17. Guillaumin, M. & Petit, C. *Annls. Génét.* **3**, 9-15 (1961).
18. Petit, C. *Annls. Génét.* **6**, 24-35 (1963).
19. Fitch, W. M. & Atchley, W. R. *Science* **228**, 1169-1175 (1985).
20. Syvanen, M. *A. Rev. Genet.* **18**, 271-293 (1984).
21. Levis, R., Dunsmuir, P. & Rubin, G. M. *Cell* **21**, 581-579 (1980).
22. Dunsmuir, P., Brorein, W. J., Simon, M. A. & Rubin, G. M. *Cell* **21**, 575-579 (1980).
23. Ilyin, Y. V., Chmeliauskaite, V. G. & Georgiev, G. P. *Nucleic Acids Res.* **8**, 3439-3457 (1980).
24. Tchurikov, N. A. *et al. Cold Spring Harb. Symp. quant. Biol.* **45**, 655-665 (1981).

Haemonectin, a bone marrow adhesion protein specific for cells of granulocyte lineage

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There is substantial evidence that the haematopoietic microenvironment is crucial to the growth and differentiation of haematopoietic cells. This microenvironment is composed of stromal cells, soluble factors and extracellular matrix (ECM). We have shown that a complex extract of bone marrow ECM can stimulate the growth and differentiation of haematopoietic cells *in vitro*[1]. Furthermore, the use of inhibitors[2] or stimulators[3] of ECM synthesis in long-term marrow culture affects cell proliferation. On a molecular level, however, the interactions between ECM and haematopoietic cells are not well understood. We have investigated the adhesion between specific bone marrow ECM components and haematopoietic cells, and found a protein, 'haemonectin', of relative molecular mass 60,000 in bone marrow ECM which is a lineage- and organ-specific attachment molecule for cells of granulocyte lineage. This specificity distinguishes haemonectin from previously described adhesion proteins which have a wider tissue distribution and cell type specificity.

To determine whether components of bone marrow ECM promote selective attachment of specific populations of haematopoietic cells, we used murine marrow cells to compare the adhesive properties of marrow ECM and fibronectin. Fibronectin promotes the attachment of erythroid precursor cells[4,5] and is present in the bone marrow[6]. Unfractionated murine marrow cells (C57/BL6) were plated in serum-free conditions in tissue culture dishes pre-coated with fibronectin or bone marrow ECM extract[1], and the attached cells removed after incubation by trypsin treatment and counted. Total cell attachment on the two substrates was similar, but the myeloid to erythroid ratio was 1.25 on fibronectin-coated dishes and 3.11 on bone marrow ECM-coated dishes. This suggested that bone marrow ECM contains factors which selectively promote attachment of cells of granulocytic origin.

The proteins comprising the bone marrow ECM are shown on an SDS-polyacrylamide gel in Fig. 1, lane 2. To test which might promote the selective attachment of granulocyte cell types, the marrow-derived ECM proteins were transferred to nitrocellulose after SDS polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with ^{51}Cr-labelled unfractionated bone marrow cells. Chromium-labelled marrow cells adhered strongly to a protein of relative molecular mass (M_r) 60,000 (60K) in marrow ECM (Fig. 1, lane 4). We have termed this protein haemonectin. No adhesion was seen to the protein standards (Fig. 1, lane 3). After fixation to the nitrocellulose and staining with haematoxylin, light microscopy showed that most cells had nuclear morphology consistent with a granulocyte origin.

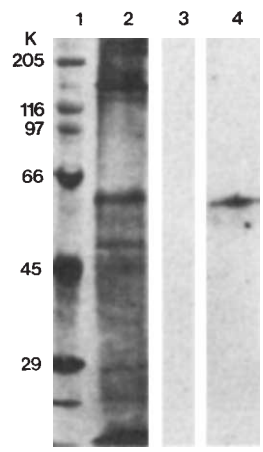

Fig. 1 Cellular attachment to haemonectin. Cell-attachment assay shown on 10% SDS-PAGE[12]: lane 1, M_r standards visualized with silver stain, values indicated to the left; lane 2, bone marrow ECM extract visualized with silver stain. Note the intense protein band at M_r ~60K. Lanes 3 and 4, fluorograms of standards and marrow ECM after transfer to nitrocellulose, non-specific cell-binding site blockade, and incubation with ^{51}Cr-labelled bone marrow cells. No cell attachment is seen to protein standards in lane 3 but a distinct band is seen in lane 4 which corresponds to M_r ~60K. See Table 1 for differential counts of attached and unfractionated input marrow cells.

Methods. Marrow was scraped from rabbit femurs and ECM prepared as described[1]. ECM and molecular weight standards were separated by 10% SDS-PAGE[12]. Lanes 1 and 2 were silver-stained[13] and duplicate lanes were transferred to nitrocellulose[14] using a modified transfer buffer (10% v/v methanol with 0.001 M SDS, 0.024 M Tris, 0.192 M glycine, *p*H 8.3). Efficient transfer was confirmed by Coomassie staining. After completion of electrophoretic transfer (200 mA for 16 h), the nitrocellulose was washed with 2.5% Triton X-100 in PBS for 30 min to promote renaturation of transferred proteins[15], rinsed in PBS and blocked with 5% w/v evaporated milk protein ('Blotto', see ref. 16) in PBS overnight at 4 °C. Bone marrow cells were flushed from femurs and tibias of C57/BL6 mice and debris removed by unit gravity sedimentation. The cells were centrifuged at 500*g* for 6 min and resuspended in Fisher's medium (500 μl) containing 0.1% BSA, and labelled using ^{51}Cr at 1 μCi/10^6 cells (New England Nuclear) for 30 min at 37 °C to a specific activity of 0.03 c.p.m. per cell. The labelled cells were washed in 50 ml fresh medium, resuspended in Fisher's medium containing 0.1% albumin to a concentration of 1.2×10^6 cells per ml and incubated over the nitrocellulose blot using 1.0×10^6 cells cm^{-2} of nitrocellulose paper for 1 h at 37 °C. The blot was rinsed 5 times with PBS. After fixation with 5% v/v glutaraldehyde in PBS for 3 min, the blots were dried and visualized by fluorography (Kodak) for 48 h. For studies of cell morphology, the 60K band was located by comparison with standards and cut from the nitrocellulose strip without fixation and trypsinized. The cell suspension was then cytocentrifuged and stained.

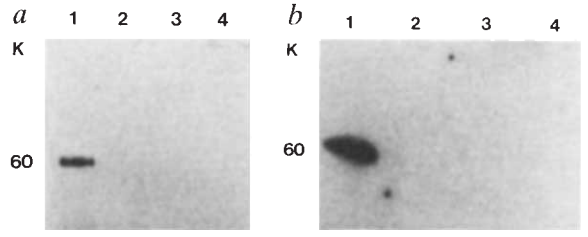

Fig. 2 Organ distribution of haemonectin. *a*, Immunoblot of bone marrow and other organ matrices using anti-haemonecton antiserum. Lane 1, bone marrow ECM; lane 2, spleen ECM; lane 3, mammary ECM; lane 4, kidney ECM. No immunological reactivity is seen in spleen, mammary or kidney ECMs. *b*, Cell attachment assay of bone marrow and non-haematopoietic tissue. Lane 1, bone marrow ECM; lane 2, spleen ECM; lane 3, mammary ECM; lane 4, kidney ECM. Attachment is seen to the 60K component of bone marrow ECM but not to components present in other ECMs.
Methods. For immunoblotting, ECMs from rabbit marrow, and rat spleen, mammary gland and kidney were prepared as described for bone marrow[1] and equal microgram quantities were separated by 10% SDS-PAGE and transferred as described. For the cell attachment assay, Western blots of equal microgram quantities of bone marrow, spleen, mammary gland and kidney ECMs were prepared, incubated with ^{51}Cr-labelled unfractionated marrow cells, and visualized by fluorography as in Fig. 1*a*.

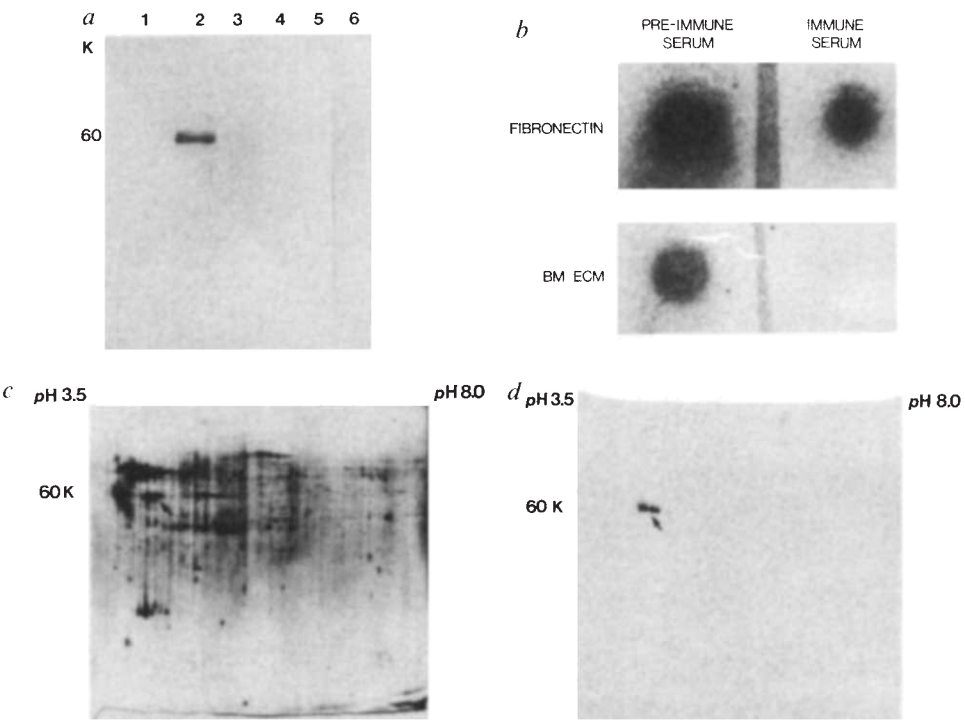

Fig. 3 Specificity of anti-haemonectin antiserum. *a*, Bone marrow ECM was compared with fibronectin, laminin, type IV collagen and vitronectin by immunoblotting using a 1:500 dilution of either pre-immune serum (lane 1) or immune serum (lanes 2–6). Lanes 1 and 2, bone marrow ECM; lane 3, fibronectin; lane 4, laminin; lane 5, type IV collagen; lane 6, vitronectin. The antibody reacts exclusively with the 60K component of bone marrow ECM. *b*, Antibody inhibition of cellular attachment. ^{51}Cr-labelled marrow cells were incubated with fibronectin or whole marrow ECM immobilized on nitrocellulose in the presence of pre-immune serum or anti-haemonectin antiserum (1:100). Adhesion is detected by fluorography. Little inhibition of adhesion to fibronectin is seen, but adhesion to marrow ECM is abolished by immune but not pre-immune serum. *c*, Two-dimensional gel electrophoresis, silver stain. *d*, Duplicate two-dimensional gel stained with anti-haemonectin by the immunoperoxidase technique as in Fig. 3*a*; anti-haemonectin recognizes only a single group of 4 closely spaced isoforms migrating at M_r 60K and *p*I 4.5, denoted by arrows.

Methods. Bone marrow ECM prepared as described, vitronectin (10 μg) (Calbiochem) and 50 μg each of human fibronectin (Collaborative), laminin (prepared as described[17]) and collagen type IV (ref. 18) were separated by 10% SDS-PAGE and transferred to nitrocellulose. The non-specific antibody-binding sites were blocked. The blot was then incubated with primary antiserum (pre-immune or immune) in a 1:500 dilution in TBS (Tris-buffered saline)-Blotto for 2 h at 20 °C. The anti-haemonectin antiserum was prepared by cutting the 60K band from a preparative gel, mixing 1:1 with complete Freund's adjuvant and injecting into the footpads of a guinea pig. The animal was boosted at week 3 and bled at week 4. The blot was then stained using a peroxidase-linked second antibody as described[14]. Antibody was used to block cellular attachment to fibronectin (10 μg) or whole bone marrow ECM, immobilized on nitrocellulose paper and incubated with ^{51}Cr-labelled bone marrow cells as above in the presence of either pre-immune guinea pig serum (1:100) or anti-haemonectin antiserum (1:100) for 1 h at 37 °C. The blots were then rinsed and visualized by fluorography. Two-dimensional gel electrophoresis was performed as described[19].

Removal from the nitrocellulose and Romanowski staining confirmed that more than 90% of the cells had granulocyte morphology (Table 1).

To investigate the relationship of haemonectin with other ECM adhesion proteins, anti-haemonectin antiserum was raised in a guinea pig. The specificity of the antiserum was demonstrated by an immunoblot which showed reaction with the 60K component from bone marrow, but not with the defined ECM components fibronectin, laminin, type IV collagen, or vitronectin (Fig. 3*a*). The anti-haemonectin antibody also blocked cell attachment to the bone marrow ECM. Adhesion of radiolabelled bone marrow cells to fibronectin was minimally affected by anti-haemonectin antiserum but adhesion to bone marrow matrix was abrogated (Fig. 3*b*). We conclude that haemonectin is the major granulocyte attachment protein in our marrow ECM preparation and is immunologically unrelated to the defined ECM adhesion proteins fibronectin, laminin, type IV collagen and vitronectin. We further characterized haemonectin by two-dimensional gel electrophoresis and immunoblotting of marrow ECM and found (Fig. 3*c* and *d*) that anti-haemonectin recognizes only a single group of four closely spaced isoforms migrating at M_r ~60K and *pI* ~4.5.

As granulopoiesis takes place primarily in bone marrow, we studied the organ distribution of haemonectin. Haemonectin could not be detected in matrix preparations from spleen, mammary gland and kidney when examined by immunoblotting (Fig. 2*a*, lanes 2–4). This organ distribution was confirmed using the cell-attachment assay (Fig. 2*b*): cell attachment occurred only at the 60K band in bone marrow ECM and there was no attachment to any components in the matrices of the other tissues. Indirect immunofluorescence with anti-haemonectin antiserum was used to study the tissue distribution of haemonec-

Table 1 Morphology of haemonectin-adherent cells

	Granulocytic (%)			Erythrocytic (%)		
	Immature*	Mature†	Total	Immature‡	Mature§	Total
Adherent cells	38	53	91	1	4	5
Input cells	30	27	57	4	31	35

Differential counts of attached and unfractionated input marrow cells. For this morphological quantitation, cells adhering to the 60K band were removed by treatment with trypsin, cyto-centrifuged and studied under Wright staining. Cells were identified using standard morphological criteria for murine haematopoietic cells[11].

* Myeloblasts, progranulocytes and myelocytes.

† Metamyelocytes, bands, and PMNs.

‡ Proerythroblasts and early erythroblasts.

§ Intermediate and late erythroblasts. The remaining cells, constituting less than 5% of the total, had lymphocytic morphology, or were not identified.

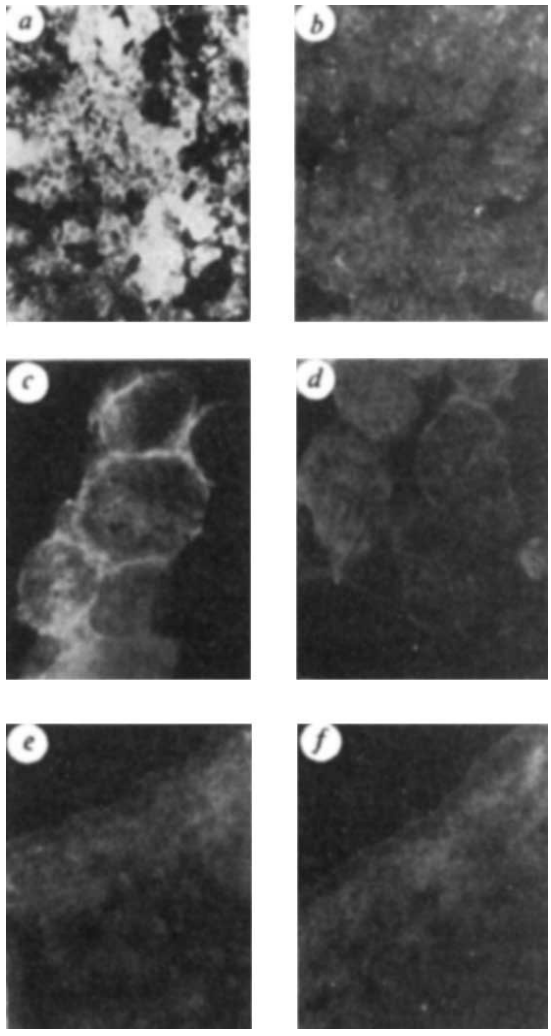

Fig. 4 Immunofluorescent localization of haemonectin *in vivo*. *a*, Bone marrow, anti-haemonectin; *b*, bone marrow, pre-immune serum; *c*, bone spicules, anti-haemonectin; *d*, bone spicules, pre-immune serum; *e*, spleen, anti-haemonectin; *f*, spleen, pre-immune serum. Note positive staining in bone marrow and endosteal surface of bone spicules.

Methods. Bone marrow was flushed from a C57/BL6 mouse femur with PBS and centrifuged at 500*g* for 6 min. The pellet was snap-frozen in liquid nitrogen, and stored at −70 °C. For spleen tissue, C57/BL6 murine spleen was cut into small pieces and snap-frozen. Cryostat sections (8 μm thick) were mounted on polylysine-coated slides. For immunofluorescent staining the sections were fixed with 1% paraformaldehyde in PBS, *p*H 7.4, for 15 min at 20 °C then washed with 0.1 M glycine in PBS, *p*H 7.4, 5 times for 5 min. The sections were then incubated with immune or pre-immune serum diluted 1:10 in PBS containing 0.1% albumin for 1 h, then with a 1:60 dilution of rhodamine isothiocyanate-conjugated goat anti-guinea pig IgG (Cooper Biomedical) for 1 h at 20 °C. After rinsing, the sections were covered with PBS containing 20% glycerol, coverslipped and viewed with an epi-illuminated fluorescence microscope. Equal exposure times were used for immune and pre-immune stained sections.

tin *in vivo*: sections of murine bone marrow stained with anti-haemonectin gave specific fluorescence in the pericellular and extracellular matrix with some variation in intensity (Fig. 4*a*). In addition to staining around haematopoietic cells, bright specific fluorescence was seen on the outer (endosteal) surface of bone spicules (Fig. 4*c*). No specific fluorescence was seen in the spleen sections (Fig. 4*e*) and little or no fluorescence was seen in sections stained with preimmune sera (Fig. 4*b*, *d*, *f*). Thus, by immunoblotting cell attachment assay and immunofluorescence, we have shown that haemonectin is found only in bone marrow.

Bone marrow ECM therefore contains an adhesive protein of M_r ~60K and *pI* ~4.5, which is immunologically distinct from fibronectin, laminin, type IV collagen and vitronectin, has lineage specificity for granulocytes, and is limited to bone marrow; these features distinguish haemonectin from previously described attachment proteins, such as fibronectin, which has been implicated in the adhesion of erythroid cells[4,5]. The lack of fibronectin-mediated attachment found in our cell attachment assay could result from lesser binding affinity between fibronectin and erythroid cells, as compared to haemonectin-granulocytic cell binding, because under the less stringent conditions of the adhesion inhibition experiment (Fig. 3*b*) fibronectin-mediated attachment was evident. Similarly, the lack of detectable cell binding to ECM components other than to a haemonectin confirms the findings of Giancotti *et al.*[7], who found that haematopoietic cells do not attach to laminin, types I, III and IV collagens, sternal cartilage proteoglycan, or serum-spreading factor (vitronectin).

Components of the microenvironment are responsible for the granulocyte predominance of bone marrow haematopoiesis and erythroid predominance of spleen[8]. Because of its specificity for granulocyte cells and its location in bone marrow, haemonectin could explain the granulocyte predominance of haematopoiesis in this tissue. Also, the location of haemonectin could account for the homing of donor haematopoietic cells to the bone marrow in marrow transplantation. Our finding by immunofluorescence of large amounts of haemonectin along the endosteal surface of bone, an area known to contain high concentrations of stem cells[9,10], may bear upon this. We are currently studying the attachment of haematopoietic stem and progenitor cells to haemonectin. Peripheral blood granulocytes are only one tenth as efficient as marrow granulocyte precursors in binding to haemonectin (unpublished observations), suggesting that loss of binding to haemonectin may cause release of mature granulocytes into the circulation, a mechanism similar to that proposed for the release of mature erythrocytes from bone marrow fibronectin[4,5]. Our experiments indicate that the haematopoietic microenvironment contains adhesion-promoting, lineage-specific ECM components in addition to the soluble factors and stromal cells previously described, and that these may be important in haematopoietic growth and differentiation.

We thank Dr Samir Hanash for technical assistance with two-dimensional gel electrophoresis and Drs Stephen Weiss, Mary Zeigler and Joanne Blum for discussion. This work was supported by the Veterans Administration, NIH, the American Cancer Society and the American Heart Association. M.W.L. is a Scholar of the Leukemia Society of America.

Received 15 June; accepted 9 September 1987.

1. Campbell, A. D., Long, M. & Wicha, M. S. *J. clin. Invest.* **75,** 2085–2090 (1985).
2. Zuckerman, K. S. *et al. J. clin. Invest.* **75,** 970–975 (1985).
3. Spooncer, E., Gallagher, J. T., Krishna, F. & Dexter, T. M. *J. Cell Biol.* **96,** 510–514 (1983).
4. Patel, V. P. & Lodish, H. F. *Science* **224,** 996–998 (1984).
5. Patel, V. P. & Lodish, H. F. *J. Cell Biol.* **102,** 449–456 (1986).
6. Zuckerman, K. S. & Wicha, M. S. *Blood* **61,** 540–547 (1983).
7. Giancotti, F. G., Comoglio, P. M. & Tarone, G. *J. Cell Biol.* **103,** 429–437 (1986).
8. Wolf, N. S. & Trentin, J. J. *J. exp. med.* **127,** 205–214 (1968).
9. Lord, B. I., Testa, N. G. & Hendry, J. H. *Blood* **46,** 65–72 (1975).
10. Carsten, A. L. & Bond, V. P. *Nature* **219,** 1082–1084 (1968).
11. Chervenick, P. A., Boggs, D. R., Marsh, J. C., Cartwright, G. E. & Wintrobe, M. M. *Am. J. Physiol.* **215,** 353–360 (1968).
12. Laemmli, U. K. *Nature* **227,** 680–685 (1970).
13. Morrissey, J. H. *Analyt. Biochem.* **117,** 307–310 (1981).
14. Towbin, H., Staehelin, T. & Gordon, J. *Proc. natn. Acad. Sci. U.S.A.* **76,** 4350–4355 (1979).
15. Clarke, S. *Biochim. biophys. Acta* **670,** 195–202 (1981).
16. Johnson, D. A., Gautsch, J. A., Sportsman, J. R. & Elden, J. H. *Gene analyt. Tech.* **1,** 3–8 (1984).
17. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. *J. biol. Chem.* **252,** 1102–1106 (1977).
18. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I. & Foidart, J. M. *J. biol. Chem.* **254,** 9933–9939 (1979).
19. Hanash, S. M., Baier, L. J., McCurry, L. & Schwartz, S. A. *Proc. natn. Acad. Sci. U.S.A.* **83,** 807–811 (1986).