12. S. Feng and E. C. Holland, *Nature* **334**, 165 (1988); J. A. Garcia *et al.*, *EMBO J.* **8**, 765 (1989).
13. B. Berkhout and K.-T. Jeang, *J. Virol.* **63**, 5501 (1989); S. Roy, N. T. Parkin, C. Rosen, J. Iticitch, N. Sonenberg, *ibid.* **64**, 1402 (1990).
14. L. J. Seigel *et al.*, *Virology* **148**, 226 (1986); M. Newstein, E. J. Stanbridge, G. Casey, P. R. Shank, *J. Virol.* **64**, 4565 (1990).
15. C. E. Hart *et al.*, *Science* **246**, 488 (1989).
16. A. Gatignol, A. Kumar, A. Rabson, K.-T. Jeang, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7828 (1989); R. Gaynor, E. Soultanakis, M. Kuwabara, J. Garcia, D. S. Sigman, *ibid.*, p. 4858; R. A. Marciniak, M. A. Garcia-Blanco, P. A. Sharp, *ibid.* **87**, 3624 (1990).
17. H. Singh, J. H. LeBowitz, A. S. Baldwin, P. A. Sharp, *Cell* **52**, 415 (1988); C. R. Vinson, K. L. LaMarco, P. F. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988); H. Singh, R. G. Clerc, J. H. LeBowitz, *BioTechniques* **7**, 252 (1989).
18. J. M. Short, J. M. Fernandez, J. A. Sorge, W. D. Huse, *Nucleic Acids Res.* **16**, 7583 (1988).
19. *Escherichia coli* XL1 blue was infected with 1×10^6 plaque-forming units of λ ZAP HeLa cDNA library (Stratagene) and plated. After 3 hours at 42°C, the plates were overlaid with nitrocellulose filters previously soaked in 10 mM IPTG and then incubated for 6 to 8 hours at 37°C. Filters were then immersed sequentially for 5 min in 6 M, 3 M, 1.5 M, and 0.75 M guanidine-HCl in binding buffer [50 mM tris-HCl, (pH 7.5); 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol]. Finally, the filters were equilibrated with binding buffer alone. For the binding assays, we incubated the filters for 60 min in 2.5% nonfat milk in binding buffer and then probed with ^{32}P-labeled TAR RNA (50 fmol/ml) in the presence of poly(dI-dC) (10 μg/ml) and yeast RNA (10 μg/ml). We used 1×10^6 cpm per filter with an incubation time of 1 hour at room temperature and then washed the filters extensively in binding buffer.
20. S. M. Hanley *et al.*, *Genes Dev.* **3**, 1534 (1989).
21. K.-T. Jeang *et al.*, *J. Virol.* **61**, 1559 (1987).
22. L. Laimins, P. Gruss, R. Pozzatti, G. Khoury, *ibid.* **49**, 183 (1984).
23. J. Brady, K.-T. Jeang, J. Duvall, G. Khoury, *ibid.* **61**, 2175 (1987).
24. D. H. Gubzda, J. Hess, J. Small, J. E. Clements, *Mol. Cell. Biol.* **9**, 2728 (1989).
25. R. Chiu, M. Imagawa, R. J. Imbra, J. R. Bockoven, M. Karin, *Nature* **329**, 648 (1987).
26. A. Gatignol and K.-T. Jeang, unpublished data.
27. H. Nashimoto and H. Uchida, *Mol. Gen. Genet.* **201**, 25 (1985); J. C. A. Bardwell *et al.*, *EMBO J.* **8**, 3401 (1989).
28. C. A. Kozak, unpublished observations.
29. R. D. Marshall, *Annu. Rev. Biochem.* **41**, 673 (1972); E. Bause, *Biochem. J.* **209**, 331 (1983).
30. The sequence has been deposited with GenBank (accession number M60801).
31. We thank M. Martin and W. Leonard for critical readings of the manuscript, and C. Buckler for computer analysis. Supported in part by the intramural AIDS antiviral targeted program from the office of the director of the NIH.

13 November 1990; accepted 5 February 1991

In Vitro and in Vivo Consequences of VLA-2 Expression on Rhabdomyosarcoma Cells

BOSCO M. C. CHAN, NARIAKI MATSUURA, YOSHIKAZU TAKADA, BRUCE R. ZETTER, MARTIN E. HEMLER*

Cloned integrin α_2 subunit complementary DNA was expressed on human rhabdomyosarcoma (RD) cells to give a functional VLA-2 ($\alpha_2\beta_1$) adhesion receptor. The VLA-2–positive RDA2 cells not only showed increased adhesion to collagen and laminin in vitro, but also formed substantially more metastatic tumor colonies in nude mice after either intravenous or subcutaneous injection. These results show that a specific adhesion receptor (VLA-2) can markedly enhance both experimental and spontaneous metastasis. In contrast to the metastasis results, there was no difference in either the in vitro growth rate or apparent in vivo tumorigenicity of RD and RDA2 cells.

MEMBERS OF THE INTEGRIN FAMILY of adhesion receptors, comprised of at least 15 distinct αβ-subunit heterodimers (*1, 2*), mediate cell binding to major components of the extracellular matrix (ECM). For example, among the β_1 subfamily of integrins (VLA proteins), VLA-1, -2, and -3 mediate cell binding to collagen, VLA-3, -4, and -5 bind fibronectin, and VLA-1, -2, and -6 bind laminin (*2*). Integrins in the β_1 subfamily may be involved in tumor cell metastasis because the dissemination of tumor cells and their subsequent growth in secondary sites require extensive interaction with ECM proteins, both in the vascular basement membrane and interstitial stroma at the secondary site (*3*).

As evidence of a potential role for β_1 integrins in metastasis, monoclonal antibodies (MAbs) to VLA proteins can block cell migration and invasion through basement membranes in vitro (*4*), and VLA protein expression has been variably correlated with invasiveness in vitro (*5, 6*). Also, small synthetic peptides derived from cell adhesion molecules can block both cellular invasiveness in vitro and experimental metastasis in vivo, presumably by acting as ligand analogs competing for adhesion receptor binding sites (*7*). However, in vivo studies have not yet identified the specific adhesion receptor or receptors important for metastasis. Without addressing the issue of metastasis, other investigators have found that cell transformation (*8, 9*) and increased tumorigenicity (*10*) correlate with alterations in β_1 integrin expression.

Because cells usually express multiple integrins with overlapping ligand specificities, MAb blocking studies and correlational changes in integrin profiles are difficult to interpret. Also, the adhesion receptors that facilitate cell growth at a primary tumor site (that is, show tumorigenicity) are not necessarily the same as those involved in dissemination to tissue sites distant from the primary tumor (that is, metastasis).

This study focuses on the in vitro and in vivo roles of VLA-2, an adhesion receptor that usually binds both collagen and laminin, but on some cell types only binds collagen (*11*). To fully evaluate the in vivo effects of VLA-2, we examined not only tumorigenicity, but also both "spontaneous" and "experimental" metastasis. In the former, a tumor cell migrates into and through surrounding tissue, traverses a nearby vascular wall (or lymphatic channel), travels through the circulation, extravasates by again migrating through a vascular wall, and finally begins to grow in a new tissue location. In "experimental metastasis," tumor cells are injected intravenously and then escape from the circulation and colonize a tissue site, in a model system that mimics the latter steps of spontaneous metastasis.

To study the functions of VLA-2, we introduced the full-length cDNA clone for the α_2 subunit (*12, 13*) into the rhabdomyosarcoma tumor cell line RD by means of the mammalian cell expression vector pFneo (*14*). The expression of VLA-2 or transfected RD (RDA2) was demonstrated by immunoprecipitation (Fig. 1B) and by immunofluorescence staining (Fig. 1A). Although β_1 and other α subunits are present in both RD and RDA2 cells, α_2 expression was observed only in the RDA2 cells. Because the α_2 gene product was coprecipitated with the β_1 subunit (Fig. 1B) and the amount of β_1 expressed at the cell surface increased (Fig. 1A) over that of RD cells, the α_2 subunit must have associated with the endogenous β_1 subunit of RD cells. Flow cytometry experiments showed that transfection of the α_2 gene caused no alteration in the surface levels of VLA-1, -4, -5 and -6, normally found on RD cells. Together these results support the previous suggestion (*15*) that a pool of excess β_1 subunit is available for association if the amount of α should increase. Also, our results agree with findings from other integrin transfection studies which showed that α or β subunits from the β_2 and β_3 integrin

B. M. C. Chan, Y. Takada, M. E. Hemler, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.
N. Matsuura and B. R. Zetter, Department of Surgery, Children's Hospital, and Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.

subfamilies were expressed at the cell surface only if the correct complementary subunit was available for assocation (*16*).

The RDA2 cells bound to collagen and laminin better than did RD cells (Fig. 2, A and B), whereas binding to fibronectin (Fig. 2C) was similar for RD and RDA2. Collagen and laminin binding by RDA2 cells, but not by RD cells, was susceptible to inhibition by MAbs to VLA-2 (Fig. 3). Visual inspection of bound cells revealed that RDA2 spread more than RD cells on collagen and laminin, whereas both cell types spread similarly on fibronectin.

To assess the in vivo effect of VLA-2 expression on RD cells, we first used an experimental metastasis system, in which 10^6 RD or RDA2 cells were injected into the tail veins of nude mice (Fig. 4). After 8 weeks of observation, more tumors were found in the lungs and other tissues of mice injected with RDA2 cells (mean, 56.4; median, 29 foci per mouse) compared to RD cells (mean, 2.6; median, 2 foci per mouse). The tumors in each group were of equivalent size, suggesting that VLA-2 expression does not affect the in vivo growth properties of RD cells. Rather, VLA-2 expression on RD cells most likely altered adhesion to vascular determinants soon after intravenous injection. Importantly, no RD tumor growth was observed in any but lung sites, whereas RDA2 cells also yielded tumors in the bone, lymph nodes, and adrenal glands (Fig. 4, inset).

Also, we compared the capability of RD and RDA2 cells to form spontaneous metastases. After subcutaneous injection of RD cells, no detectable tumors (0/6 mice) were observed in any secondary sites, whereas mice injected subcutaneously with RDA2 cells had a total of five secondary lung tumor foci (in 4/6 mice). These results indicate that VLA-2 expression can also markedly enhance spontaneous metastasis.

In contrast to the notable differences in metastasis between RD and RDA2 cells, there was no difference in growth rate (assessed by [^{3}H]thymidine uptake) for RDA2, mock-transfected RD, and untransfected RD cells. Also, there was no apparent difference in the tumorigenicity of RDA2 and RD cells because each formed tumors at primary subcutaneous injection sites in 6/6 cases, and the tumors were of similar size and grew at similar rates.

Possible explanations for increased RDA2 cell metastases are that (i) VLA-2 expression causes preferential RDA2 adhesion to basement membrane (or other ligand-containing sites) or (ii) RDA2 cells might be less sensitive to clearance by natural killer cells. The latter explanation may be unlikely, because expression of VLA-2 (and other VLA proteins) correlates with increased susceptibility to lysis mediated by natural killer cells (*6*). Thus, if nude mouse natural killer cell activity were blocked, RDA2 cells conceivably could form even more tumor foci relative to the RD cells. Also, we have observed no propensity for either RD or RDA2 cells to form cell aggregates while in suspension,

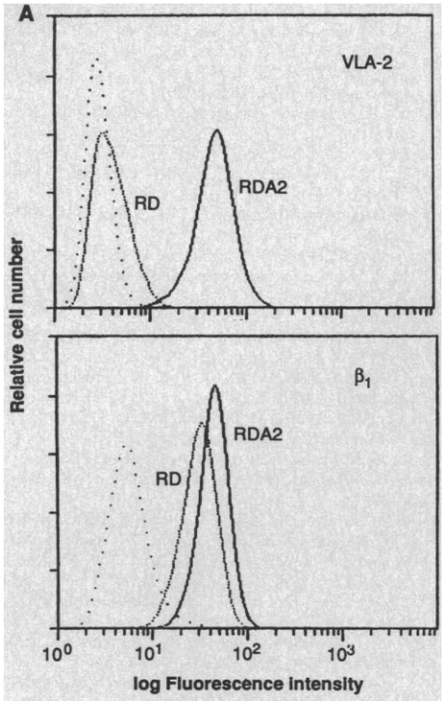


Fig. 1. Expression of VLA-2 ($\alpha_2\beta_1$) by RD cells. By means of the Lipofectin reagent (Bethesda Research Laboratories), RD cells were transfected with 10 μg of the plasmid pFneo (*14*), containing α_2 cDNA (*12, 13*). After 4 weeks in selective media [containing 2 mg of Geneticin per milliliter (Gibco)], α_2-positive cells (called RDA2) were further enriched by means of magnetic beads (Dynal). (**A**) Flow cytometric analyses of RD and RDA2 cells were performed with the anti–VLA-2 MAb 5E8 (*19*) (upper panel), the VLA-β_1 MAb A-1A5 (*22*) (lower panel), and the MAb P3 (*23*) as a negative control (dotted line, both panels). (**B**) For analysis of VLA-2 by immunoprecipitation, RD (lanes a to c) and RDA2 cells (lanes d to f) that were ^{125}I-labeled on the cell surface were solubilized in phosphate-buffered saline (PBS) containing 1% NP-40, by established procedures (*22*). Cell extracts were immunoprecipitated with the MAb J-2A2 (*24*) as a negative control (lanes a and d); the MAb 5E8 (*9*) anti–VLA-2, (lanes b and e); and the MAb A-1A5 (*22*) anti-VLA β_1(lanes c and f). Precipitated samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), on 6% gels under nonreducing conditions.

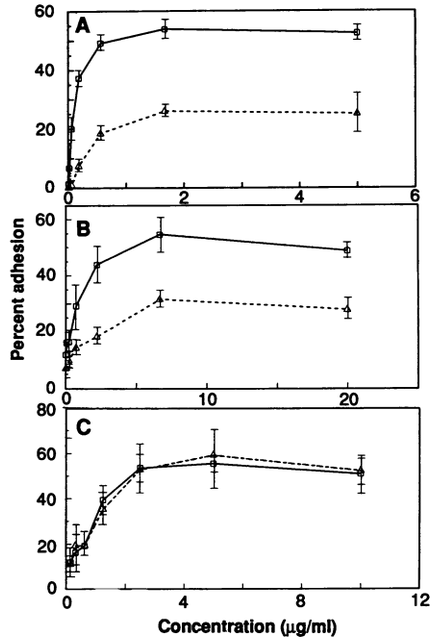


Fig. 2. Binding of RD (- - -) and RDA2 (——) cells to collagen (**A**), laminin (**B**), and fibronectin (**C**). Cell adhesion assay procedures (*13*) were carried out with ^{51}Cr-labeled cells [5×10^4 cells in 0.1 ml of RPMI media with 1% bovine serum albumin (BSA)] added to 96-well microtiter plates that had been coated with various concentrations of matrix proteins and then blocked with BSA. After a 20-min incubation at 37°C, unbound cells were removed by gentle washing with RPMI. Radioactivity corresponding to bound cells was used to calculate percent cell adhesion after subtraction of nonspecific binding to BSA-coated wells (usually less than 10%). Each data point represents the average of triplicates ± 1 SD. The basal binding by RD cells to collagen, laminin, and fibronectin is due to endogenous VLA-1, -6, -4, and -5.

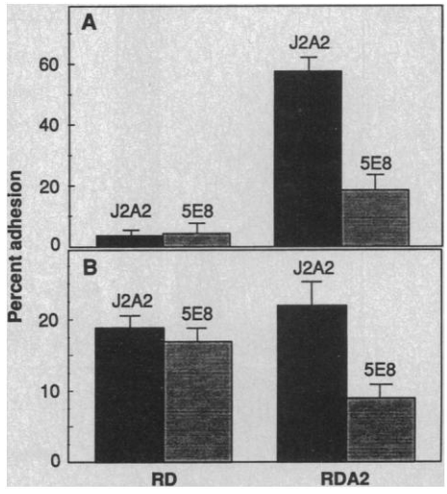


Fig. 3. Inhibition of RD and RDA2 cell binding to collagen (**A**) and laminin (**B**). Binding to collagen (0.1 μg/ml) and laminin (3 μg/ml) was as in Fig. 2 except that either the control MAb J-2A2 or the anti–VLA-2 MAb 5E8 was present at ~5 to 20 μg/ml. Data shown are from triplicate determinations, ±1 SD.

suggesting that mechanical trapping of aggregates is not an explanation for the observed differences in metastasis.

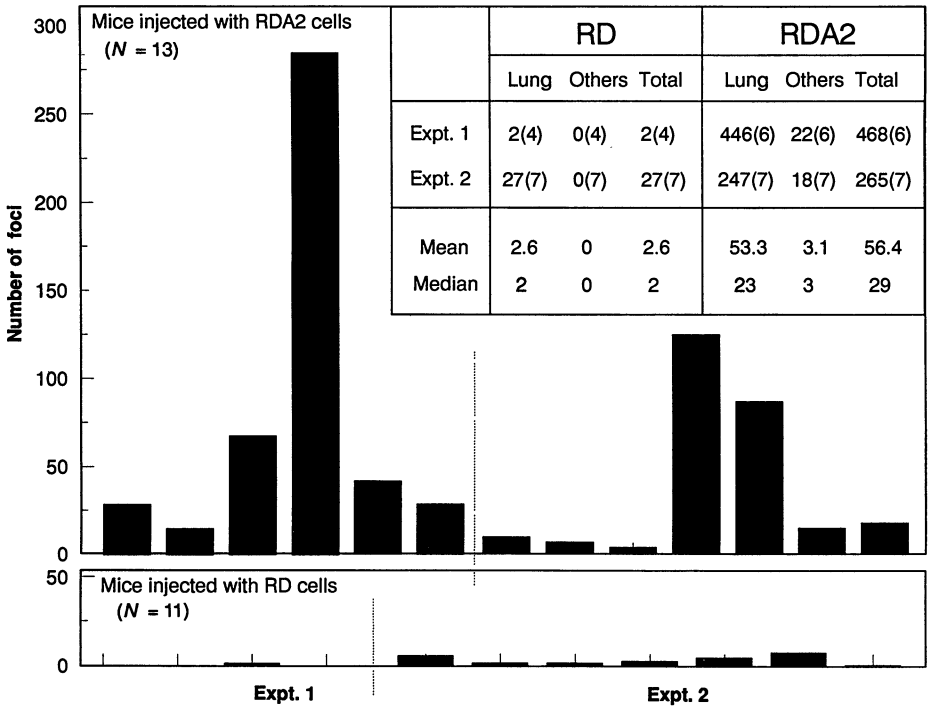
Our in vitro and in vivo studies together suggest that binding of VLA-2 to collagen, laminin, or both contributes to one or more steps in the metastatic process. In particular, our experimental metastasis results show that the influence of VLA-2 can occur after cells have entered the circulation, in a mechanism perhaps involving adhesion to collagen or laminin, or both, in the subendothelium, during extravasation. Earlier studies have shown that laminin may potentiate tumor metastasis (*17*), possibly by a mechanism that involves laminin-induced activation of metalloproteinase activity (*18*). It is not known whether a similar mechanism might be mediated through VLA-2, but if so, it might contribute to VLA-2-mediated invasiveness. In this regard, another integrin, VLA-5, transmits a signal leading to induction of matrix-degrading enzymes (*19*).

Whereas the α_2-transfected RD cell is an artificially created system for analyzing the role of VLA-2 in metastasis, there are tumor cells that naturally express VLA-2. Thus we predict that the up-regulated expression of VLA-2 on many lung tumors (*9*) and on an osteosarcoma cell line (*5*) could contribute to the invasiveness of those cells.

Once the exact peptide sequences recognized by VLA-2 in collagen and laminin are determined, small peptides, or drugs mimicking these peptides, could perhaps be used therapeutically to block tumor cell metastasis. Besides binding to collagen and laminin, it is also conceivable that VLA-2 might mediate cell-cell interactions (*20*), which may contribute to the in vivo results obtained here. The interaction of other integrins (LFA-1 and VLA-4) with their cell surface ligands (ICAM-1 and VCAM-1) has been suggested to contribute to tumor cell metastasis (*21*), although direct evidence is still lacking.

A previous study showed that overexpression of a fibronectin receptor ($\alpha_5\beta_1$, VLA-5) was accompanied by reduced tumorigenicity of transformed cells (*10*), but the issue of metastasis was not addressed. The present study failed to show any apparent effect of VLA-2 on growth rate or tumorigenicity, but showed that VLA-2 receptors can enhance metastasis. Together these studies affirm that individual integrins can differentially influence the tumorigenic and metastatic properties of cancer cells.

In conclusion, the results presented here represent a direct demonstration that a specific adhesion receptor can influence the frequency and distribution of metastatic colonies in vivo in both experimental and spontaneous systems. Also, this work establishes a precedent suggesting that other adhesion receptors might similarly be evaluated for their contributions to the complex process of tumor cell localization.



	RD			RDA2		
	Lung	Others	Total	Lung	Others	Total
Expt. 1	2(4)	0(4)	2(4)	446(6)	22(6)	468(6)
Expt. 2	27(7)	0(7)	27(7)	247(7)	18(7)	265(7)
Mean	2.6	0	2.6	53.3	3.1	56.4
Median	2	0	2	23	3	29

Fig. 4. Experimental metastasis by RD and RDA2 cells in nude mice. Cells (1×10^6 per mouse) were injected into the tail veins of *nu/nu* CD-1 mice [experiment (Expt.) 1] or *nu/nu* Balb/c mice (Expt. 2). After 8 weeks, the total number of tumors in the lungs and other tissues was determined for each mouse (vertical bars). The inserted table indicates the number of tumors observed in lungs, other locations, and the total, for experiments 1 and 2. The number of mice used in each experiment is indicated in parentheses. The category of "Others" includes bone, adrenal glands, and lymph nodes. The data from both experiments 1 and 2 were used to obtain mean and median tumors per mouse.

REFERENCES AND NOTES

1. R. O. Hynes, *Cell* **48**, 549 (1987); E. Ruoslahti and M. D. Pierschbacher, *Science* **238**, 491 (1987); C. A. Buck and A. F. Horwitz, *Annu. Rev. Cell Biol.* **3**, 179 (1987); R. S. Larson and T. A. Springer, *Immunol. Rev.* **114**, 181 (1990).
2. M. E. Hemler, *Annu. Rev. Immunol.* **8**, 365 (1990).
3. B. R. Zetter, *N. Engl. J. Med.* **322**, 605 (1990).
4. K. M. Yamada *et al.*, *Cancer Res.* **50**, 4485 (1990).
5. S. Dedhar and R. Saulnier, *J. Cell Biol.* **110**, 481 (1990).
6. A. Anichini *et al.*, *Int. J. Cancer*, **46**, 508 (1990).
7. M. J. Humphries *et al.*, *Science* **233**, 467 (1986); K. R. Gehlsen *et al.*, *J. Cell Biol.* **106**, 925 (1988); Y. Iwamoto *et al.*, *Science* **238**, 1132 (1987).
8. L. C. Plantefaber and R. O. Hynes, *Cell* **56**, 281 (1989); S. K. Akiyama *et al.*, *Cancer Res.* **50**, 1601 (1990); S. Saga, W.-T. Chen, K. M. Yamada, *ibid.* **48**, 5510 (1988); J. Peltonen *et al.*, *J. Clin. Invest.* **84**, 1916 (1989); M. Pignatelli, M. E. F. Smith, W. F. Bodmer, *Br. J. Cancer* **61**, 636 (1990).
9. S. Zylstra *et al.*, *Cancer Res.* **46**, 6446 (1986).
10. F. G. Giancotti and E. Ruoslahti, *Cell* **60**, 849 (1990).
11. S. A. Santoro, *ibid.* **46**, 913 (1986); Y. Takada *et al.*, *J. Cell. Biochem.* **37**, 385 (1988); W. Staatz, S. M. Rajpara, E. A. Wayner, W. G. Carter, S. A. Santoro, *J. Cell Biol.* **108**, 1917 (1989); R. H. Kramer and N. Marks, *J. Biol. Chem.* **264**, 4684 (1989); M. J. Elices and M. E. Hemler, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9906 (1989); L. R. Languino *et al.*, *J. Cell Biol.* **109**, 2455 (1989); D. Kirchhofer, L. R. Languino, E. Ruoslahti, M. D. Pierschbacher, *J. Biol. Chem.* **265**, 615 (1990).
12. Y. Takada and M. E. Hemler, *J. Cell Biol.* **109**, 397 (1989).
13. M. J. Elices *et al.*, *Cell* **60**, 577 (1990).
14. P. Ohashi *et al.*, *Nature* **316**, 606 (1985); T. Saito, *et al.*, *ibid.* **325**, 125 (1987); H. Band *et al.*, *J. Immunol.* **142**, 3267 (1989).
15. J. Heino *et al.*, *J. Biol. Chem.* **264**, 380 (1989).
16. S. C. Bodary, M. A. Napier, J. W. McLean, *ibid.*, p. 18859; T. E. O'Toole *et al.*, *Blood* **74**, 14 (1989); M. L. Hibbs *et al.*, *J. Clin. Invest.* **85**, 674 (1990); J. M. Wilson *et al.*, *Science* **248**, 1413 (1990).
17. V. P. Terranova, L. A. Liotta, R. G. Russo, G. R. Martin, *Cancer Res.* **42**, 2265 (1982); V. P. Terranova *et al.*, *Science* **226**, 982 (1984).
18. T. Turpeenniemi-Hujanen *et al.*, *J. Biol. Chem.* **261**, 1883 (1986); T. Kanemoto *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2279 (1990).
19. Z. Werb *et al.*, *J. Cell Biol.* **109**, 877 (1989).
20. H. Larjava *et al.*, *ibid.* **110**, 803 (1990).
21. F. F. Roossien, D. de Rijk, A. Bikker, E. Roos, *ibid.* **108**, 1979 (1989); J. P. Johnson, B. G. Stade, B. Holzmann, W. Schwable, G. Riethmuller, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 641 (1989); P. Natali *et al.*, *Cancer Res.* **50**, 1271 (1990); G. E. Rice and M. P. Bevilacqua, *Science* **246**, 1303 (1989).
22. M. E. Hemler, C. F. Ware, J. L. Strominger, *J. Immunol.* **131**, 334 (1983).
23. J. F. Kearney, A. Radbruch, B. Liesegang, K. Rajewsky, *ibid.* **123**, 1548 (1979).
24. M. E. Hemler and J. L. Strominger, *ibid.* **129**, 2734 (1982).

25. We thank R. Bankert (Roswell Park Memorial Institute, Buffalo, NY) for providing the MAb 5E8. Supported by National Institute of General Medical Science grant GM 38903 to M.E.H., National Cancer Institute grant CA 37393 to B.R.Z., and a Medical Research Council of Canada Postdoctoral Fellowship to B.M.C.C.

24 September 1990; accepted 14 December 1990