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Immunology

Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family

(cell surface protein/polymerase chain reaction/immunohistology)

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ABSTRACT **Recently, hematopoietic stem cells were purified to homogeneity from mouse bone marrow. The protein structure of Sca-1, the cell surface antigen used in the isolation of hematopoietic stem cells, is described here. It is shown that the Sca-1 antigen is a member of the Ly-6 antigen family. The anti-Sca-1 antibody was used in immunohistochemistry experiments to define the structures in several tissues that had previously been shown to contain Ly-6 antigens. In thymus, spleen, and kidney, specific staining of parenchymal cells can be demonstrated, whereas only vasculature reacts with anti-Sca-1 in brain, heart, and liver and possibly in lung.**

Peripheral blood cells are known to be derived from a self-renewing population of hematopoietic stem cells residing in the bone marrow. Many attempts have been made to identify mouse hematopoietic stem cells based on the expression of cell surface markers detected by monoclonal antibodies (mAbs). In previous studies (1) the mouse hematopoietic stem cell was found to express low levels of Thy-1 antigen (Thy-1^{lo}) and to be "lineage-negative" (Lin^-)—i.e., not express markers characteristic of B cells (B220), granulocytes (Gr-1), myelomonocytic cells (Mac-1), and T lymphocytes (CD4 and CD8). More recently, a new mAb, anti-Sca-1, was used to purify stem cells from the Thy-1^{lo}, Lin^- bone marrow subpopulation (2). By injecting irradiated hosts with selected cells, it was shown that the Thy-1^{lo}, Lin^-, Sca-1^+ (but not the Thy-1^{lo}, Lin^-, Sca-1^-) population of bone marrow cells are highly purified, and perhaps homogeneous, pluripotent stem cells. They read out with nearly unit efficiency in assays for primitive myeloerythroid and thymic progenitors, and 25–30 cells are sufficient to permit lethally irradiated hosts to survive and be restored in all blood-cell lineages. Moreover, the Thy-1^{lo}, Lin^-, Sca-1^+ subpopulation appeared to contain all stem cells present in the bone marrow. Here we show the biochemical and immunohistological characterization of the Sca-1 antigen and show that Sca-1 found on lymphocytes is a member of the Ly-6 antigen family. In addition, we show that the antigen recognized by anti-Sca-1 on bone marrow stem cells is identical to that recognized on peripheral lymphocytes.

Ly-6 antigens are encoded by a multigene family and are expressed on both lymphoid and nonlymphoid cells. We have used anti-Sca-1 in immunoperoxidase staining experiments on frozen tissue sections to determine which anatomic structures in nonlymphoid tissues express Ly-6A.2 antigen.

MATERIALS AND METHODS

Cells. Single-cell suspensions of C57BL/6 or BALB/c splenocytes, peripheral lymph node cells, and mesenteric lymph node cells were prepared by mincing the tissues through a steel wire mesh. Erythrocytes were lysed by incubating the cell suspensions in 0.33% NaCl. The 7B4 cell line is a fusion product of BW5147 and C57BL/6 peripheral lymph node T lymphocytes; this cell line has lost the expression of Thy-1.2 but still expresses Thy-1.1 (C. Guidos, personal communication). For Con A stimulation of C57BL/6 or BALB/c lymphocytes, cells were incubated at a density of 0.5×10^6 cells per ml for 48 hr in medium containing Con A at 4 μg/ml. All cells were cultured in RPMI-1640 medium containing 7% fetal bovine serum.

mAbs. mAb anti-Sca-1 (rat IgG2a; ref. 2) was used as a culture supernatant for immunofluorescence studies and in a purified form for immunoprecipitation studies. Anti-Ly-6A.2 mAbs 34.11.3 (mouse IgG2a; ref. 3) and 3E7 [rat IgG2a; detects the TAP (T-cell activation protein) antigen; K. Rock, Harvard Medical School] were used as ascites. mAb 53.2.1 (rat IgG2a) is directed against Thy-1.2 (American Type Culture Collection). Mel-14 (rat IgG2a) detects the mouse lymph node-specific homing receptor (10). mAbs R7D4 (rat IgG2a directed against an idiotype of the mouse B-cell lymphoma 38C13; ref. 5) and 19XE5 (mouse IgG2a; anti-Thy-1.1; R. Nowinski, Genetics Systems, Seattle) were used as negative controls. MAR 18.5 (mouse IgG2a anti-rat κ chain; American Type Culture Collection) was used in immunoprecipitation studies.

Immunofluorescence. Lymph node cells were incubated with mAb R7D4, anti-Sca-1, 34.11.3, or 19XE5, washed, and incubated with fluoresceinated goat anti-rat or goat anti-mouse immunoglobulin (Caltag, South San Francisco, CA). T lymphocytes were stained with biotinylated 53.2.1 mAb and Texas red-avidin. Thy-1^- cells were excluded from analysis by Texas red gates. Samples were analyzed with a dual-laser fluorescence-activated cell sorter (FACS, Becton Dickinson) modified as described (6) and made available through the FACS shared users group at Stanford University.

Immunoprecipitation. Splenocytes ($20–50 \times 10^6$) were labeled with ^{125}I by using lactoperoxidase. For metabolic labeling, 7B4 cells were washed in methionine-free Dulbecco's modified Eagle's medium (DMEM), resuspended in 1 ml of methionine-free DMEM containing 0.5 mCi (18.5 MBq) of [^{35}S]methionine and 7% dialyzed fetal bovine serum, and incubated for 20 min at 37°C. Immunoprecipitations were carried out with protein A-Sepharose and MAR 18.5 antibody.

Polymerase Chain Reaction. Poly(A)$^+$ mRNA was isolated by lysing 2×10^3 Thy-1^{lo}, Lin^-, Sca-1^+ bone marrow cells, 10^4 Thy-1^{lo}, Lin^-, Sca-1^- bone marrow cells, or 2×10^3 BW5147 thymoma cells in 500 μl of 0.5 M NaCl/1% SDS/10 mM Tris, pH 7.5/1 mM EDTA containing proteinase K at 200 μg/ml. After an incubation of 1 hr at 37°C, a small amount of oligo(dT)-cellulose (Pharmacia) was added and the suspension was agitated for 1 hr at room temperature. The poly(A)$^+$ RNA was pelleted by centrifugation, washed twice in 500 μl of 0.5 M NaCl/0.2% SDS/10 mM Tris, pH 7.5/1 mM

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Abbreviations: mAb, monoclonal antibody; TAP, T-cell activation protein.

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EDTA/1 mM phenylmethanesulfonyl fluoride, and given a final wash in the same buffer without SDS or phenylmethanesulfonyl fluoride. Single-strand cDNA was synthesized in a 100-μl volume with avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources, Tampa, FL). One-tenth of the single-strand cDNA preparation was subsequently incubated with two oligonucleotides derived from opposite strands of Ly-6 cDNA (4) and subjected to a polymerase chain reaction (Perkin–Elmer Cetus) consisting of 30 cycles. The oligonucleotide sequences were derived from the Ly-6 cDNA sequence (4) and contained nucleotides 81–100 (5'-CT-CTG-AGG-ATG-GAC-ACT-TCT-3') and 465–485 (5'-GGT-CTG-CAG-GAG-GAC-TGA-GC-3'). One-tenth of the amplified material was analyzed by agarose gel electrophoresis. Bone marrow cells were isolated as described (2).

Immunohistochemistry. Cryostat sections of C57BL/6 organs were incubated with anti-Sca-1 or control antibodies and stained as described (7).

RESULTS

Surface Expression of Sca-1 Increases upon Con A Activation of T Lymphocytes. The cell surface markers Thy-1 and Sca-1 are two antigens expressed on mouse hematopoietic stem cells in the bone marrow; both antigens are also found on lymphocytes and on cells in other tissues (1, 2, 7). C57BL/6 T lymphocytes cultured in medium containing Con A showed a strong increase in the expression of Sca-1 compared with nonactivated lymphocytes (Fig. 1 *A* and *B*). Unstimulated BALB/c-derived T lymphocytes expressed fewer Sca-1 determinants than found on C57BL/6 cells (Fig. 1*C*). After Con A stimulation, BALB/c T lymphocytes expressed levels of Sca-1 similar to those on activated C57BL/6 cells (Fig. 1*D*). Thus, Sca-1 appears to be a T-lymphocyte activation antigen. The reaction of anti-Sca-1 with activated C57BL/6 T cells appeared to mirror the kinetics of expression of Ly-6A.2 antigens, which are found on C57BL/6 (*Ly-6^b* haplotype) cells (Fig. 1 *A* and *B*).

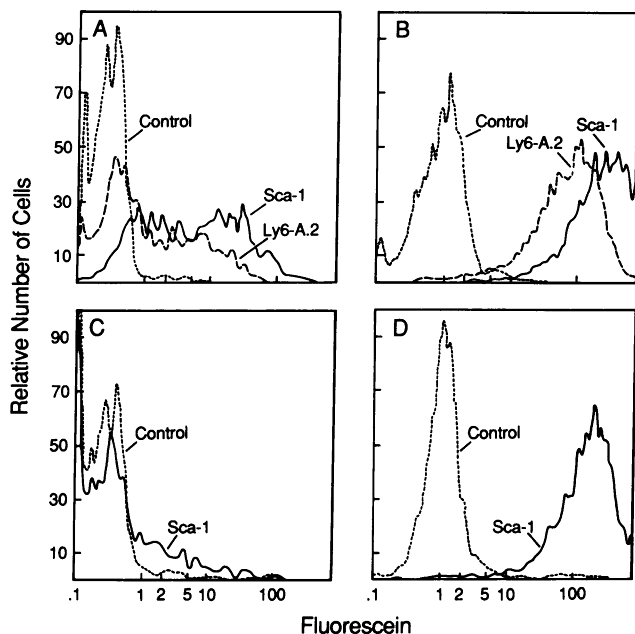

FIG. 1. Expression of Sca-1 antigens on C57BL/6 and BALB/c peripheral lymphocytes increases after activation by Con A. (*A*) C57BL/6 T lymphocytes. (*B*) C57BL/6 T lymphocytes after Con A stimulation. (*C*) BALB/c T lymphocytes. (*D*) BALB/c T lymphocytes after Con A stimulation. R7D4 and 19XE5 were used as negative control antibodies; 34.11.3 was used to detect Ly-6A.2.

Ly-6E.1 antigens are found on BALB/c (*Ly-6^a* haplotype) cells and are commonly thought to be the allelic form of Ly-6A.2. While Ly-6A.2 antigens are found on both nonstimulated and stimulated T cells, Ly-6E.1 antigens are only weakly expressed on nonstimulated T cells and become strongly expressed only after stimulation (ref. 8 and A. Bothwell, personal communication). Therefore, the reaction of anti-Sca-1 on BALB/c T lymphocytes is similar to that described for anti-Ly-6E.1 antibodies.

Anti-Sca-1 and Anti-Ly-6A.2 Antibodies Precipitate Identical Molecules. The finding that Sca-1 and Ly-6 expression increased in Con A-activated lymphocytes prompted us to compare the biochemical properties of these antigens. Con A-stimulated C57BL/6 splenic lymphocytes were surface-radioiodinated and lysates were immunoprecipitated with anti-Sca-1, 34.11.3 (anti-Ly-6A.2), 53.2.1 (anti-Thy-1.2), and 19XE5 (anti-Thy-1.1) mAbs. The precipitated material was analyzed by SDS/PAGE under nonreducing conditions (Fig. 2, lanes A–D) and reducing conditions (lanes E–H). Sca-1 appeared to have a molecular mass of 8 kDa under nonreducing conditions and of 18 kDa under reducing conditions, indicating the presence of intrachain disulfide bonds. In both cases, the Sca-1 molecule migrated at a position identical to the Ly-6A.2 antigen precipitated by 34-11-3.

To define further the relation between Sca-1 and Ly-6 antigens, sequential immunoprecipitation (preclear) experiments were carried out. The part of the lysate from which Sca-1 was isolated was precipitated twice more with anti-Sca-1, and the lysate from which 34.11.3 was isolated was reprecipitated with 34.11.3. No material was precipitated with anti-Sca-1 in the third precipitation (Fig. 2, lane I), indicating that all Sca-1 had been removed. When the lysate precleared with anti-Sca-1 was subjected to precipitation with 34.11.3, no material was precipitated (lane K), indicating that anti-Sca-1 can effectively remove the antigen recognized by 34.11.3.

We were unable to remove all Ly-6A.2 by using 34.11.3; in the third round of precipitation a small amount of 18-kDa material could still be isolated from the lysate with 34.11.3 (Fig. 2, lane J), suggesting that the 34.11.3 antibody has a relatively low affinity for the Ly-6A.2 antigen in immunoprecipitation experiments. When the lysate precleared with 34.11.3 was precipitated with Sca-1 antibody, a band was found at 18 kDa (lane L). Considering the low affinity of 34.11.3 for its antigen, this could indicate that anti-Sca-1

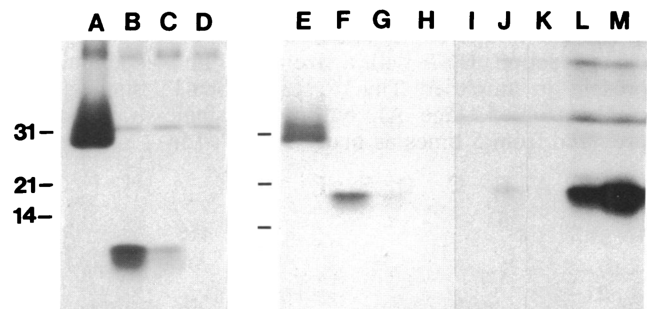

FIG. 2. SDS/PAGE, under nonreducing (lanes A–D) or reducing (lanes E–M) conditions, of immunoprecipitated material from radioiodinated, Con A-stimulated C57BL/6 lymphocytes. Antibodies used are indicated. Lanes: A, 53.2.1 (anti-Thy-1.2); B, anti-Sca-1; C, 34.11.3 (anti-Ly-6A.2); D, 19XE5 (anti-Thy-1.1); E, 53.2.1; F, first precipitation with anti-Sca-1; G, first precipitation with 34.11.3; H, 19XE5; I, third precipitation with anti-Sca-1; J, third precipitation with 34.11.3; K, precipitate made with 34.11.3 from anti-Sca-1-precleared material; L, precipitate made with anti-Sca-1 from 34.11.3-precleared material; M, precipitate made with anti-Sca-1 from 19XE5-precleared material. Lanes E–H were exposed for 6 hr; lanes A–D and J–M were exposed for 24 hr. Markers indicate size in kilodaltons.

precipitated the residual Ly-6A.2 molecules present in the 34.11.3-precleared lysates. However, based on these results it cannot be determined whether the material precipitated by anti-Sca-1 from the 34.11.3-precleared lysate was Ly-6A.2 that was not removed by 34.11.3, or a distinct 18-kDa molecule. We therefore decided to use an antibody with a higher affinity for the Ly-6A.2 antigen than 34.11.3. The TAP antigen is identical to the Ly-6A.2 antigen (9). We used the anti-TAP mAb 3E7 for preclearing experiments on 7B4 cells. This cell line is a T-cell hybridoma that expresses high levels of Sca-1 (C. Guidos, personal communication). A lysate of metabolically labeled 7B4 cells was split into three aliquots and precipitations were carried out with R7D4 (negative control), 3E7, and anti-Sca-1. The 3E7 and anti-Sca-1 mAbs precipitated molecules with identical molecular weights (Fig. 3, lanes B and C) and were able to remove their antigens completely from lysates after three rounds of precipitation (lanes D and E). No Sca-1 material could be precipitated from 3E7-precleared lysate, nor could 3E7 precipitate any Ly-6A.2 material from lysate precleared with anti-Sca-1 (lanes F and G). After the immunoprecipitations on the 7B4 lysates were carried out, a final precipitation was performed on each part of the lysate with the Mel-14 antibody (10). The three aliquots of the 7B4 lysate yielded equal amounts of the 90-kDa glycoprotein gp90^{Mel-14}, indicating that the removal of 18-kDa material from the lysates during preclearing was not due to a nonspecific depletion of the lysates. From these experiments we conclude that anti-Sca-1 antibody reacts with molecules bearing the Ly-6A.2 antigen.

Sca-1$^+$ Stem Cells, but not Sca-1$^-$ Bone Marrow Cells, Express Ly-6 mRNA. While the results shown above clearly demonstrated that the Sca-1 antigen isolated from murine lymphocytes is a member of the Ly-6 family, the possibility remained that the reaction of anti-Sca-1 with mouse stem cells was due to a determinant shared by an unrelated molecule on stem cells and Ly-6 antigens on peripheral lymphocytes. The amount of mouse stem cells that can be purified from bone marrow is limited and prohibits the use of protein biochemistry to identify the molecules recognized by anti-Sca-1 on this population. We therefore decided to study the expression of mRNA encoding Ly-6 antigens in 2×10^3 Thy-1^{lo}, Lin$^-$, Sca-1$^+$ and 10^4 Thy-1^{lo}, Lin$^-$, Sca-1$^-$ bone marrow cells. Poly(A)$^+$ RNA was isolated and reverse-transcribed into single-strand cDNA. A polymerase chain reaction was performed using oligonucleotides derived from Ly-6 cDNA. The oligonucleotide probes span 403 base pairs (bp) in the cDNA clone. After 30 cycles of the polymerase chain reaction, DNA with a size of 400 bp was abundantly present in amplified Thy-1^{lo}, Lin$^-$, Sca-1$^+$ single-strand cDNA (Fig. 4, lane A), but not in single-strand cDNA prepared from 5 times as many Thy-1^{lo}, Lin$^-$, Sca-1$^-$ cells (lane B). The 400-bp material found in lane A comigrated with a band derived from amplified BW5147 (Ly-6A.2$^+$) thymoma cell cDNA (lane C). In addition, restriction enzyme analysis with *Bst*XI of material amplified from Thy-1^{lo}, Lin$^-$, Sca-1$^+$ cells yielded bands of 100 and 300 bp (lane D), predicted by the Ly-6 cDNA sequence and identical to those derived from BW5147 single-strand cDNA (lane E). These results clearly show the correlation between the presence of Sca-1 antigen on the cell surface and the expression of Ly-6 mRNA in mouse stem cells. We conclude that the antigen recognized by anti-Sca-1 on mouse hematopoietic stem cells is a member of the Ly-6 family.

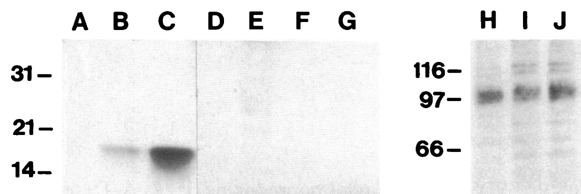

FIG. 3. Anti-Sca-1 and anti-Ly-6A.2 antibodies can specifically remove identical antigens from [^{35}S]methionine-labeled 7B4 cells. Lanes: A, 53.2.1 (negative control); B, 3E7 (anti-Ly-6A.2); C, anti-Sca-1; D, third precipitation with 3E7; E, third precipitation with anti-Sca-1; F, anti-Sca-1 precipitate from lysate precleared with 3E7; G, 3E7 precipitate from lysate precleared with anti-Sca-1; H, Mel-14 precipitate from lysate precleared with R7D4; I, Mel-14 precipitate from lysate precleared with anti-Sca-1; J, Mel-14 precipitate from lysate precleared with 3E7. The gels were run under reducing conditions. Lanes A–G, 18% acrylamide gel; lanes H–J, 7% acrylamide gel. Markers indicate size in kilodaltons.

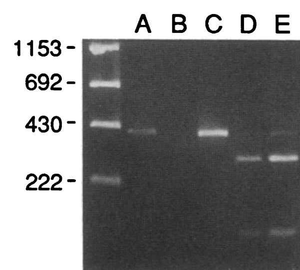

FIG. 4. Ly-6A.2 mRNA is present in Thy-1^{lo}, Lin$^-$, Sca-1$^+$ but not Thy-1^{lo}, Lin$^-$, Sca-1$^-$ mouse bone marrow cells. Ten percent of total single-strand cDNA synthesized from 2×10^3 Thy-1^{lo}, Lin$^-$, Sca-1$^+$ bone marrow cells (lane A), 10^4 Thy-1^{lo}, Lin$^-$, Sca-1$^-$ bone marrow cells (lane B), and 2×10^3 BW5147 thymoma cells (lane C) was subjected to polymerase chain reaction using Ly-6-specific oligonucleotides. Products were visualized with ethidium bromide. Lanes D and E show *Bst*XI digestion products of material run in lanes A and C, respectively. Size markers are in leftmost lane; lengths (bp) are indicated.

Tissue Distribution of Sca-1/Ly-6A.2. In heart (Fig. 5*A*), brain (Fig. 5*B*), and liver (data not shown), the reaction of anti-Sca-1 seemed to be restricted to the vasculature within these organs. Due to the high vascularization in the lung we were unable to determine whether anti-Sca-1 reacted with vasculature only, or also with parenchyma in this tissue (data not shown). Anti-Sca-1 on kidney sections (Fig. 5*C*) showed reactivity with the majority of cortical tubules. Though difficult to discern in frozen tissue sections, the reactivity of anti-Sca-1 appeared to be confined mainly to distal tubules in the cortex. The reactivity on glomeruli was restricted to the glomerular capillaries. The medullary cords in the kidney were mostly positive. The reactivity of anti-Sca-1 on thymus and spleen sections has been described (7).

DISCUSSION

The purification of mouse hematopoietic stem cells to homogeneity has been a long-term objective for researchers studying the regulation of growth and differentiation within the hematopoietic system. With purified stem cells, *in vitro* experiments could be performed to define further the role growth factors, growth factor receptors, and the bone marrow environment play in the maturation and differentiation of hematopoietic cells. Ultimately, it will be possible to determine the factors that regulate the differentiation of stem-cell progeny into distinct lineages. In a previous report (2), we demonstrated that the mAb anti-Sca-1 reacts with mouse hematopoietic stem cells and that it can be used, along with other antibodies, to purify hematopoietic stem cells to homogeneity.

Expression of the Sca-1 antigen was shown not to be restricted to the mouse bone marrow, as cells carrying this antigen could be found within the thymic medulla and spleen (7). In this report we show the molecular characterization of Sca-1 and describe the expression of this antigen on mouse tissues. Lymphocytes stimulated with Con A showed an

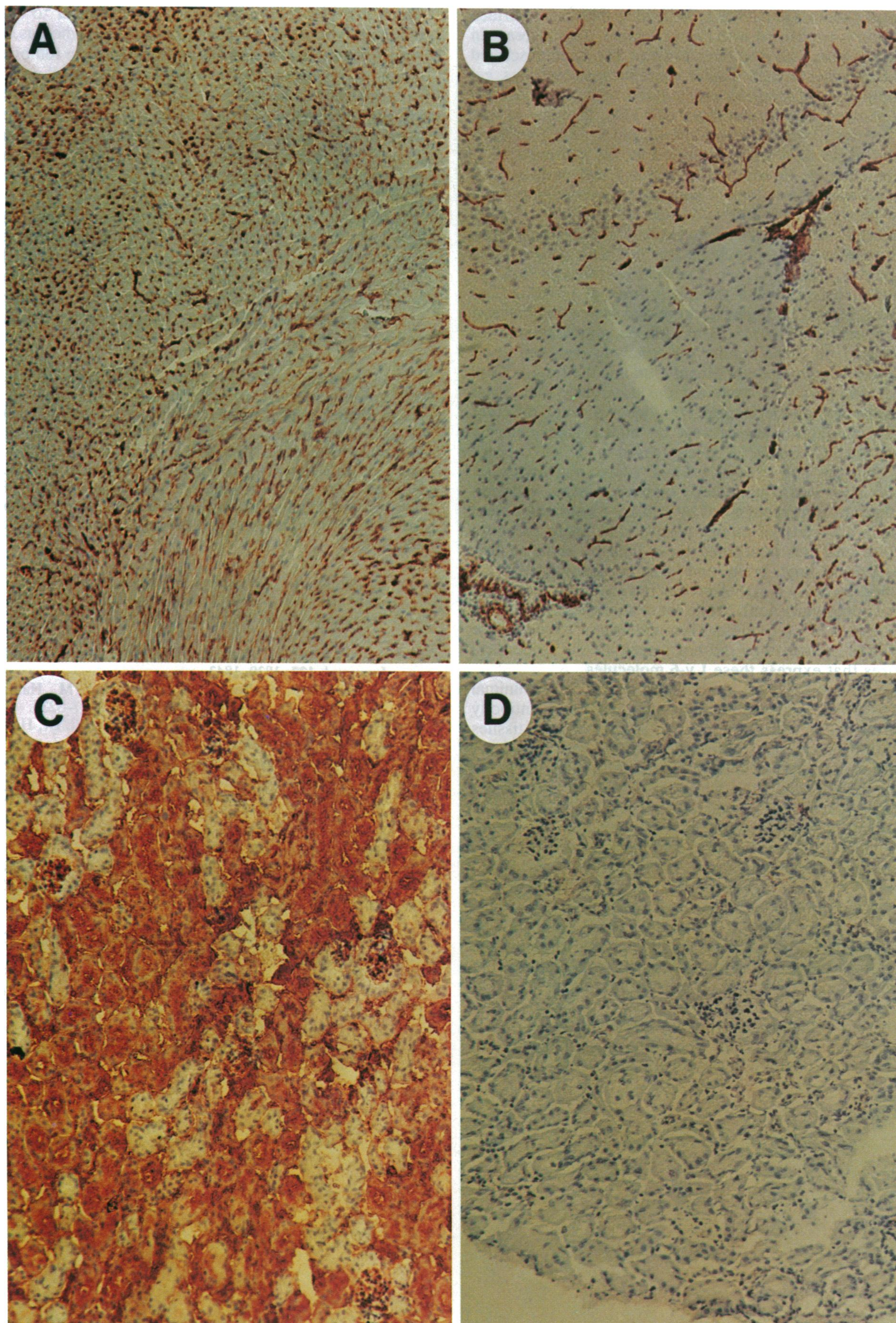

FIG. 5. Immunoperoxidase staining of frozen tissue sections. (*A*) Anti-Sca-1 on heart. (*B*) Anti-Sca-1 on brain. (*C*) Anti-Sca-1 on kidney. (*D*) R7D4 on kidney.

increased expression of Sca-1, indicating that Sca-1 surface expression is correlated with cell activation. This increase in expression after Con A stimulation had also been described for Ly-6 antigens (4, 8, 9). Under nonreducing conditions, gel electrophoresis of antigens precipitated with anti-Sca-1 antibody showed a molecular mass of 8 kDa, identical to that found for the Ly-6A.2 antigen. Sca-1 and Ly-6A.2 antigens migrated at 18 kDa when analyzed in the presence of 2-mercaptoethanol. In addition, anti-Sca-1 and anti-Ly-6A.2 antibodies were shown to preclear each other's antigens from lysates prepared from iodinated and metabolically labeled cells of the *Ly-6*^{b} haplotype. The reactivity of anti-Sca-1 with Con A-stimulated lymphocytes, but not with nonactivated lymphocytes, from BALB/c (*Ly-6*^{a}) mice indicates that this antibody also reacts with the allelic form of Ly-6A.2, Ly-6E.1. The Ly-6E.1 antigen is expressed only on stimulated lymphocytes (ref. 8 and A. Bothwell, personal communication). Based on these results we conclude that anti-Sca-1 reacts with a common determinant found on the Ly-6A.2 and Ly-6E.1 antigens. We excluded the possibility that Sca-1 antigens found on peripheral lymphocytes were different from the Sca-1 antigens present on mouse stem cells by showing that Ly-6 mRNA is expressed in Thy-1^{lo}, Lin^{-}, Sca-1^{+}, but not Thy-1^{lo}, Lin^{-}, Sca-1^{-}, bone marrow cells.

Previously, cytotoxic antibody inhibition assays had indicated that antigens recognized by anti-Ly-6 antibodies were present in a variety of mouse tissues (11). More recently, RNA blot analyses confirmed these data by showing that spleen, kidney, lung, brain, and heart contain mRNAs that can hybridize to Ly-6 cDNA probes (ref. 9 and A. Bothwell, personal communication). While these studies indicated that the expression of Ly-6 antigens is not restricted to cells of the lymphocyte lineage, they did not determine the anatomical structures that express these Ly-6 molecules.

While 3E7 and 34.11.3 antibodies showed no or minimal staining on frozen tissue sections, the anti-Sca-1 antibody reacted strongly with components of the various tissues studied. In three organs a specific staining with anti-Sca-1 antibody was found: in spleen the red pulp area showed a stronger staining than the white pulp area; in thymus the medulla but not the cortex reacted strongly; and in kidney the majority of the tubules were strongly reactive. In brain, heart, and liver the reaction of the antibody was restricted to blood vessels. It therefore seems likely that the brain, heart, and liver RNA that can hybridize to Ly-6 cDNA probes originates in the vasculature and not in the parenchymal cells of these tissues. Sufficient anatomical detail could not be obtained in frozen tissue sections of the lung to determine whether the strong staining found with all components except the terminal and larger bronchi was limited to the vascular components.

So far, no antigenic determinant has been found that is specific for mouse stem cells. Those antigens that have been valuable in the positive selection for stem cells—namely, Thy-1 (1), H-2 (12), and Ly-6 (2)—all appear to share the characteristic of a broad tissue distribution, whereas antigens with a limited tissue distribution were used to remove more-committed progenitor cells during the stem-cell purification experiments. Although the search for a stem-cell-specific antigen undoubtedly will continue, it is clear that by selecting cells expressing a combination of widely distributed antigens a very small subpopulation of bone marrow stem cells ($\approx$0.05% of total bone marrow cells; ref. 2) can be purified to homogeneity.

It is intriguing that both the stem-cell antigens, Thy-1 and Sca-1 (Ly-6), are linked to the lipid bilayer through a phosphatidylinositol bond (13) and seem to be involved in an activation pathway (9, 14). It remains to be determined whether this is merely coincidental or whether this reflects similarities in the function these antigens serve on the stem-cell membrane.

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