HEMCAM, an Adhesion Molecule Expressed by c-kit$^+$ Hemopoietic Progenitors

O. Vainio,* D. Dunon,‡ F. Aïssi,‡ J.-P. Dangy,* K.M. McNagny,§ and B.A. Imhof*

*Basel Institute for Immunology, CH-4058 Basel, Switzerland; ‡Laboratoire de Biologie Moléculaire et Cellulaire du Développement, URA-CNRS 1135, Universite Pierre et Marie Curie, Paris, France; and §European Molecular Biology Laboratory, Heidelberg, Germany

Abstract. We have characterized the adhesion molecule HEMCAM, which is expressed by hemopoietic progenitors of embryonic bone marrow. HEMCAM belongs to the immunoglobulin superfamily and consists of the V-V-C2-C2-C2 Ig domains. There are three mRNA splice variants. One has a short cytoplasmic tail; another has a long tail; while the third seems to lack transmembrane and cytoplasmic regions. Except for the NH_2-terminal sequence, HEMCAM is identical to gicerin, a molecule involved in neurite outgrowth and Wilm's kidney tumor progression in the chicken and it is significantly homologous with MUC18 a molecule involved in melanoma progression and metastasis in human beings.

In the bone marrow the HEMCAM$^+$ cell population contains c-kit$^+$ subsets. HEMCAM$^+$ cells coexpressing the receptor tyrosine kinase c-kit give rise to T cells at a frequency of 0.17 when injected intrathymically in congenic animals. As HEMCAM$^+$, c-kit$^+$ cells differentiate into myeloid and erythroid CFU's the double-positive cell population seems to contain precursors for multiple lineages. HEMCAM promotes cell–cell adhesion of transfected cells. Cross-linking of murine HEMCAM leads to cell spreading of T-lymphocyte progenitors adhering to the vascular adhesion molecules, PECAM-1 and VCAM-1. Thus, HEMCAM is likely to be involved in cellular adhesion and homing processes.

A large proportion of immunoglobulin superfamily (IgSF)[1] molecules serve as adhesion receptors on the cell surface. In the recent literature there has emerged a prominent subgroup with a characteristic V-V-C2-C2-C2 Ig domain structure. Important representative members of this subgroup of molecules are B-CAM (human), Lutheran blood group antigen (human), BEN/DM-GRASP/SC1 (chicken), ALCAM (human), KG-CAM (rat), irreC-rst (drosophila), Neurolin (goldfish), Gicerin (chicken), and MUC18 (human) (3–5, 26, 28, 38, 41, 45, 47, 57, 60). Although most of these molecules are involved in adhesion and migration processes of neural cells, several of them are also found directly or indirectly associated with cells of the immune system, with epithelial cells, and/or with vascular endothelium.

BEN is expressed by embryonic hemopoietic progenitor cells from the bone marrow and immature thymocytes, but it is absent from most mature leukocytes (8). BEN-positive bone marrow cells develop macrophage, granulocyte, thrombocyte, and erythrocyte colonies when allowed to differentiate in vitro in the presence of appropriate factors. Mature resting peripheral T-lymphocytes are virtually negative for BEN expression, however this can be overcome by mitogenic or allogeneic stimulation of these cells; thus BEN is a T cell activation marker (7). ALCAM (activated leukocyte-cell adhesion molecule), the human homologue of BEN, has recently also been identified as an adhesion ligand for CD6 (3) that functions as an accessory protein in T cell activation; antibodies against CD6 can prevent transplant rejection of kidney and bone marrow (2, 25, 48).

Gicerin is transiently expressed in the developing nervous system and kidney (57, 58). Cloning and transfection experiments have demonstrated that the adhesive interactions mediated by gicerin include homophilic (gicerin-gicerin) or heterophilic contacts between gicerin and neurite outgrowth factor (NOF), a 700-kD extracellular matrix glycoprotein of the laminin family (18). Antibodies against

Please address all correspondence to O. Vainio, Department of Microbiology, Turku University, Kiinamyllynkatu 13, SF-20520 Turku, Finland. Tel.: 358 2 333 7400. Fax: 358 2 233 0008; or B.A. Imhof, Centre Medical Universitaire, Department of Pathology, Geneva University, 1 rue Michel-Servet, CH-1211 Geneva, Switzerland. Tel.: 41 22 702 5745. Fax: 41 22 702 5746.

1. *Abbreviations used in this paper*: 2-ME, 2-mercaptoethanol; cMGF, chicken myelomonocytic growth factor; HEMCAM, hemopoietic cell adhesion molecule; IgSF, immunoglobulin superfamily; NOF, neurite outgrowth factor; RT, room temperature; SCF, steam cell factor.

mouse laminin react with the 215-kD and 195-kD B type chains but not with the 330-kD putative A chain of NOF (61, 63). Immunohistochemical analysis suggests that both homophilic and heterophilic gicerin adhesion may be of importance during ontogeny of the kidney (58). After kidney development has been completed, gicerin expression is suppressed in most cell types in kidney. As neoplastic renal tissue re-expresses gicerin, it is a potential indicator for tumor tissue (58). Such de novo expression by tumors has also been found for MUC18, another V-V-C2-C2-C2 molecule (28). MUC18 expression is gradually increased during tumor progression of human melanomas, and its adhesive property might be involved in tumor cell migration and metastasis.

To initiate T cell differentiation, hemopoietic stem cells must migrate to the thymus (37, 39). Several studies have shown that hemopoietic progenitors are transported via the blood to the vicinity of the thymus when adhesion molecules result in the attachment to the thymic endothelium. Both α6 integrin and CD44 expressed on vascular endothelium are involved in this process (10, 51). Extravasation and migration of progenitors in the perivascular space through the perithymic mesenchyme towards the thymic epithelium requires interaction with extracellular matrix molecules (52). Another adhesion step between the progenitor T cell and the thymic epithelium is thought to occur before the progenitor cell enters the thymus. Identification of the adhesion molecules involved in thymic homing requires the isolation of hemopoietic progenitor cells from the tissue in which they are most abundant.

Attempts to characterize hemopoietic stem cells have been made with mouse and human tissue. In the adult mouse, bone marrow stem cells, which can reconstitute all hemopoietic cell lineages, are of the phenotype Thy-1^{lo} Sca-1^{+} and lineage marker negative (lin^{-}) (20). A similar stem cell population was isolated from human bone marrow although the type of marker molecules was different ($CD34^{+}$, lin^{-}) (20). In both cases, the proportion of stem cells to total leukocytes is very low (<0.05%)(20) although it is significantly increased in embryonic tissue. The bone marrow and the blood of human embryos contains $CD34^{+}$ cells that give rise to lymphocytes in SCID mice with human thymus transplants (40). From mouse blood at embryonic day 15.5 (E15.5), it was recently possible to separate pro-thymocytes (Thy-1^{+}, c-kit^{lo} and $CD3^{-}$ cells) from pluripotent hemopoietic progenitors (Thy-1^{-}, c-kit^{+}) (49). With birds, unlike mammals, one can obtain experimental access to precisely staged embryos. Hemopoietic stem cells emerge in chicken bone marrow as early as E10, and the percentage of pro-T cells that can migrate to the thymus peaks at E13 (11). In the present work, we measured the T cell differentiation potential of hemopoietic progenitors by intrathymic injection into congenic animals. Using this method, we show that an E13 embryonic bone marrow population expressing c-kit and the antigen recognized by a monoclonal antibody (mAb) c264 gives rise to T cells at frequencies of 0.17. The mAb c264 facilitated the cloning and sequencing of the antigen which we call hemopoietic cell adhesion molecule (HEMCAM). This adhesion protein of the V-V-C2-C2-C2 IgSF subgroup was highly homologous to the human melanoma progression molecule MUC18 and gicerin.

Materials and Methods

Animals

Embryonated eggs from H.B19 strain of White Leghorn chickens were produced at the Basel Institute Chicken Facility at Gipf-Oberfrick (Switzerland). Fertilized eggs were incubated at 38°C and 80% humidity in a ventilated incubator. The two congenic lines, H.B19ov^{+} and H.B19ov^{-}, differ in the expression of the ov antigen on thymocytes and T cells. The ov antigen is recognized by mAb 11A9 (11, 12, 19).

Monoclonal Antibodies

A hybridoma producing mAb c264 (IgG2b) was obtained after fusion of Sp2/0 myeloma cells with lymph node cells from a BALB/c mouse immunized with a mixture of E15 and E16 thymocytes from H.B15, H.B19, and H.B21 chicken embryos as previously described (31). Anti-HEMCAM antibody c236 (IgG2a) which works better for histochemistry and immunoprecipitation than c264 was made against soluble HEMCAM protein. To obtain a chicken c-kit–specific mAb, a mouse was immunized with Sp2/0 cells, Sp/chkit4A4, transfected with chicken c-kit cDNA. Other mAb have been described earlier: anti–Bu-1a, a B cell marker (L22) (44), anti-CD4 (2-6) (31), anti-CD8 (11-39) (30), anti-ov (11A9) (19), anti-γδ TCR (TCR1), anti-αβ1 TCR (TCR2), anti-αβ2 TCR (TCR3), and anti-CD3 (CT3) were purchased from Southern Biotechnology (Birmingham, AL). FITC (5(6)-Carboxyfluorescein-*N*-hydroxysuccinimidester [FLUOS]; Boehringer Mannheim, Germany), biotin (Biotin-*N*-Hydroxysuccinimidester, NHS-D-Biotin; Calbiochem, La Jolla, C A) and phycoerythrin (R-Phycoerythrin, Molecular Probes, Leiden, The Netherlands) conjugation of mAb were done in our laboratory according to the manufacturers' instructions.

Histochemistry

Our indirect method uses peroxidase as the detection reagent. Frozen tissue sections (5 μm) were fixed in acetone for 5 min at room temperature (RT), rehydrated with PBS containing 1% bovine serum albumin (PBS/BSA), and loaded with antibody c236 (hybridoma supernatant). After 60 min incubation at RT, the sections were washed twice with PBS/BSA and incubated for 30 min with goat anti–mouse IgG (H+L) antibody coupled to biotin (dilution 1:400; Southern Biotechnology). The biotin indirectly bound to the antigen was detected by a bridging technique using a commercial kit (ABC standard, PK 6100; Vector Labs , Burlingame, CA). Briefly, avidin was allowed to bind to the biotinylated secondary antibody, and the remaining free-binding sites of ligated biotin were used by biotinylated peroxidase. This indirect labeling intensifies the signal without increasing background staining. Labeling was developed for 30 min by 3, 3'-Diaminobenzidine (0.05% in 50 mM Tris buffer, pH 7.5; both from Sigma Chem. Co., St. Louis, MO) after addition of 0.005% H_2O_2. The contrast of the immuno-staining was further intensified by exposing the section to a solution of 0.5% $CuSO_4$/0.9% NaCl for 10 min. Tissues were counterstained with Meyer's hemalum solution (Merck, Darmstadt, Germany). Sections were mounted in Mowiol and analyzed under a Zeiss Axiophot microscope equipped with a CCD video camera (Zeiss, Oberkochen, Germany) and a Sony videoprinter UP-5000P.

Immunoprecipitation

Thymocytes (5×10^7, viability >95%) were surface-labeled with 1 mCi $Na^{125}I$ (Amersham, Buckinghamshire, UK) by the lactoperoxidase catalyzed method (22). The cells were lysed in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% NaN_3, 1 mM pefabloc (Boehringer Mannheim), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml trypsin inhibitor, 20 mM ε-amino-*n*-caproic-acid, 2 μg/ml antipain, 20 mM iodoacetamide (all reagents from Sigma), and 1% NP-40 (Calbiochem) for 45 min at 4°C. After centrifugation (10,000 *g*, 45 min at 4°C), the lysate was immunoprecipitated by a solid phase method (SPIT) (59), and absorbed molecules were eluted with 1% SDS in 50 mM Tris-HCl with 5% 2-mercaptoethanol (2-ME). For glycolytic enzyme digestions, the immunopreciptates were eluted with 0.5% SDS, 0.1 M 2-ME for 20 min at 80°C. The reaction conditions were adjusted according to manufacturer's instructions and the immunoprecipitates were incubated overnight at 37°C in the presence of 50 U/ml *N*-glycanase (Genzyme, Boston, MA). Undigested controls were treated similarly but without the addition of enzymes. Samples were prepared for electrophoresis by addition of 6× sample buffer containing 2-ME. Immunoprecipitates were electrophoresed on 7.5% SDS PAGE.

Immunofluorescence and Cell Sorting

For single, two-, or three-color analysis cells (10^6) were incubated with hybridoma supernatants or purified mAb, washed, and incubated with FITC-conjugated anti-mouse Ig isotype specific antibodies (Southern Biotechnology). After washing, the cells were blocked with normal mouse serum and then stained with PE-conjugated mAb. For three-color analysis the cells were washed again and incubated with a biotin-conjugated mAb followed by streptavidin-Tricolor reagent (Caltag Labs, South San Francisco, CA). A FACScan was used for immunofluorescence analysis, a FACStar Plus (Becton Dickinson, Mountain View, CA) was used for sorting.

Intrathymic Injection and Differentiation of Embryonic Bone Marrow Cells

To prepare bone marrow cells from congenic ov^+ E13.5 donor animals, cells were flushed from the cavity of the isolated femurs and tibiae with a 25G 5/8-inch long syringe containing medium (DMEM containing 10% FCS), washed twice in PBS, counted, and adjusted to the required cell concentration (11). The recipient, 14-day-old ov^- congenic chicks were irradiated with 600 rad from a ^{137}Cs source (110 rad/min) (Gamma Cell Irradiator, Atomic Energy of Canada, Ottawa, Canada) about 6 h before receiving the donor bone marrow cells. Before intrathymic injection, the recipients were anesthetized with an i.m. injection of 0.4 ml ketamine (Imalgene 500, Rhone Merieux, France), diluted 1:10 in PBS followed by a short inhalation of halothane (HOECHST, Frankfurt, Germany). A midline incision was made in the skin on the dorsal side of the neck to expose the upper thymic lobes on each side. The donor bone marrow cells were injected into two upper lobes on each side. Each lobe was injected with 10 μl cell suspension in PBS (3–6 × 105 cells/thymus lobe) in a 1-ml syringe (Insulin syringe, Becton Dickinson, San Jose, CA) placed into a Tridek Stepper (Tridek, Brookfield, CT). The incision was closed with three wound clips (Autoclip, Clay Adams, Becton Dickinson Primary Care, Sparks, MD). After the operation, the chickens were kept under an infrared lamp until they were fully conscious. The animals recovered rapidly and no special care was necessary for housing. 2–4 wk after chimera construction the chickens were sacrificed and cells from the injected thymus lobes isolated. Donor-derived cells were identified by immunofluorescence with the mAb 11A9 (isotype IgM) directed against the ov^+-antigen.

cDNA Cloning and Sequencing

mRNA from E13.5 H.B19ov^+ thymocytes was prepared by a FastTrack mRNA isolation kit (Invitrogen, De Schelp, Netherlands). A library of cDNA was constructed from 15 μg of the mRNA in the COS cell expression vector pCDA3 (Invitrogen). The library consisted of 2.2×10^6 primary clones, was amplified once, and had an average cDNA size of 1.0 kb. Transfection of adherent COS.7 cells with the library and screening of the transfected cells with mAb was done as described earlier (64). Briefly, COS.7 cells grown in a 75-cm^2 tissue culture flask (Falcon 3111; Becton Dickinson, Franklin Lakes, NJ) at ~80% confluence were trypsinized by incubation in PBS/0.02% EDTA/0.25% trypsin and 4×10^5 cells were transfected in 3 ml Hepes-buffered DMEM (HBD) containing 500 μg/ml DEAE-dextran, 100 μM chloroquine (Sigma), and 3 μg DNA for 2 h at 37°C. The cells were washed in HBD, cultured on tissue culture slides (Lab-Tek, Chamber Slide, Nunc, Naperville, IL) in DMEM/10% FCS for 48 h, rinsed in PBS, fixed on culture slides with ice-cold acetone/methanol (1/1 vol) for 2 min, washed twice with PBS, and blocked with PBS/5% FCS for 30 min followed by 1 h incubation with c264 mAb. The slides were washed three times with PBS/0.05% Tween 20 (PBST) followed by a 1-h incubation with peroxidase-conjugated goat anti-mouse Ig (DAKO, Glostrup, Denmark) diluted 1:200 in PBS/5% FSC. Enzyme activity was visualized with a 1:20 dilution of a 4-mg/ml stock of 9-amino-3-ethylcarbazol in *N,N*-dimethylformamide in 0.1 m NaAc (pH 4.8) containing 0.1% H_2O_2 for 30–60 min. Slides were washed and screened under an inverted microscope for positive cells, which were scraped into a small drop of water and picked with a hand-held pipette (Drummond, 509, Scientific Company, Broomall, PA). Individual picked cells were treated with 200 μl proteinase K (100 μg/ml) in 0.6% SDS and 10 mM EDTA for 30 min at 37°C, and a Hirt extraction was performed. Plasmid DNA was precipitated and transformed into *E. coli* strain Top 10F' by electroporation. Plasmids from pools of 10 colonies were recovered on spinbind columns (QIAprep Spin Plasmid Kit, Qiagen, Chatsworth, CA) and retransfected into COS.7 cells. A mAb c264-reactive cDNA pool was identified, and after another round of electroporation and retransfection a single plasmid clone, pc264, was isolated. Sequenase (Version 1.0; USB, Cleveland, OH) was used for sequencing pc264 on both strands. The cDNA sequence has been submitted to the EMBL database with accession numbers YO8854 (soluble form), YO8855 (long cytoplasmic form), and YO8856 (short cytoplasmic form). A search in the EMBL data base showed that this sequence corresponded to a recently isolated cDNA coding for gicerin. However, our sequence differed from the gicerin sequence at the 5′ and 3′ extremities (57, 58). At position 310 of gicerin sequence, one C is replaced by one T changing a proline in leucine. At position 326, one C was missing whereas at position 466 another C was inserted changing the reading frame between amino acid residues 43 and 77. To confirm our cDNA sequence, we sequenced RT-PCR products from thymocytes and we determined the exact NH_2-terminal amino acid sequence of the c264 protein. By microsequencing, the 28 NH_2-terminal residues were determined to be RLEVYMPAVLEVEIGSTARLECSFSIPG. At position 1644 and 1754 of the gicerin sequence, one G and one T are replaced by one A and one C, respectively. The CITI 2 (Paris, France), UWGCG (Genetics Computer Group, Madison, WI), and Prosite pattern search (EMBL, Heidelberg) software packages were used for the analysis of the sequence data. Assignment of the HEMCAM immunoglobulin domains was performed using the rules as described (1).

Semiquantitative PCR

From day 9 onwards embryonic tissues were carefully microdissected and total RNA was prepared. For cDNA synthesis, standard protocols and universal primers were used (51). Semiquantitative PCR was done as described by Keller et al. (24, 51) and Ruiz et al. (24, 51). The amount of cDNA synthesized was calibrated from the relative expression level of β actin as a standard. The two actin oligonucleotide primers, 4611 and 4612, generated a band of 646 nucleotides (13). A series of 10-fold dilution was used to standardize the cDNA. The oligonucleotides covering the coding sequence of HEMCAM were: 11169 (HEMCAM, from nucleotide 195) ATGGCTGGGGGGCGACGGGCG; 11260 (HEMCAM, from nucleotide 609) GGAGAGCCGCACCGAGCTCTACACC; 11261 (HEMCAM, from nucleotide 634, antisense) GGTGTAGAGCTCGGTGCGGCTCTCC; 11188 (HEMCAM, from nucleotide 1097) GGAGAGAGCTGGGGGACAGCTGG; 11106 (HEMCAM, from nucleotide 1363, antisense) GCTTGACGGGGCTGTGGGC; 11898 (HEMCAM, from nucleotide 1597) TGCAAGGCCATCGCCTTC; 11902 (HEMCAM, from nucleotide 2100, antisense) AAGCACTTAGGAGATCGC; GIC1 (HEMCAM, from nucleotide 2448, antisense) AAAAGAAACGTCCCATCAGC; 4611 (5′ of actin, from nucleotide 3598) TACCACAATGTACCCTGGC; and 4612 (3′ of actin, from nucleotide 4246, antisense) CTCGTCTTGTTTTATGCGC. PCR reaction mixtures in 30 μl with 1 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) were denatured by heating to 96°C for 5 min, and then subjected to 30 rounds of amplification using a Biometra Protocol thermocycler under the following conditions: 96°C for 15 s, 50°C for 40 s, and 72°C for 1 min for cDNA amplification. Final extension was done at 72°C for 10 min. Amplified DNA fragments were gel purified and cloned into pCR II (Invitrogen). Sequences were determined from denatured double-stranded recombinant plasmid DNA using Sequenase (USB) in the chain termination reaction.

Generation of Soluble Recombinant HEMCAM

The vector pCDA3 containing the cloned HEMCAM cDNA was used as a PCR template for the amplification of the sequence encoding the extracellular domains of HEMCAM. The PCR primers were: 5′-ATTAGAGCTCACCATGGCTGGGGGGCGACGGGC-3′ (5′ primer) and 5′-TATACTTACCTCCTTTGCTCTCCGATGGCTTTTG-3′ (3′ primer). The amplified product was purified and inserted into the vector pHT4 containing the mouse Igκ constant region as a fusion partner, and the junctional regions were analyzed by nucleotide sequence analysis. The vector was cloned into competent *E. coli* K803 cells then transfected into J558L myeloma cells by protoplast fusion. Supernatants of the transfected mycophenolic acid and xanthine resistant cell clones were screened by ELISA with goat anti-mouse cκ reagents (Southern Biotechnology). Supernatants were purified by affinity chromatography with a monoclonal rat anti-mouse Cκ antibody (clone 187.1). The purified molecules were equilibrated with PBS and used for NH_2-terminal amino acid sequencing and for immunizations of mice in order to produce mAb. For sequencing, the protein was transferred to Immobilon-P membrane (Millipore, Bedford, MA) and loaded into the cartridge of 475A protein sequencer (Applied Biosystems, Foster City, CA).

Polyclonal Antibody

Soluble recombinant HEMCAM protein was injected into a rabbit at 100 μg in complete Freund's adjuvant followed by three injections of 100 μg protein each in incomplete adjuvant. The specific serum, but not the pre-immune serum, reacted with chicken thymocytes and HEMCAM-transfected L cells at a dilution of 1:3,000, and it cross-reacted with mouse pro-T lymphocytes at 1:1,000. The polyclonal antibodies were purified on protein A columns, and anti-mouse κ IgG was removed by absorbing the purified antibodies on a κ containing affinity column.

Transfection of L Cells

The liposome-mediated transfection method was used to introduce pc264 DNA into mouse L cells for stable expression of HEMCAM. A day before transfection, L cells (3×10^5 in 2 ml DMEM, 10% FCS) were plated on a six-well plate (Costar, Cambridge, MA). Plasmid DNA (5 μg) was mixed with 20 μl lipofectin (Lipofectamine, GIBCO BRL, Gaithersburg, MD) in a total volume of 100 μl H_{20} and left at room temperature for 30 min. Then, 800 μl Opti-MEM medium (GIBCO BRL) was added to the DNA-liposome mixture, and after rinsing the L cells once with prewarmed Opti-MEM medium, the mixture was overlayed on them and incubated at 37°C 5% CO_2 for 5 h. After incubation 1 ml DMEM, 20% FCS was added to the well. At 24 h after transfection, the medium was replaced with fresh DMEM, 10% FCS. At 72 h after transfection, the cells were trypsinized and plated at a 1:20 dilution into 96-well plates in DMEM, 10% FCS, and 1 mg/ml G418 (Geneticin, GIBCO BRL). Growing G418-resistant clones were tested for HEMCAM surface expression by flow cytometry.

Progenitor Cell Assays

Plasma clot assays for colony forming units were performed as described previously (9). Briefly, sorted cells were seeded in 1.2 ml of "Media Mix" (33) composed of DMEM (GIBCO BRL), 10% FCS (Sigma), 2.5% chicken serum (Sigma), 0.15% $NaHCO_3$, 56 μg/ml conalbumin (Sigma), 80 mM 2-mercaptoethanol, 0.9 μg/ml insulin, and penicillin-streptomycin (GIBCO BRL). 120 μl of citrated bovine plasma (GIBCO BRL) and 10 μl thrombin (100 i.u./ml, Sigma) were added rapidly, and two 600-μl aliquots from this mixture were seeded in a 24-well tissue culture plate (Nunc). Cultures were incubated for 3 d at 37°C, harvested, and air dried onto microscope slides according to the methods described previously for collagen cultures (27). Slides were stained for hemoglobin using diaminobenzidine (32), and counterstained with May-Grünwald-Giemsa; colony types were assessed by morphology. In some experiments, cultures were supplemented with anemic serum (46) as a source of erythropoietin, chicken myelomonocytic growth factor (cMGF) (29), or stem cell factor (SCF) (55). Liquid culture experiments were performed as above, but bovine plasma and thrombin were omitted. The frequence of eosinophils and erythroid cells in liquid culture experiments were determined by sensitive, in situ stains for eosinophil-peroxidase (15, 23) and hemoglobins (36).

Cell Aggregation and Spreading

Adherent HEMCAM-transfected L cells were rapidly trypsinized and removed from culture flasks by moderately vigorous pipetting with PBS. Cells were aggregated at 2×10^6 cells/ml in DMEM (GIBCO BRL) containing 10% FCS at 37°C in popypropylene tubes (Falcon, Becton Dickinson, Basel, Switzerland). Particles were enumerated at intervals in aliquots taken after gently turning the tube in order to resuspend the sedimented aggregates. Aliquots were taken by pipettes with widened tips in order to avoid shear forces during aggregate collection. Aggregates were counted in a Malassez chamber. Untransfected L cells were labeled by the fluorescent dye BCECF/AM (Calbiochem, San Diego, CA) by adding 2 μl stock solution (1 mg/ml in DMSO) to 1 ml cell suspension in full medium and incubated at 37°C for 20 min. After one wash the cells were ready to use for aggregation.

Purified recombinant HEMCAM, PECAM-1, and VCAM-1 were directly coated onto microtiter wells (Costar Corp., Cambridge, MA) at 10 μg/ml in 50 ml D-PBS for 2 h at room temperature (43). The wells were blocked with 20% BSA for 1 h at room temperature and washed with D-PBS. FTF1 mouse pro-T cells (a generous gift of R. Palacios, Houston, TX) were loaded in full medium (42) to the coated wells. The plates were then put under a microscope (Zeiss) equipped with a heated humid chamber at 37°C and a video-recording system (Panasonic).

Results

The Antigen Recognized by mAb c264 Is Expressed on Hemopoietic and Vascular Endothelial Cells

To follow migration and differentiation of chicken T cell precursors, mAb were raised against surface molecules of embryonic thymocytes (E15). At this stage of development many pro-T lymphocytes have already migrated from the bone marrow, the site of emergence, to the embryonic thymus (6, 11). One of the antibodies, c264, was selected because it detected an antigen on embryonic bone marrow cells, spleen cells, and thymocytes (Fig. 1). In embryonic bone marrow and spleen, many cell types, including erythrocyte precursors, expressed the antigen recognized by mAb c264 (not shown). In the adult thymus, the c264 antigen is highly expressed by 80% of $CD4^-$, $CD8^-$

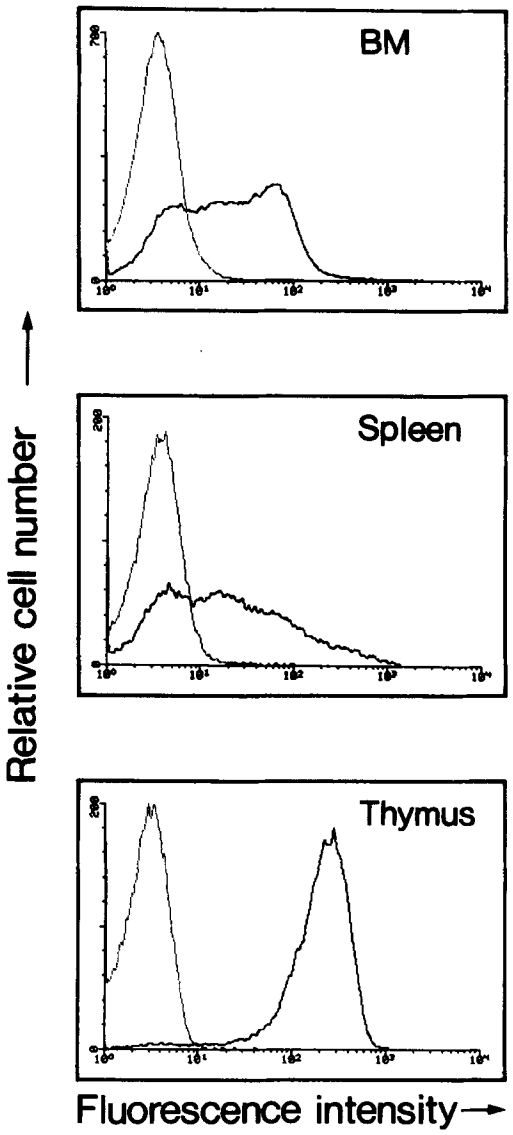

Figure 1. Distribution of $c264^+$ cells in embryonic tissues. E13.5 H.B19 bone marrow, spleen, and thymus cells were stained with mAb c264 followed by an FITC-conjugated second antibody specific for the mouse Ig isotype. The thick curves show staining with c264 antibody, the faint curves with a control antibody.

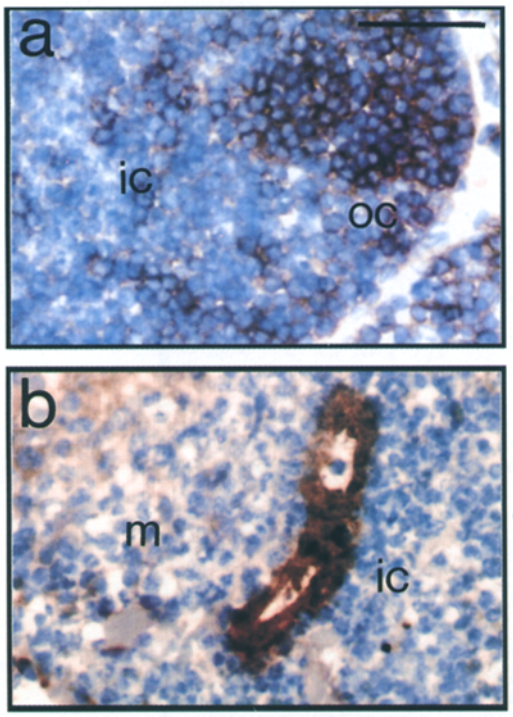

Figure 2. Immunohistochemistry of c264 antigen expression in the thymus. Frozen tissue sections of the thymus from a hatched chick were stained by mAb c238 specific for c264 antigen and best suitable for histochemistry. The bound antibody was visualized by a staining kit containing a peroxidase-conjugated reagent. The immune reaction appears in brown, the counterstaining of the tissue in blue. (*a*) The cortical region of the thymus contained c264-antigen–positive thymocytes. (*b*) Thymocytes in the corticomedullary junction were negative for c264 antigen expression but the vascular endothelium of thymic blood vessels were stained. (*ic*) inner cortex; (*oc*) outer cortex; (*m*) medulla. Bar represents 50 μm.

(double negative) as well as on all double positive thymocytes. The c264 antigen was also expressed albeit at lower levels, by $CD4^-$, $CD8^+$ cells but was absent from $CD4^+$, $CD8^-$ single positive cells (data not shown). The antigen was expressed at 100-fold lower levels, as determined by relative fluorescence intensity, on cells of the B lymphocyte lineage (data not shown). For immunohistochemistry, we used mAb c238 because it recognized the C264 antigen and it was most suitable for histochemistry. It reacted most strongly with thymocytes in the outer cortex of the thymus of newly hatched chicks (Fig. 2 *a*). Staining of cells in the inner cortex was clearly weaker, and most medullary thymocytes appeared to be c264-negative. The antigen was also expressed at high levels by vascular endothelial cells, as exemplified by thymic capillary endothelial cell staining (Fig. 2 *b*). Thus, mAb c264 seems to be specific for an antigen that is most prominently expressed on cells of the T lymphocyte lineage and on vascular endothelium. The expression of the c264 antigen was not exclusively restricted to the thymus since it was also found in other tissues such as spleen, bursa of Fabricius, gut, muscle, and brain of 3-wk-old chicks (Fig. 3). In all these tissues the antigen is expressed by vascular endothelium, although it was also found in epithelial cells of the bursa (Fig 3, *b* and *c*) and in myocytes (Fig. 3 *e*). In the brain however, only vascular endothelium was stained by the antibody (Fig. 3 *f*). Antibody c264 precipitates a molecule with an apparent molecular mass of 98 kD, under both reducing (Fig. 4) and nonreducing conditions (not shown). After treatment with N-glycanase, the core protein migrated at ~84 kD; thus, 14 kD are due to N-linked glycosylation.

Table I. Growth and Myeloid/Erythroid Differentiation Assay in Liquid Medium

Cell type \ Growth factors	SCF cMGF	SCF EPO	SCF cMGF EPO
	Cell No $\times 10^5$		
HEMCAM+/c-kit$^+$	10.5	8.7	6.5
HEMCAM+/c-kit$^-$	0.6	0.6	0.5
HEMCAM−/c-kit$^-$	0.1	0.1	0.1

Embryonic E13.5 bone marrow cells were sorted for the expression level of HEMCAM and c-kit. The sorted cells were then cultured in liquid suspension at 10^5 cells per well in the presence of hemopoietic growth factors; stem cell factor (SCF), chicken erythropoietin (EPO), and chicken myelomonocytic growth factor (cMGF). The number of cells after culturing for 5 d is indicated.

T Cell Differentiation of c264-positive Bone Marrow Cells

As the c264 antigen is highly expressed by immature thymocytes as well as by embryonic bone marrow cells, we assayed the c264 positive bone marrow cells for hemopoietic precursors able to differentiate into T cells. To this end, we

Table II. Colony Differentiation Assay in Semisolid Medium

Cell type 5×10^2	Growth factors	Type of colony							
		Mac	Gran	Mac/Gran	Myl/Mac	Myl	Ery/Thro	BFU-E	Stromal
HEMCAM$^+$/c-kit$^+$	SCF/EPO	49	23	6	9	2	26	1+	0
	SCF/cMGF	37	13	3	8	5	6	2	0
HEMCAM$^+$/c-kit$^-$	SCF/EPO	6*	1	1	0	1	2	1	0
	SCF/cMGF	5*	1	0	0	1	0	1	0
HEMCAM$^-$/c-kit$^-$	SCF/EPO	1*	0	0	0	0	0	1	1
	SCF/cMGF	1*	0	0	0	0	0	0	0

Embryonic E13.5 bone marrow cells were sorted for the expression level of HEMCAM and c-kit. These cells were placed into semisolid medium containing bovine plasma and the following growth factors; stem cell factor (SCF), chicken erythropoietin (EPO), and chicken myelomonocytic growth factor (cMGF). Cultures were incubated for 3 d, harvested, dried, stained by May-Grünwald Giemsa solutions to visualize myeloid cells, and diaminobenzidine to stain erythroid cells. Colonies were then counted and analyzed by microscopy. Colonies contained: Macrophages (Mac), Granulocytes (Gran), Macrophages and Granulocytes (Mac/Gran), Immature Myeloid cells and Macrophages (Myl/Mac), Immature Myeloid cells (Myl), Erythrocytes and Thrombocytes (Ery/Thro), Erythroid burst forming cells (BFU-E), and Stromal cells (Stromal). *small mature colonies; + immature colonies.

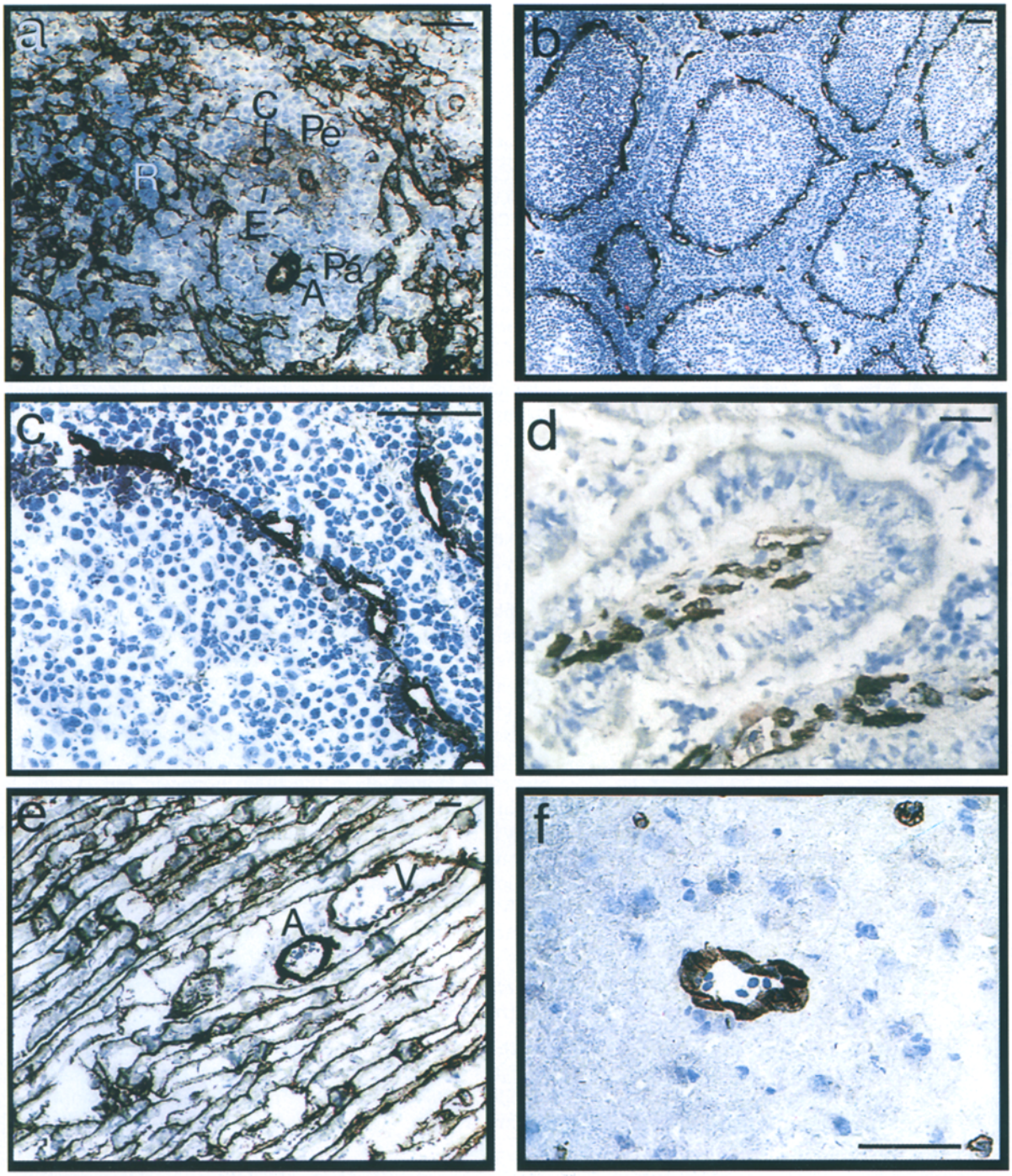

Figure 3. c264 antigen expression in various tissues. Frozen tissue sections of a 3-wk-old chick were stained by mAb c238 as described for Fig. 1. (*a*) Spleen, it showed prominent staining of blood vessels; capillaries (*C*), arterioles (*A*). The T-lymphoid tissue in periarteriolar lymphoid tissue, PALS (*Pa*), and the B-lymphoid tissue in perielipsoidal lymphoid tissue, PELS (*Pe*), are not stained. The stroma in the red pulp (*R*), mainly sinuses, are strongly stained. (*b*) Bursa of Fabricius, note the strong staining in the cortico-medullary junction. (*c*) Bursa of Fabricius at 4× higher magnification. All the cortico-medullary capillaries and the thin epithelial monolayer are stained. (*d*) Intestinal villus, the blood vessels in the lamina propria are stained. (*e*) Skeletal muscle, the arterioles (*A*), and the venules (*V*) are strongly stained. All plasma membranes of myocytes are stained (*f*). Brain, all the blood vessels are stained, neural tissue was generally negative. Bars represent 50 μm.

sorted c264$^+$ and c264$^-$ E13.5 bone marrow cells (H.B19ov$^+$ animals) and injected them intrathymically into 14-d-old H.B19ov$^-$ congenic animals. Thymus reconstitution by the ov$^+$ donor bone marrow cells was measured by flow cytometry with the anti-ov mAb 11A9 at 2 wk after injection. In a representative experiment, 500 sorted c264$^+$ cells led to 5% thymocyte chimerism while up to 10,000 c264$^-$ cells induced no chimerism (Fig. 5). To obtain a similar recon-

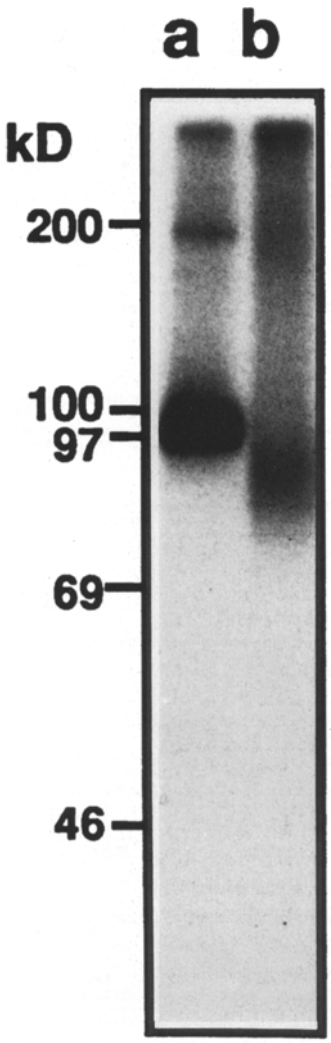

Figure 4. Biochemical analysis of c264 antigen. The thymocytes from a three-wk-old chicken of the strain H.B19 were ^{125}I-labeled, and the lysate precipitated with mAb c236 obtained by immunization with soluble recombinant HEMCAM protein. Immunoprecipitates were analyzed by SDS-PAGE on a 7.5% gel under reducing conditions (*a*). In *b*, the precipitated protein was digested by N-glycanase before analysis by SDS-PAGE. The molecular weight standards are indicated on the left.

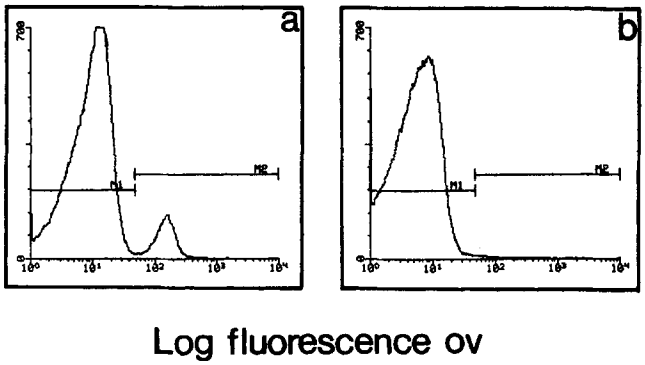

Figure 5. Thymus reconstitution by intrathymic injection of sorted c264$^+$ embryonic bone marow cell populations. (*a*) Demonstration of ov chimerism in the thymus two weeks after intrathymic injection of 500 H.B19ov$^+$ c264$^+$ bone marrow cells into irradiated 14-d-old H.B19ov$^-$ recipients. Ov-positive cells in injected thymus lobes were detected by immunofluorescence staining with mAb 11A9. (*b*) Demonstration of the absence of thymus chimerism when 10,000 c264$^-$ cells were injected.

stitution level with total bone marrow, about 15,000 cells were needed (not shown).

In the mouse and human systems, it has been demonstrated that early hemopoietic progenitors can be greatly enriched on the basis of expression of c-kit, a tyrosine kinase-type receptor for hemopoietic stem cell factor (35). Recently, we generated an anti–chicken c-kit mAb (see Materials and Methods). We sorted c264-positive cells on the basis of the expression of c-kit. Strikingly, we found that virtually all c-kit positive cells in the bone marrow expressed the c264 antigen (Fig. 6 *a*). Sorting of c264$^+$, c-kit$^+$ double positive cells resulted in a population that was highly enriched for early progenitor cells and extremely potent in the T cell differentiation assay. In limiting dilution experiments, injection of as few as three c264$^+$, c-kit$^+$ bone marrow cells resulted in clear T cell chimerism in a substantial fraction of recipients. The estimated frequency of T cell precursors in the c264$^+$, c-kit$^+$ double positive population was ~0.17 (Fig. 6 *b*).

To test whether the c264 antigen$^+$, c-kit$^+$ bone marrow cell population also contained progenitors for the myeloid and/or the erythroid lineages, we assayed growth and differentiation in vitro in the presence of chicken stem cell factor (SCF), myelocytic growth factor (cMGF), and erythropoietin (EPO). All the combinations stimulated proliferation of the sorted c264$^+$, c-kit$^+$ cells but not single positives or double negatives (Table I). Culturing of 1×10^5 double positive cells for 5 d resulted in $6.5–10.5 \times 10^5$ differentiated cells of all myeloid or erythroid lineages (Table I and not shown). When the double positive cells were cultured under semi-solid growth conditions, there was a high frequency of mixed colonies (Table II). These results suggest that c264 antigen and c-kit are present on hemopoietic progenitors of all lineages.

The c264 Antigen Is Homologous to the Human Melanoma Progression Marker MUC18

Since embryonic thymocytes showed the highest expression level of the c264 antigen, a cDNA library was made from E13.5 thymus polyA$^+$ mRNA. The library was constructed into the pCDA3 vector, which allowed expression of cDNA in eukaryotic cells (COS 7). Using expression screening with mAb c264, we isolated one clone from transiently transfected COS cells. This clone has an open reading frame of 1,878 bp that corresponded to a recently isolated cDNA encoding gicerin, a molecule involved in neurite outgrowth. Among nucleotide differences, at position 326 of the gicerin sequence, one C was missing whereas at position 466 another C was inserted changing the reading frame between amino acid residues 43 and 77 (Fig. 7 and not shown) (56, 57). We confirmed the cDNA sequence by NH_2 terminus amino acid sequencing of the recombinant soluble protein produced in J558L myeloma cells (28 amino acid residues). The leader peptide was unusually long (35 amino acids) and it contained six cysteine residues. In the NH_2 terminus c264 sequence a new putative N-linked glycosylation site was found.

The predicted amino acid sequence of the c264 protein identified this molecule as a member of the type I transmembrane protein of the IgSF, with five extracelluar Ig domains, two of them of the V-like and three of the C2-like, in the order V-V-C2-C2-C2. The extracellular domains showed nine putative N-glycosylation and seven

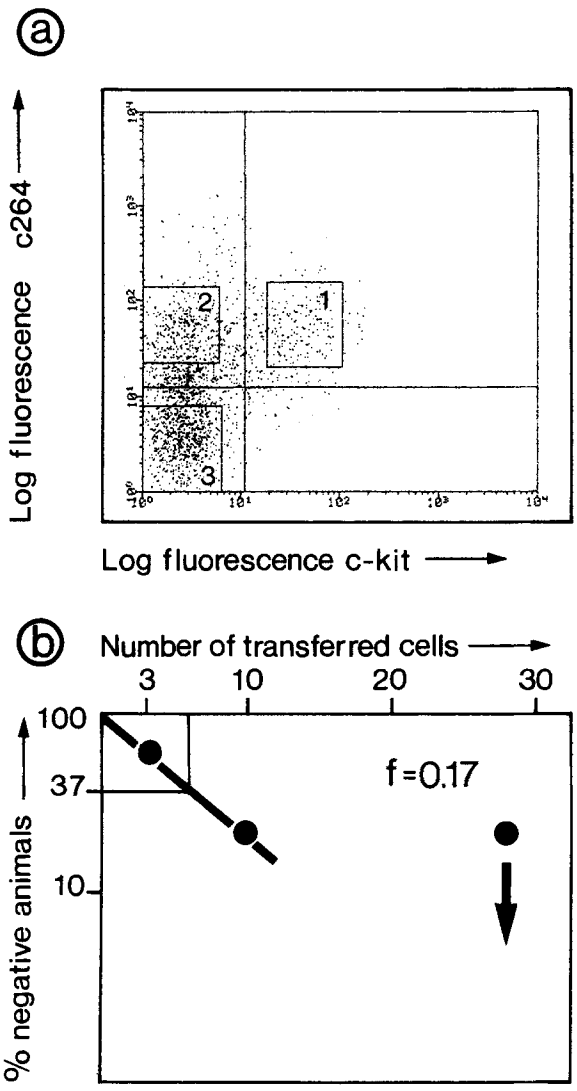

Figure 6. Thymus reconstitution after intrathymic injection of $c264^+$ c-kit^+ bone marrow cells. (*a*) Bone marrow cells derived from E13.5 H.B19 animals stained for c-kit and c264 antigen. Populations 1, 2, and 3 define $c264^+$ c-kit^+, $c264^+$ c-kit^- and $c264^-$ c-kit^- cells, respectively. (*b*) Frequency of T cell precursors in $c264^+$ c-kit^+ E13.5 bone marrow population. Titrated numbers of $c264^+$ c-kit^+ ov^+ cells were injected intrathymically into ov^- recipients and the thymuses were assayed two weeks later for ov^+ cells by flow cytometry.

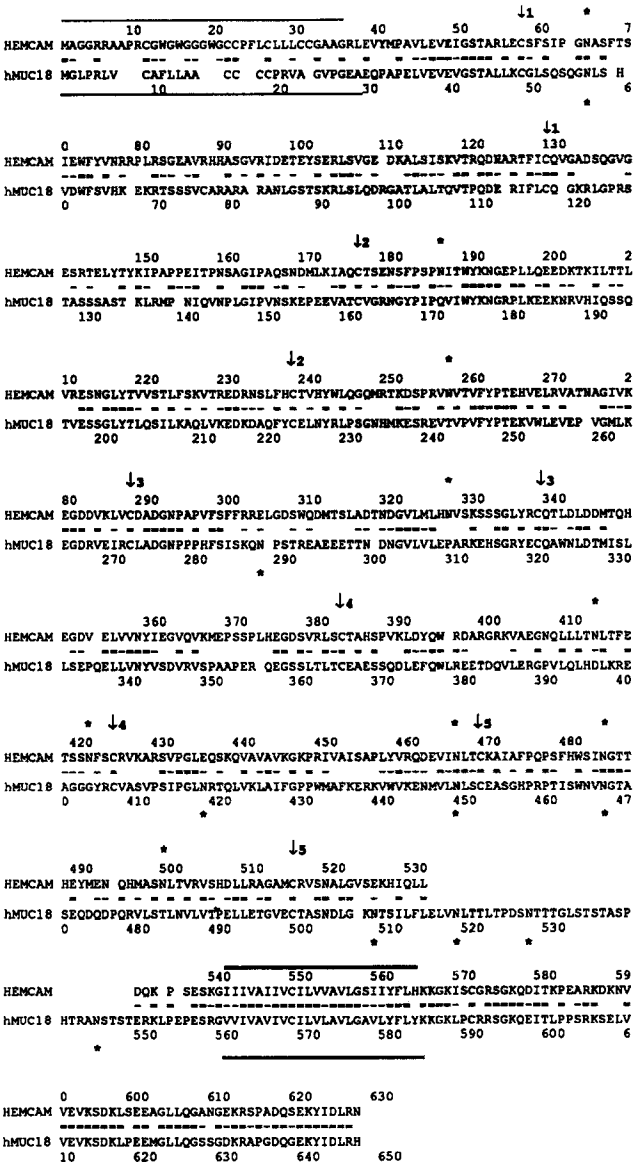

Figure 7. Alignment of amino acid sequences of chicken HEMCAM and human MUC18 precursors. Gaps have been introduced to maximize alignment scores. The thin solid line indicates the leader peptides, the thick solid line the putative transmembrane domain. Numbered arrows above the sequences show the conserved cystein residues of each Ig domain. Stars show potential N-linked glycosylation sites. Identical or similar amino acid residues are indicated by a double or a single bar, respectively. The long gap in HEMCAM sequence starting from residue 531 corresponds to amino acids in human MUC18 sequence encoded by exon 13.

myristylation sites. Among the IgSF molecules in the EMBL data base MUC18 showed high homology (39%) with the c264 protein (Fig. 7). MUC18 is a human member of the V-V-C2-C2-C2 family and is a marker for human melanoma tumor progression (28). Significant but lower homology was also found with other five Ig-domain proteins from various species: B-CAM (27%, human), ALCAM (25%, human), BEN (25%, chicken), and neurolin (28%, fish). There was much higher homology between chicken c264 and human MUC18 in the transmembrane (66%) and cytoplasmic regions (69%). Exon 13 of MUC18, which encodes 34 amino acid residues located between the last Ig domain and the transmembrane region is not present in c264 (Fig. 7). Because of the expression by hemopoietic cells, the structural similarity and the high sequence homology to MUC18, we call the c264 protein HEMCAM (Hemopoietic, MUC18 related, Cell Adhesion Molecule). It was interesting to note that HEMCAM showed higher sequence homology to MUC18 in the NH_2-terminal region than gicerin to MUC18.

As two different cytoplasmic sequences have been observed in gicerin and HEMCAM, we searched for the presence of cDNA splicing variants (56) with reverse transcriptase PCR (RT-PCR) on several tissues. Three differ-

Table III. Expression of HEMCAM Transcripts during Chicken Development

Days of development	9	10	11	12	13	14	15	17	35
Bone marrow	nd	++	++	++	++	++	++	±	±
Thymus	+	++	++	++++	++++	++	+++	++++	++
Spleen	nd	++	+	+	±	±	±	±	±
Bursa	nd	+	++	+++	+	+	nd	++	+++
Intestine	nd	+	+	+	+	+	++	++	−
Liver	nd	++	++	++	++	++	++	+++	++
Muscle	nd	++	nd	nd	+++	nd	nd	++++	+++
Brain	nd	++	nd	nd	nd	nd	nd	++	++
Blood cells	nd	±	±	nd	++	nd	nd	++	+
Kidney	nd	nd	nd	nd	++	nd	nd	nd	+

The pattern has been established with a semiqualitative PCR-technique using oligonucleotide primers 11188 and 11106 which allow amplification of an invariant part of HEMCAM transcripts (see Materials and Methods). The PCR products have been analyzed by agarose gels and the intensity of the appearing bands is indicated by a grading from (−) no band to a (++++) band at saturation levels.

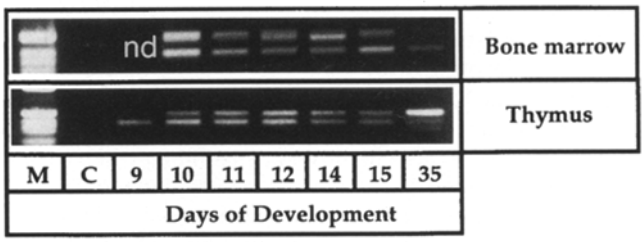

Figure 9. Expression of HEMCAM mRNA encoding the cytoplasmic variants during embryogenesis. RT-PCR analysis of embryonic bone marrow and thymus from day 9 (E9) of development onwards was done with a combination of two primers depicting mRNA coding for the long or short cytoplasmic forms (11898-11902). After 30 cycles of amplification, PCR products were separated on a 1.2% agarose gel. The short form appears as the lower band at 384 bp and the long form at 503 bp. (*M*) DNA molecular weight markers 1 kb ladder. (*C*) Control sample containing the primers but no templates. *nd*, not determined.

ent mRNA forms encoding putative RNA splicing variants were identified in the 3′ end (Fig. 8). The first transcript corresponded to the HEMCAM sequence with a long cytoplasmic tail. Another encoded a short cytoplasmic variant that was identical with the COOH-terminal end of the gicerin sequence. The third transcript would lead to a soluble protein truncated just before the transmembrane region.

During development, HEMCAM is expressed as early as E10 in several organs, including bone marrow, thymus, spleen, bursa, intestine, liver, and brain, as determined by RT-PCR with primers detecting all variants (Fig. 9 and Table III). The mRNA splice variants encoding the short and the long cytoplasmic tails were present in equal levels at all stages of development in the bone marrow (Fig. 9). In the developing thymus however the short variant predominated during early embryonic stages, while the long form was present in later stages and predominated in thymus of hatched chicks. Two PKC phosphorylation sites at positions 573 (RSGK) and 594 (KSDK) are conserved in human MUC18. Although there is a conserved tyrosine residue at the cytoplasmic tail, neither HEMCAM nor MUC18 has a potential tyrosine phosphorylation site, as defined by the Prosite pattern data base.

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          481
Long      SINGTTHEYMENQHMASNLTVRVSHDLLRAGAMCRVSNALGVSEKHIQLLDQKPSESKGI  540
Short     ..............................................................  540
Soluble   .....VRAGRVLWAALGMGAVRGC                                       504

          541                         +     *           +
Long      IIVAIIVCILVVAVLGSIIYFLHKKGKISCGRSGKQDITKPEARKDKNVVEVKSDKLSEE  600
Short     ..........................................ARNTSI                584
Soluble                                                                   504

          601         *   +
Long      AGLLQGANGEKRSPADQSEKYIDLRN                                      626
Short                                                                     584
Soluble                                                                   504
```

Figure 8. Predicted amino acid sequences of three mRNA splicing variants of HEMCAM. The sequences shown start from residues 481. "Long" and "short" indicate the long and short forms of the cytoplasmic tails of HEMCAM; soluble corresponds to the putative soluble form of HEMCAM ending at residue 504. +, *, and · indicate possible phosphorylation sites for protein kinase C, casein kinase II, and calmodulin kinase II, respectively.

HEMCAM Confers Cell Spreading and Cell Adhesion by Homophilic Interaction

HEMCAM cDNA coding for the short and the long cytoplasmic variants were transfected into mouse L cells. Both forms of HEMCAM led to cell–cell adhesion in a suspension assay (Fig. 10 *A*). In transfected cell lines, the average size of aggregates was four cells, while in control, untransfected cell lines, the average was 1.2 cells. The largest aggregates were 30 cells in the transfected lines, while aggregates larger than four or five cells were never seen in the controls. Soluble recombinant HEMCAM molecules and anti-HEMCAM antibodies blocked cell adhesion confirming that the adhesion was HEMCAM specific (Fig 10 *B*). And finally, the aggregation of transfected L cells was mediated by homophilic interaction of HEMCAM molecules, since untransfected cells were not found in aggregates formed by HEMCAM expressing cells (Fig. 10 *C*).

A mouse pro-T lymphocyte cell line (FTF1) bound to recombinant chicken HEMCAM, mouse PECAM-1, and mouse VCAM-1, all molecules found on vascular endothelia (21). When HEMCAM of adherent pro-T cells was cross-linked by a purified polyclonal antibody that recognizes chicken and mouse HEMCAM (see Materials and Methods), the cells exhibited significant spreading on PECAM-1–, VCAM-1–, and HEMCAM–coated surfaces but not on control BSA (bovine serum albumin) (Fig. 10 *D*). Thus, HEMCAM seems to be able to mediate adhesion and induce cell spreading, probably by activating cytoskeletal elements after signal transduction.

Discussion

We have characterized and cloned HEMCAM, a molecule expressed by hemopoietic progenitor cells in embryonic bone marrow, thymocytes, and capillary endothelial cells. HEMCAM-expressing cells sorted from bone marrow were enriched in progenitors able to differentiate into T cells when transferred into the appropriate thymic microenvironment. An extremely high frequency of T cell precursors of 0.17 was found among embryonic bone marrow cells expressing both HEMCAM and the receptor tyrosine

kinase c-kit. Three putative splice variants of HEMCAM were observed; two have transmembrane domains with cytoplasmic tails of 63 or 21 amino acids, and one lacks both transmembrane and cytoplasmic regions. HEMCAM which is almost identical to gicerin, a molecule involved in neural outgrowth (57), is also homologous to human MUC18, a molecule expressed by highly metastatic melanoma cells (28). The function of HEMCAM seems to be mediation of cell–cell adhesion as well as cell spreading of pro-T lymphocytes. We concluded that HEMCAM could be involved in the colonization of the thymus by hemopoietic progenitor cells.

Although progenitor cells represent an extremely small population in the mouse, <0.05% of leukocytes in adult bone marrow, a much higher frequency was found in fetal tissues (e.g., 8% of all leukocytes in fetal blood [20, 34, 49]). Embryonic bone marrow cells, which are easily obtainable in the chicken, are also highly enriched in hemopoietic progenitors (11). Here we have characterized the phenotype of these progenitor cells, and, as expected from mouse and human data, hemopoietic progenitors expressed c-kit. More importantly, we showed that the c-kit$^+$ progenitors coexpressed the adhesion molecule HEMCAM.

Three V-V-C2-C2-C2 IgSF molecules are known in human beings: BCAM, ALCAM, and MUC18. Two forms of BCAM are generated by alternative splicing; BCAM and the Lutheran blood group antigen. (5, 38). BEN, DM-GRASP, and SC1 are homologues of ALCAM in the chicken, or KG-CAM in the rat and neurolin in the fish (Fig. 11). HEMCAM differs from gicerin by a stretch of 35 amino acids at the NH_2 terminus. However, this difference is due to an insertion and a deletion of one nucleotide at each side of the borders of this stretch, leading to a frame shift. The few nucleotide substitutions observed between the two sequences make it likely that HEMCAM and gicerin are the same molecular entity. The high GC percentage in the NH_2 terminus region may account for the possible errors encountered in the gicerin sequence (57). Two different clues suggest that our nucleotide sequence is the correct one: first, the determined amino acid sequence of our recombinant HEMCAM protein fits with the cDNA sequence and second, the amino acids 43 to 77 of HEMCAM and not those of gicerin are homologous to MUC18, a human metastatic melanoma marker (28), at the same level as the rest of the extracellular part of the HEMCAM or gicerin proteins. Overall, the HEMCAM amino acid sequence is homologous to 39% with MUC18. Although the homology was relatively low in the extracellular part, 32–40% for each Ig domain, the transmembrane and the cytoplasmic domains were highly homologous, 66% and 69%, respectively. This suggests that intracellular ligands of HEMCAM, or proteins with which HEMCAM forms complexes in the membrane should be well conserved during evolution, while extracellular ligands might have diverged. Two of the potential PKC phosphorylation sites of the HEMCAM long form, at positions 573 and 594, were conserved in human MUC18 further strengthening the possible functional homology of these proteins. The main difference between MUC18 and HEMCAM is the absence in HEMCAM of a stretch of 34 amino acids located between the last Ig domain and the transmembrane domain of MUC18. These residues are encoded by exon 13 of

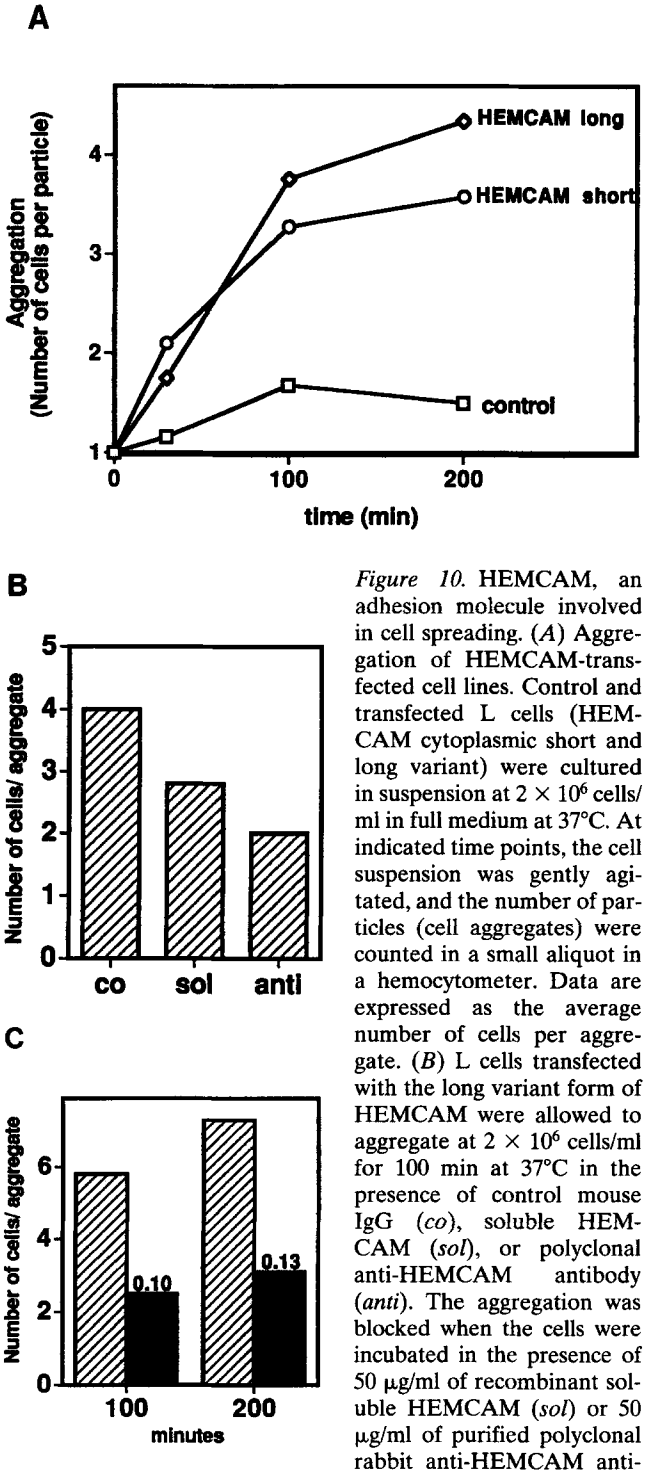
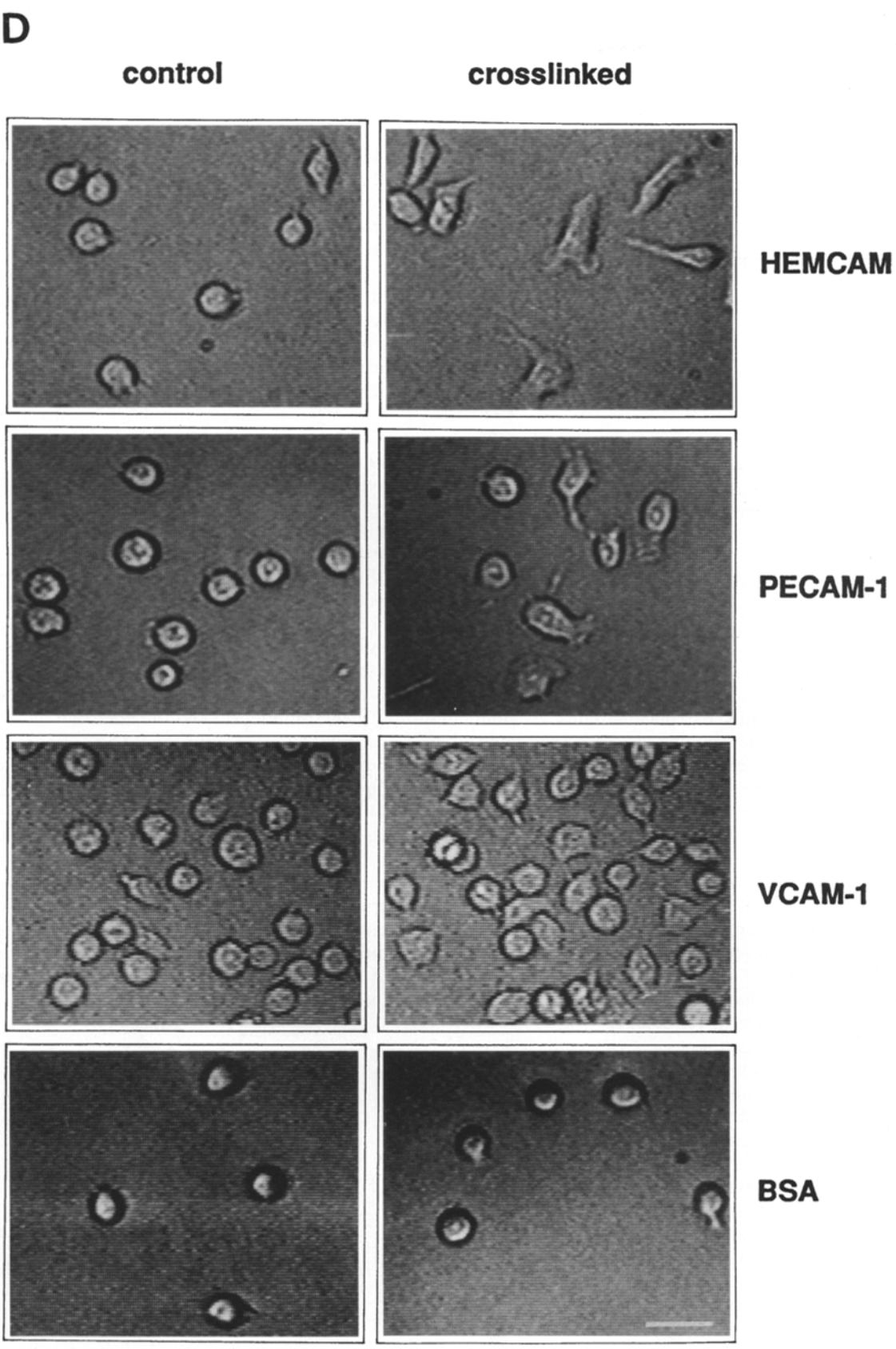


Figure 10. HEMCAM, an adhesion molecule involved in cell spreading. (*A*) Aggregation of HEMCAM-transfected cell lines. Control and transfected L cells (HEMCAM cytoplasmic short and long variant) were cultured in suspension at 2×10^6 cells/ml in full medium at 37°C. At indicated time points, the cell suspension was gently agitated, and the number of particles (cell aggregates) were counted in a small aliquot in a hemocytometer. Data are expressed as the average number of cells per aggregate. (*B*) L cells transfected with the long variant form of HEMCAM were allowed to aggregate at 2×10^6 cells/ml for 100 min at 37°C in the presence of control mouse IgG (*co*), soluble HEMCAM (*sol*), or polyclonal anti-HEMCAM antibody (*anti*). The aggregation was blocked when the cells were incubated in the presence of 50 μg/ml of recombinant soluble HEMCAM (*sol*) or 50 μg/ml of purified polyclonal rabbit anti-HEMCAM antibody (*anti*). (*C*) HEMCAM mediates L cell aggregation by homophilic interaction. L cells, transfected with the long variant form of HEMCAM were allowed to aggregate at 2×10^6 cells/ml for 100 or 200 min, respectively (*hatched bars*). In a simultaneous experiment transfected L cells at 2×10^6 cells/ml were mixed with nontransfected control L cells at 2×10^6 cells/ml which have been previously labeled with the fluorescent dye BCECF. The average number of cells per aggregate was assessed by a microscope equipped by epifluorescence (*solid bars*). In 30 aggregates the number of fluorescent and unstained cells were counted and expressed as an index given on top of the bars, it is calculated as the number of labeled cells (control L cells) divided by the number of unlabeled cells (transfected L cells). Note that the aggre-



gates in 1:1 cell mixtures were smaller and contained mostly transfected cells. (*D*) Soluble recombinant HEMCAM, PECAM-1, VCAM-1, or control BSA were coated onto culture dishes. FTF1 pro-T lymphocytes were allowed to adhere to the coated dishes for 20 min at 37°C, and the nonadhering cells were removed. The spreading of adherent cells was observed over 2 h by video-microscopy; photographs were taken at the end of this observation time (*control*). Then, polyclonal anti-HEMCAM antibody was added at 50 μg/ml to cross-link HEMCAM. Cell spreading occured after a further 15–20 min at 37°C, and photographs were taken at 20 min after (*crosslinked*). Bar, 10 μm.

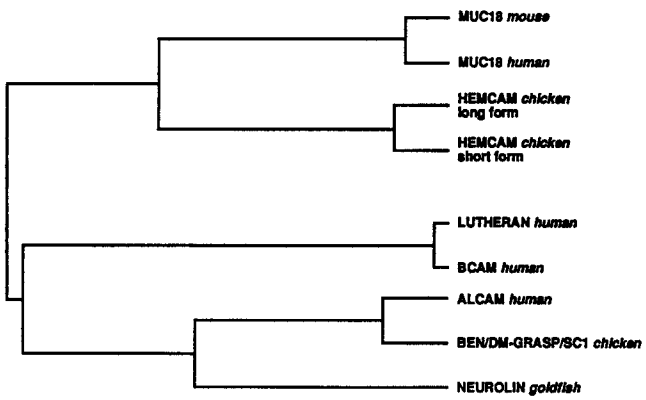


Figure 11. Evolutionary tree of proteins belonging to the family of V-V-C2-C2-C2- IgSF.

MUC18 (53). In fact, these 34 amino acids correspond exactly to a mucin repeat found in human molecule SMUC-40 (16). Such structural differences have been previously encountered in homologous molecules in different species. For example, in the mouse MAdCAM-1, a large mucin domain replaced an Ig domain present in human MAdCAM-1 (54). Thus, taken together, our data suggest that HEMCAM is the chicken homologue of human MUC18.

We identified three HEMCAM mRNA splice variants. Two of them encode transmembrane proteins that vary in their cytoplasmic tail. This may be of importance for the protein function since the cytoplasmic domain of the long form has three protein kinase C (PKC) and two casein kinase II (CKII) phosphorylation sites, the short form has only one of these PKC sites in common along with a calmodulin kinase 2 phosphorylation site. The capacity for differential phosphorylation of the short and long cytoplasmic forms of HEMCAM might have consequences for signal transduction into the cell and/or inside out signaling leading to different ligand specificities. This is further emphasized by the fact that the two forms have temporally different expression patterns in the thymus. It remains to be determined whether or not differentiating thymocytes and thymic vascular endothelial cells express both forms of HEMCAM. The nucleotide sequence missing from the short cytoplasmic HEMCAM transcript corresponds exactly to exon 15 of MUC18. It suggests that MUC18 and HEMCAM genes have the same structure. In this context the generation of the two alternative transcripts is easy to understand. The transcript encoding the short cytoplasmic form would be generated by direct junction of the exons homologous to exons 14 and 16 of MUC18, while the transcript encoding the soluble form would result in the absence of splicing of the intron homologous to intron 11 of MUC18 (53).

As HEMCAM is expressed by hemopoietic progenitor cells and has mostly disappeared in differentiated T cells, we think this molecule might contribute to a specific mechanism allowing hemopoietic progenitors to colonize the thymus. As it is an adhesion molecule and it belongs to the V-V-C2-C2-C2 family, HEMCAM would likely be involved in an adhesion process allowing hemopoietic progenitor cells to attach to the luminal plasma membrane of the vascular endothelial cells. As it is a variant of gicerin, HEMCAM should exhibit similar adhesion specificities; e.g., homophilic interactions and heterophilic interaction with NOF, a laminin-like molecule (18). Interestingly, HEMCAM is expressed on vascular endothelium, which may allow progenitor/endothelial cell adhesion. Moreover, as is the case with many other adhesion molecules of the IgSF family (14), a physiological soluble form of HEMCAM can be produced. Soluble HEMCAM may play a role in the regulation of thymus homing, inhibiting progenitor/endothelial cell adhesion by competitive binding to its ligand on the plasma membrane. It remains to be determined whether the two cytoplasmic domains are involved in inside out signaling leading to different ligand specificity. For example, it has recently been shown that alternatively spliced tails of PECAM-1 determine whether the molecule interacts in a homophilic or heterophilic manner (62). At present, we are testing such a regulation of ligand specificity with HEMCAM. The strength of HEMCAM adhesion is weak in comparison to adhesion with selectins, integrins, and other IgSF proteins such as ICAMs, VCAM-1, and PECAM-1. The main role of HEMCAM, whether it is a proper adhesion molecule or whether it acts as a modulator of adhesion through association with other adhesion molecules or they are involved in outside-in signaling, remains to be tested.

Like HEMCAM, two molecules involved in thymus homing of pro-T cells, α6 integrin and the adhesion molecule CD44 play a role in tumor metastasis. The α6β1 integrins are mediators of metastatic melanoma cell adhesion to the vascular endothelium (50), and the expression of a variant of CD44 confers metastatic potential to rat carcinoma cells (17). Gicerin, the homologue of HEMCAM has been described in tumorigenesis (58). Both HEMCAM and its human homologue MUC18 participate in organogenesis during development. At the adult stage their expression is restricted to a few tissues, but it may be induced during tumor progression (28). Understanding the normal function of these molecules ought to contribute to the understanding of their malfunction in tumors.

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