricted T cells. These APCs may be important participants in the regulation of host immune responses. This APC activity may explain several phenomena of cytotoxic T lymphocyte (CTL) priming in vivo and might be exploited for eliciting CTL responses to protein vaccines.

ANTIGENS IN THE EXTRACELLULAR fluid are taken up by specialized APCs, processed in an endosomal compartment, and subsequently displayed in association with class II MHC molecules (*1*). In contrast, endogenously synthesized Ags, of virtually all cells, are processed in a distinct intracellular compartment and are subsequently displayed in association with class I MHC molecules (*2, 3*). These two pathways for Ag processing and presentation are segregated, which influences the specificity of immune responses (*3–5*). This segregation helps determine the host response to a particular pathogen. For example, as a consequence of the segregated pathways, cytolytic responses are selectively targeted to virally infected or transformed cells. However, if there is absolute segregation of class I and class II MHC Ag-processing pathways, it is then unclear how the necessary collaboration between T helper cells and CTLs occurs. Previous studies have analyzed Ag presentation with APCs in conventional cytolytic assay systems. We have used a novel, class I MHC–restricted T-T hybridoma (*6*) to examine the segregation of class I and II MHC–restricted pathways of Ag presentation in APCs that reside in normal lymphoid organs.

The ability of various APCs to present an exogenous protein Ag to T cells was ana-

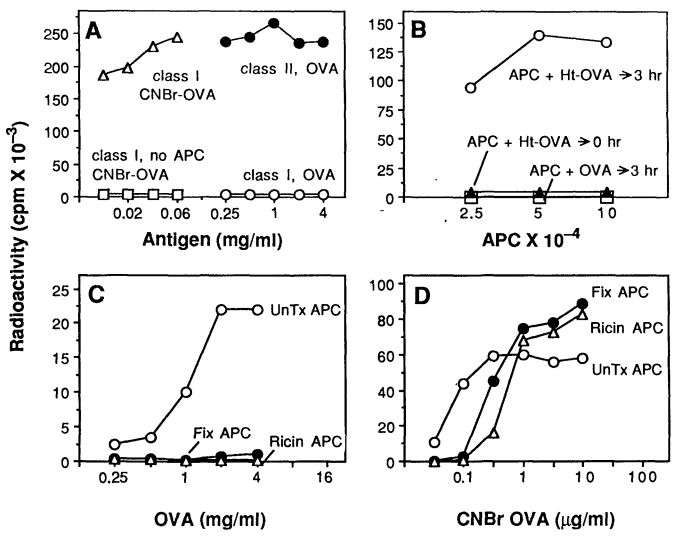

Fig. 1. Presentation of OVA by different APCs. (A), (B), (C), and (D) were assayed with T cells that are class I restricted. Some assays in (A) were also done with T cells that are class II restricted. (**A**) Class I (10^5, RF33.70) (*6*) or class II (5×10^4, DO.11.10) (*16*) MHC–restricted T-T hybridomas were cultured in the presence or absence of untreated LB27.4 APCs (5×10^4) (*17*) with native OVA or cyanogen bromide–cleaved OVA (CNBr-OVA) as described (*18*). After 18 hours of incubation at 37°C, a 100-μl aliquot of supernatant was removed from duplicate cultures and assayed for IL-2 content with HT-2 cells (*19*). (**B**) Similar to (A), except that LB27.4 APCs were incubated for 10 min at 37°C with native OVA (10 mg/ml) under isotonic or hypertonic (Ht) conditions as previously described (*5*). Cells exposed to Ht conditions were diluted in hypotonic media and incubated for 3 min at 37°C to cause osmotic lysis of pinosomes, as described (*5, 7*). After several washes at 4°C the APCs were either fixed with paraformaldehyde (*20*) immediately or after 3 hours of incubation at 37°C. The number of LB27.4 cells was titrated in cultures with RF33.70 cells in the absence of exogenous Ag. (**C**) Similar to (A) but APCs were C57BL/6 splenocytes (10^6) that were either untreated (UnTx), fixed (Fix) with glutaraldehyde (*21*), or incubated with ricin (2×10^{-9} M) for 1 hour at 37°C and washed, and the Ag added to cultures was OVA. (**D**) Similar to (C) but the Ag added to cultures was CNBr-OVA. Data points are expressed as the mean incorporation of [^{3}H]thymidine (counts per minute) into HT-2 cells. Data are representative of at least three experiments.