Polymorphism of class A scavenger receptors in C57BL/6 mice[1]

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Abstract **Scavenger receptors class A (SR-A) have been hypothesized to regulate the development of atherosclerotic lesions through recognition of modified low density lipoprotein (LDL) and macrophage adhesion to substrata. Supporting data have been collected from studies using the monoclonal antibody 2F8, an antibody developed from the BALB/c strain-derived macrophage cell line, RAW.264. Although 2F8 immunostained both cultured peritoneal macrophages (MPM) and thymic macrophages from Swiss, BALB/c, and DBA/2 mice, no immunostaining was detected in cells and tissues from C57BL/6 mice, one of the most commonly used atherosclerosis-susceptible mouse strains. Similarly, 2F8 detected SR-A protein in MPM by Western blotting in all strains except C57BL/6. However, a guinea pig antiserum developed to a fusion protein of the extracellular SR-A domain detected appropriately sized bands in all strains. Incubation with 2F8 antagonized acetylated low-density lipoprotein (AcLDL)-induced cholesterol esterification in MPM from BALB/c, Swiss, and DBA/2 strains but had no effect on MPM from C57BL/6 mice. Sequencing of SR-A cDNA from C57BL/6 mice demonstrated complete identity with published sequence in the collagen-like domain. However, four single-residue substitutions were noted in the α-helical coiled-coil domain. Site-directed mutagenesis demonstrated that a single substitution (L168S) in this domain accounted for the loss of 2F8 immunoreactivity.—Daugherty, A., S. C. Whitman, A. E. Block, and D. L. Rateri. Polymorphism of class A scavenger receptors in C57BL/6 mice. *J. Lipid Res.* 2000. 41: 1568–1577.**

Supplementary key words lipoprotein metabolism • adhesion • atherosclerosis • macrophages

Class A scavenger receptors (SR-A) were initially implicated in the development of atherosclerosis because of their ability to recognize and endocytose modified lipoproteins (1, 2). This activity results in deposition of excessive cholesterol and cholesteryl esters within macrophages. Full-length SR-A is composed of six domains, which include cytoplasmic, transmembrane, spacer, α-helical coiled-coil, collagen-like, and cysteine-rich (3). The region of the protein that recognizes the modified lipoproteins has been defined by a series of truncated and site-directed mutants (4–6). The binding region is within a specific region of the collagen-like domain, in which a cluster of lysines forms a positively charged groove that interacts with modified lipoproteins, such as acetylated low density lipoprotein (AcLDL) (6).

The hypothesized role of SR-A in atherosclerosis has expanded with the finding that this protein can mediate adhesion of macrophages to specific strata including glycated collagen and serum-coated tissue culture plastic (7, 8). This property was initially identified during the development of a monoclonal antibody, 2F8, which totally inhibited the adhesion of the mouse macrophage cell line, RAW.264, to tissue culture plastic in the absence of divalent cations. Currently, the epitope for 2F8 is unknown. Furthermore, a motif in SR-A that mediates adhesion has not been described.

Delineating mechanisms involved in atherogenesis is increasingly reliant on the use of mice (9). Early studies used inbred strains of mice. Of the inbred strains, C57BL/6 mice were the most susceptible to lesion formation when fed a modified diet (10). Contemporary studies are frequently performed with genetically modified mice that have enhanced plasma cholesterol concentrations, either endogenously or during feeding of modified diets (11–13). It is becoming increasingly evident in apolipoprotein E-deficient (apoE$^{-/-}$) mice that the background strain is important to the development of atherosclerosis in genetically engineered mice (14, 15). However, it is still commonplace for these genetically engineered mice to be backcrossed into the C57BL/6 strain for use in atherosclerosis studies.

SR-A expression can be regulated by many factors, and

Abbreviations: AcLDL, acetylated low-density lipoproteins; apoE$^{-/-}$, apolipoprotein E-deficient; DMEM, Dulbecco's modified Eagle's medium; DNA, deoxyribonucleic acid; LDL, low density lipoprotein; MPM, mouse peritoneal macrophages; RNA, ribonucleic acid; SR-A, class A scavenger receptors.

[1] The accession number of the sequence included in this article is AF203781.

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therefore its presence in atherosclerotic lesions will be dependent on the local milieu of the tissue (2). To study the effect of local regulators on SR-A expression in lesions, we used 2F8 to immunostain aortic tissues from both apoE$^{-/-}$ and LDL receptor$^{-/-}$ mice that were backcrossed 10 times into a C57BL/6 background. In both varieties of genetically engineered mice, we were unable to detect the expression of SR-A in lesions, as defined by immunoreactivity with 2F8. Subsequent investigation demonstrated that 2F8 interacted with the α-helical coiled-coil domain of SR-A and that a polymorphism in the C57BL/6 strain accounted for the inability to be recognized by this monoclonal antibody.

MATERIALS AND METHODS

Mice

Age-matched 4- to 6-week-old male mice were purchased from the National Cancer Institute (Rockville, MD). The following mouse strains were used: Swiss, BALB/c, DBA/2, and C57BL/6. Mice were fed a standard laboratory diet and given water ad libitum. All housing and procedures were approved by the Institutional Care and Use Committee (IACUC) of the University of Kentucky (Lexington, KY).

Generation of apoE$^{-/-}$ of differing backgrounds

ApoE$^{-/-}$ mice (backcrossed 10 times in C57BL/6) were bred to BALB/c. The F_1 generation was interbred and the F_2 generation was genotyped for apoE deficiency. The colony was expanded by interbreeding of apoE $^{-/-}$ mice of the F2 generation. Atherosclerotic lesions in the aortic root were sectioned and immunostained as described previously (16).

Antibodies

The following antibodies and sera were used: 2F8 (rat anti-mouse SR-A; Serotec, Raleigh, NC) (7), isotype-matched rat IgG (IgG2b; Serotec), Mac5-2 (guinea pig antiserum to mouse SR-A; gift from Dr. J. L. Witztum, UCSD) (17), rabbit antiserum to mouse macrophages (Accurate Chemical Company, Westbury, NY), nonimmune guinea pig, and rabbit serum (Sigma).

Harvest of peritoneal macrophages and thymus tissue

Mice were anesthetized and exsanguinated. Mouse peritoneal macrophages (MPM) were harvested via peritoneal lavage using sterile saline (5 ml). Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) containing heat-inactivated fetal bovine serum (10%, v/v; GIBCO-BRL), penicillin, and streptomycin, and plated in Lab-Tek eight-chamber glass slides (Nunc, Roskilde, Denmark). Cells were incubated at 37°C with 5% CO_2 and 95% air. After 48 h of incubation, medium was removed, and cells were prepared for immunocytochemistry. The cells were washed five times with phosphate-buffered saline (PBS) and fixed with ethanol for 10 min on ice.

Thymus tissue was collected as described previously (18).

Immunocytochemistry

Immunocytochemistry was performed on cells and frozen sections by using Vector (Burlingame, CA) avidin-biotin complex Elite kits as described previously (19). Immunoreactivity was visualized with the red chromagen, amino-ethyl carbazole (Biomeda, Foster City, CA). SR-A protein was detected with 2F8, and macrophages were detected with rabbit antiserum to mouse macrophages.

Western blotting

MPM were harvested as described (20), pelleted, and dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer for Mac5-2 blotting, or nonreducing buffer for 2F8 blotting. Samples were stored immediately at −20°C. Cell proteins from MPM were resolved under reducing or nonreducing conditions on a 10% (w/v) or 7.5% (w/v) SDS-polyacrylamide gel, respectively, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Acton, MA). The immunoreactivity of the proteins on the membranes was assessed with Mac5-2 or 2F8. Horseradish peroxidase-conjugated antibodies were used for detection of the primary antibodies. Supersignal chemiluminescent substrate (Pierce, Rockville, IL) was used per manufacturer instructions to detect reactivity. Luminescence was detected with a Kodak Imaging Station 440CL (Eastman Kodak, Rochester, NY).

AcLDL-induced cholesterol esterification

LDL (d = 1.019–1.063 g/ml) was prepared from fresh human plasma by differential ultracentrifugation as described previously (21). LDL acetylation was performed as described by Basu et al. (1).

Cholesteryl ester synthesis in MPM was determined by the method of Brown et al. (22) as described previously (23). Briefly, cells were incubated with the stated concentrations of AcLDL protein per milliliter and 0.1 mM [^{3}H]oleate for 5 h at 37°C in DMEM without serum. The medium was removed, and the cells were washed and subjected to lipid extraction with hexane–isopropanol 3:2 (v/v). The lipid extracts were evaporated to dryness with N_2, and subsequently redissolved in $CHCl_3$–methanol 2:1 (v/v), and separated by thin-layer chromatography. The cholesteryl ester band was isolated and radioactivity content was quantified.

RT-PCR

Ribonucleic acid (RNA) from DBA/2 and C57BL/6 mice was harvested by the SV total RNA isolation system (Promega, Madison, WI). Integrity and quantity of RNA were assessed by gel electrophoresis and spectrophotometric measurements. The Access reverse transcriptase-polymerase chain reaction (RT-PCR) system (Promega) was used to generate deoxyribonucleic acid (DNA) products. The primers used to generate full-length MSR-A were as follows: 5′-CGCGGGATGACAAAAGAGATGACAGAG-3′ and 5′-CGCGGGTTGGTTTCATAATTGTAATTT-3′. All RT-PCR DNA products were sequenced.

cRNA probe synthesis

Two biotinylated cRNA probes were generated to detect different regions of mRNA for mouse SR-A. One cRNA hybridizes to the collagen-like region spanning nucleotides 768–982 of the SR-A mRNA sequence. The cDNA template used to generate this 214-nucleotide mouse SR-A cRNA probe, provided by T. Kodama, University of Tokyo, was isolated from the mouse macrophage cell line P388D1, which is derived from DBA/2 mice. The other cRNA hybridizes to the α-helical coiled-coil region spanning nucleotides 48 to 349. The cDNA template used to generate this 302-nucleotide cRNA probe was constructed using the Lig'nScribe™ RNA polymerase promoter addition kit (Ambion, Austin, TX). Briefly, this kit uses a two-step process of ligation and PCR to generate a cDNA product containing both the PCR fragment of interest coupled to a sequence encoding the T7 polymerase promoter. Biotin-labeled cRNA probes were generated during transcription reactions using a T7 polymerase, the mouse cDNA templates, and the Renaissance RNA biotin labeling kit (New England Nuclear, Boston, MA) according to the manufacturer instructions.

Ribonuclease protection assay

Biotinylated SR-A cRNAs were hybridized to total cellular RNA in buffer (Ambion RPA III kit) at 55°C for 16 h. Samples were incubated with RNase A/T1 for 30 min at 37°C, followed by addition of an RNase inactivation/precipitation III solution. Hybridized cRNA-mRNA fragments were precipitated at −20°C, and suspended in gel loading buffer (Ambion). Protected fragments were separated on an 8 M urea-5% polyacrylamide gel. RNA fragments were transferred to positively charged nylon membranes, using a semidry electroblotter, and immobilized by UV crosslinking. Protected fragments were visualized with a streptavidin-conjugated alkaline phosphatase and CDP-Star (Ambion). The chemiluminescent signal was detected with a Kodak Digital Science™440CF Image Station.

DNA sequencing

Primer synthesis and DNA sequencing were done by the Macromolecular Structure Analysis Facility at the University of Kentucky. Primers were synthesized with an ABI 394 DNA synthesizer (Perkin-Elmer Biosystems, Foster City, CA). dRhodamine Terminator Cycle Sequencing Ready Reaction with Amplitaq DNA polymerase (Perkin-Elmer Biosystems) was used for DNA

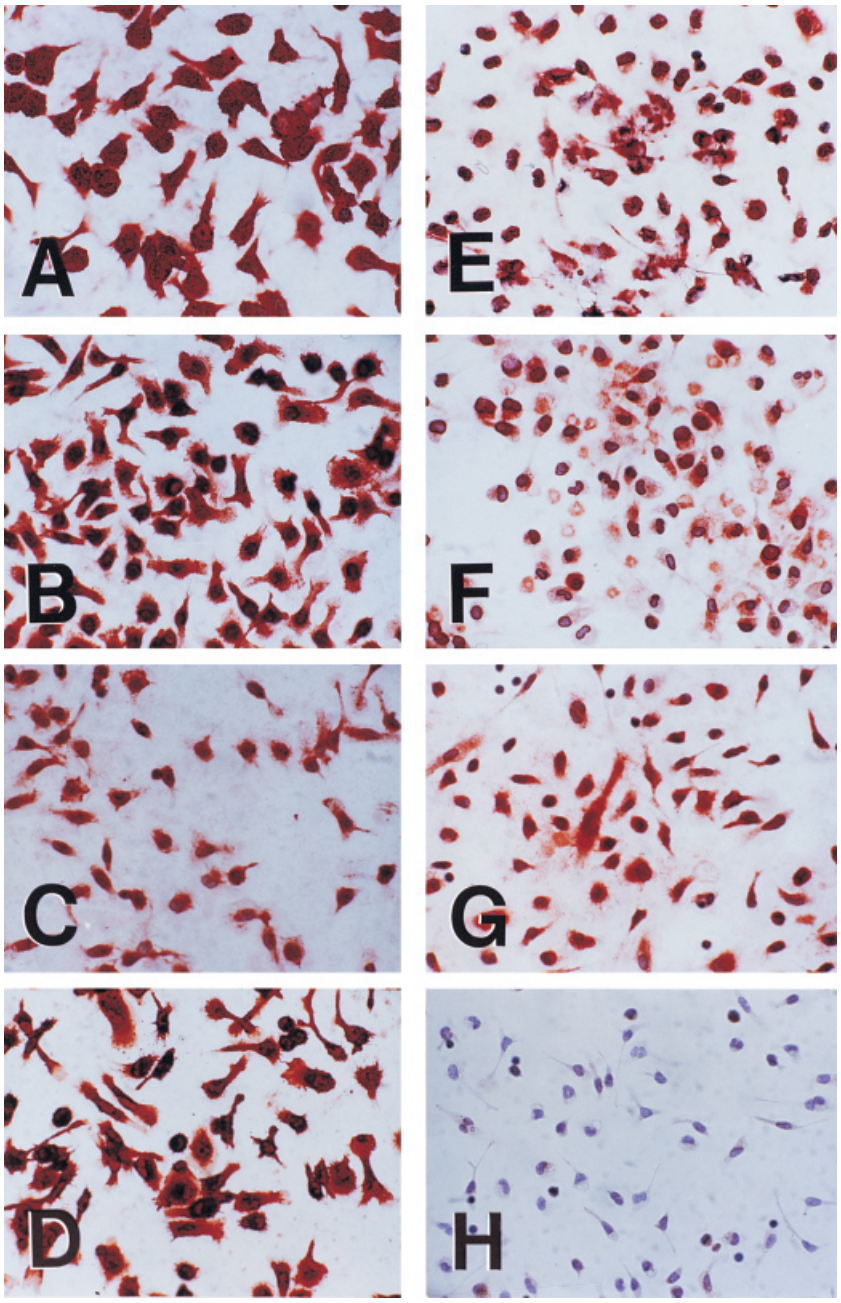

Fig. 1. 2F8 did not immunostain MPM from C57BL/6 mice. MPM were harvested and maintained in cell culture for 48 h from Swiss (A and E), BALB/c (B and F), DBA/2 (C and G), and C57BL/6 (D and H). Immunoreactive cells were visualized with red chromagen, and nuclei were counterstained with hematoxylin. Immunostaining using a rabbit antiserum to mouse macrophages indicated that all adherent cells were macrophages (A–D). The detection of SR-A using monoclonal rat antimouse 2F8 was present in Swiss, BALB/c, and DBA/2 (E–G) but absent in C57BL/6 cells (H). Cells were photographed at ×400 original magnification.

sequencing reactions. Parameters of the PCR were 98°C for 1 min; 55°C for 30 sec; and 60°C for 4 min. An ABI Prism 377 DNA sequencer (Perkin-Elmer Biosystems) was used to collect sequencing data.

Generation of fusion proteins and site-directed mutants

The extracellular region of SR-A including the α-helical coil-coiled and collagen-like domain (bp 343–1044) of the full-length mouse SR-A cDNA (kindly provided by T. Kodama) was engineered with 5′ *Sal*I and 3′ *Not*I restriction site ends by PCR. The primer set used was 5′-CGCGGGGTCGACTTTACAATTATC ATGGCA and 5′-CGCGGGCGCGGCCGCTACACTCCCCTTC TC. The resultant PCR product was subcloned into a pET23b plasmid vector and transformed into DE3 cells per manufacturer instructions (Novagen, Madison, WI). This vector generates fusion proteins with a C-terminal T7 tag and an N-terminal His tag.

GeneEditor in vitro site-directed mutagenesis system (Promega) was used to generate SR-A mutations. The following primers were used to generate mutations A120E, Q130E, L168S, and H202N, respectively: 5′-ATTATCATGGAACACATGAAG-3′; 5′-GGAGAGAATCGAAAGCATTTC-3′; 5′-TCCTTGATTTCGTC AGTCCAG-3′; and 5′-AGAAACACTGAATGTCAGAGT-3′. The extracellular domain (bp 343–1044) of DBA/2 SR-A cDNA was used as the template. The mutagenesis protocol was followed per manufacturer instructions. Positive transformants were confirmed by PCR screening and sequencing. Bacterial lysates were used in Western blotting.

RESULTS

The most widely used monoclonal antibody against mouse SR-A is 2F8, which was developed from rats injected with the BALB/c-derived macrophage cell line, RAW.264 (7). On the basis of our inability to detect SR-A using 2F8 in atherosclerotic lesions from either apoE$^{-/-}$ or LDL receptor$^{-/-}$ mice, we performed immunocytochemical analysis of tissue from a series of strains. Immunocytochemical analysis with 2F8 was performed on MPM and thymus. The former is the cell type originally used to described SR-A activity, while the latter was previously described to have one of the highest levels of expression in medullary macrophages (18). The strains of mice studied were as follows: *a*) Swiss, which is one of the most widely used strains for isolating MPM; *b*) BALB/c, which is the strain from which was derived the RAW.264 cell line that

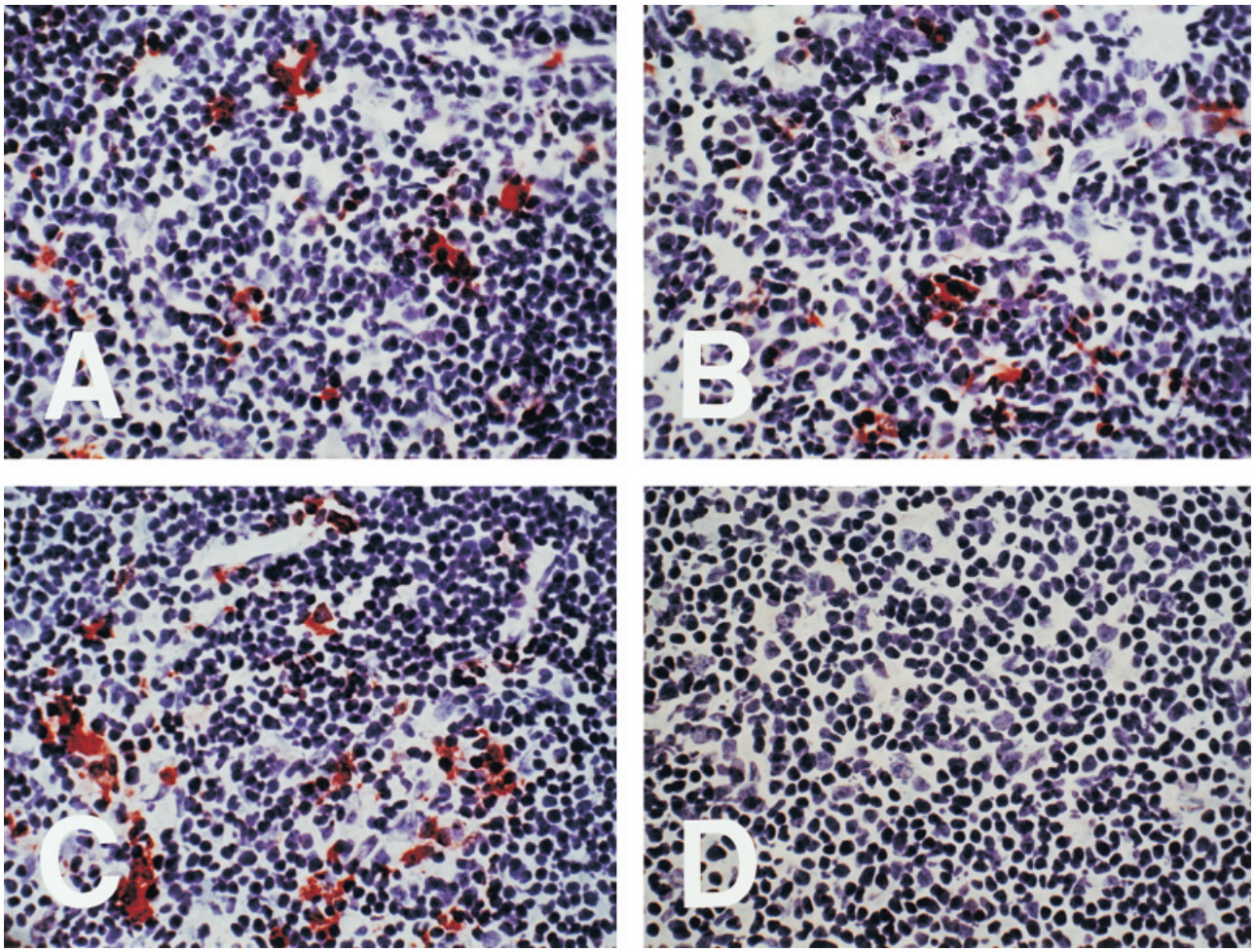

Fig. 2. 2F8 did not immunostain thymic macrophages from C57BL/6 mice. Thymus was acquired from Swiss (A), BALB/c (B), DBA/2 (C), and C57BL/6 (D) mouse strains. Immunoreactive tissues were visualized with red chromagen, and nuclei were counterstained with hematoxylin. Swiss (A), BALB/c (B), and DBA/2 (C) showed reactivity with 2F8. C57BL/6 (D) thymus tissue showed no reactivity with 2F8. Tissues were photographed at ×400 original magnification.

was used to generate 2F8; *c*) DBA/2, which is the strain that originated the P388D1 cell line, which was originally used to sequence mouse SR-A cDNA (4, 6, 24); and *d*) C57BL/6, which is the strain used in many atherosclerosis studies.

All cells that adhered to the plates after peritoneal lavage from the four strains were immunostained with the macrophage antiserum (**Fig. 1 A–D**). However, whereas MPM from Swiss, BALB/c, and DBA/2 were uniformly immunostained with 2F8, no specific immunostaining could be detected in cells from C57BL/6 (Fig. 1E and F) compared with controls that were incubated with an isotype-matched rat monoclonal antibody (data not shown). 2F8 also immunostained the BALB/c-derived cell lines J774 and RAW.264, and the DBA/2-derived cell line P388D1 (data not shown). Similarly, strong immunostaining was present in the thymic medulla of tissue from Swiss, BALB/c, and DBA/2, but none was detected in tissue from C57BL/6 (**Fig. 2**).

To determine whether the inability to detect SR-A was related to an absence of the protein, Western blotting was performed on whole-cell extracts from MPM of the four strains, using both a polyclonal antiserum against mouse SR-A, Mac5-2, and 2F8. The polyclonal antiserum, Mac5-2, clearly detected a band of the appropriate molecular weight in MPM from all four strains. In agreement with the immunocytochemical studies, 2F8 detected SR-A in all but the C57BL/6 strain (**Fig. 3**).

A further verification of the inability of 2F8 to react with MPM from C57BL/6 was derived from studies on antagonism of AcLDL-induced cholesterol esterification. 2F8 partially antagonized AcLDL-induced cholesteryl ester deposition in BALB/c in a manner similar to the effect noted previously on RAW.264 cells (7). In MPM from Swiss and DBA/2, 2F8 reduced AcLDL-induced cholesterol esterification to a level observed in the absence of lipoprotein. In contrast, 2F8 had no effect on AcLDL-induced cholesterol esterification in MPM from C57BL/6, even though the extent of cholesterol esterification was similar to that observed in cells from Swiss and DBA/2 strains (**Fig. 4**).

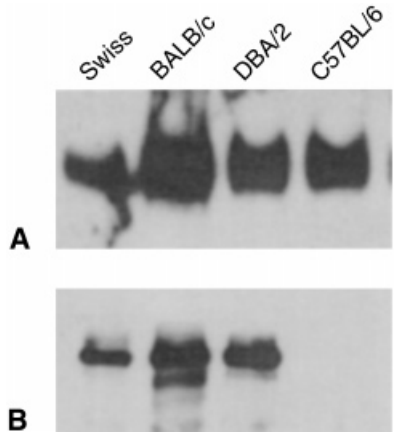

Fig. 3. SR-A in MPM from C57BL/6 was not detected by 2F8, but was by an SR-A guinea pig polyclonal antibody Mac5-2. Whole-cell extracts from the four selected mouse strains (Swiss, BALB/c, DBA/2, C57BL/6) were loaded on SDS-polyacrylamide gels based on equivalence of β-actin. Membranes were subsequently immunoblotted for SR-A using Mac5-2 (A) and 2F8 (B). Primary antibodies were detected with biotinylated anti-guinea pig and anti-rat secondary antibodies, respectively. Avidin-biotin complexes were used to enhance signal from chemiluminescent substrate.

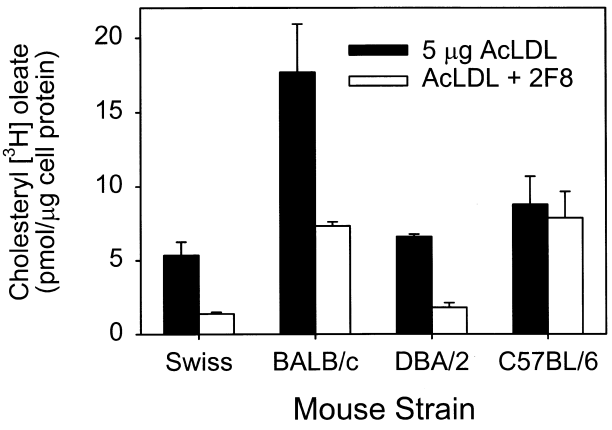

Fig. 4. 2F8 inhibits AcLDL-stimulated cholesterol esterification in MPM from all strains except C57BL/6 mice. AcLDL (5 μg/ml) stimulated cholesterol esterification was quantified in the absence (closed columns) or presence (open columns) of 2F8 (10 μg/ml).

To determine whether the sequence of C57BL/6 SR-A cDNA differed from the other strains, our initial approach was to perform ribonuclease protection assays and compare mRNA obtained from DBA/2 with C57BL/6. One probe hybridized to the collagen-like domain; while the other spanned part of the α-helical coiled-coil domain,

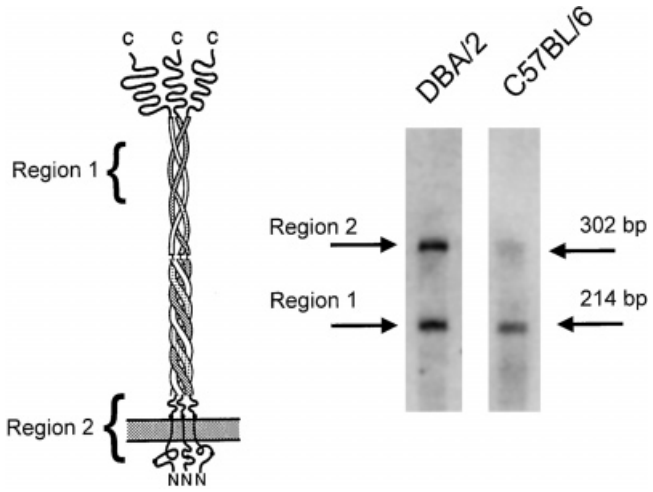

Fig. 5. SR-A mRNA from C57BL/6 mice differs from DBA/2 mice. Ribonuclease protection assays were performed using two probes. One probe (Region 1) spanned from the α-helical coiled-coil region to part of the collagen-like region (protected fragment, 214 nucleotides). The other probe (region 2) spanned the following regions: cytoplasmic tail, transmembrane, spacer, and partial α-helical coiled-coil (protected fragment, 302 nucleotides). Protected fragments for both regions were detected in mRNA from DBA/2, but only region 1 produced a protected fragment in mRNA from DBA/2 mice.

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DBA/2       ATGACAAAAGAGATGACAGAGAATCAGAGGCTCTGCCCTCATGAACGAGAGGATGCTGACTGCAGTTCAG   70
C57BL/6     ATGACAAAAGAGATGACAGAGAATCAGAGGCTCTGCCCTCATGAACGAGAGGATGCTGACTGCAGTTCAG

DBA/2       AATCCGTGAAATTTGACGCACGTTCAATGACAGCATCCCTTCCTCACAGCACTAAAAATGGCCCCTCCGT  140
C57BL/6     AATCCGTGAAATTTGACGCACGTTCAATGACAGCATCCCTTCCTCACAGCACTAAAAATGGCCCCTCCCT

DBA/2       TCAGGAGAAGTTGAAGTCCTTCAAGGCTGCCCTCATTGCTCTCTACCTCCTTGTGTTTGCAGTACTAATA  210
C57BL/6     TCAGGAGAAGTTGAAGTCCTTCAAGGCTGCCCTCATTGCTCTCTACCTCCTTGTGTTTGCAGTACTAATA

DBA/2       CCTGTTGTTGGAATAGTGACAGCTCAGCTTTTGAATTGGGAAATGAAGAACTGCTTAGTTTGTTCACGTA  280
C57BL/6     CCTGTTGTTGGAATAGTAACAGCTCAGCTTTTGAATTGGGAAATGAAGAACTGCTTAGTTTGTTCACGTA

DBA/2       ACACAAGTGATACATCTCAAGGTCCTATGGAAAAAGAAAATACCAGTAACGTGGAAATGAGATTTACAAT  350
C57BL/6     ACACAAGTGACACATCTCAAGGTCCTATGGAAAAAGAAAATACCAGTAAGGTGGAAATGAGATTTACAAT

DBA/2       TATCATGGCACACATGAAGGACATGGAGGAGAGAATCCAAAGCATTTCAAACTCAAAAGCCGACCTTATA  420
C57BL/6     TATCATGGAACACATGAAGGACATGGAGGAGAGAATCGAAAGCATTTCAAACTCAAAAGCCGACCTTATA

DBA/2       GACACGGGACGCTTCCAGAATTTCAGCATGGCAACTGACCAAAGACTTAATGATATTCTTCTGCAGTTAA  490
C57BL/6     GACACGGGACGCTTCCAGAATTTCAGCATGGCAACTGACCAAAGACTTAATGATATTCTTCTGCAGTTAA

DBA/2       ATTCCTTGATTTTGTCAGTCCAGGAACATGGGAATTCACTGGATGCAATCTCCAAGTCCTTGCAGAGTCT  540
C57BL/6     ATTCCTTGATTTCGTCAGTCCAGGAACATGGGAATTCACTGGATGCAATCTCCAAGTCCTTGCAGAGTCT

DBA/2       GAATATGACACTGCTTGATGTTCAACTCCATACAGAAACACTGCATGTCAGAGTCCGTGAATCTACAGCA  630
C57BL/6     GAATATGACACTGCTTGATGTTCAACTCCATACAGAAACACTGAATGTCAGAGTCCGTGAATCTACAGCA

DBA/2       AAGCAACAGGAGGACATCAGTAAATTGGAGGAACGTGTGTACAAAGTATCAGCAGAAGTCCAGTCTGTGA  700
C57BL/6     AAGCAACAGGAGGACATCAGTAAATTGGAGGAACGTGTGTACAAAGTATCAGCAGAAGTCCAGTCTGTGA

DBA/2       AAGAAGAACAAGCGCACGTGGAACAGGAAGTAAAACAGGAAGTGAGAGTATTGAACAACATCACCAACGA  770
C57BL/6     AAGAAGAACAAGCGCACGTGGAACAGGAAGTAAAACAGGAAGTGAGAGTATTGAACAACATCACCAACGA

DBA/2       CCTCAGACTGAAGGACTGGGAACACTCACAGACACTGAAAAACATCACCTTCATTCAAGGGCCTCCTGGA  840
C57BL/6     CCTCAGACTGAAGGACTGGGAACACTCACAGACACTGAAAAACATCACCTTCATTCAAGGGCCTCCTGGA

DBA/2       CCCCAAGGTGAAAAGGGAGACAGAGGGCTTACTGGACAAACTGGTCCACCTGGTGCTCCAGGAATAAGAG  910
C57BL/6     CCCCAAGGTGAAAAGGGAGACAGAGGGCTTACTGGACAAACTGGTCCACCTGGTGCTCCAGGAATAAGAG

DBA/2       GTATTCCAGGTGTTAAAGGTGATCGGGGACAAATTGGCTTCCCTGGAGGTCGAGGAAACCCAGGAGCACC  980
C57BL/6     GTATTCCAGGTGTTAAAGGTGATCGGGGACAAATTGGCTTCCCTGGAGGTCGAGGAAACCCAGGAGCACC

DBA/2       AGGAAAGCCAGGGAGGTCGGGATCTCCTGGACCTAAAGGACAAAAGGGAGAGAAGGGGAGTGTAGGCGGA 1050
C57BL/6     AGGAAAGCCAGGGAGGTCGGGATCTCCTGGACCTAAAGGACAAAAGGGAGAGAAGGGGAGTGTAGGCGGA

DBA/2       TCAACCCCCCTTAAGACAGTTCGACTGGTTGGTGGTAGTGGAGCCCATGAGGGCCGAGTGGAGATCTTCC 1120
C57BL/6     TCAACCCCCCTTAAGACAGTTCGACTGGTTGGTGGTAGTGGAGCCCATGAGGGCCGAGTGGAGATCTTCC

DBA/2       ACCAAGGCCAGTGGGGCACAATCTGTGATGATCGCTGGGATATACGGGCTGGACAAGTTGTCTGCCGGAG 1190
C57BL/6     ACCAAGGCCAGTGGGGCACAATCTGTGATGATCGCTGGGATATACGGGCTGGACAAGTTGTCTGCCGGAG

DBA/2       TCTAGGATACCAAGAAGTTCTAGCTGTGCACAAGAGAGCTCACTTTGGACAAGGTACTGGTCCAATATGG 1260
C57BL/6     TCTAGGATACCAAGAAGTTCTAGCTGTGCACAAGAGAGCTCACTTTGGACAAGGTACTGGTCCAATATGG

DBA/2       CTGAATGAAGTGATGTGTTTTGGAAGAGAATCATCTATTGAGAACTGTAAAATCAACCAGTGGGGAGTAC 1330
C57BL/6     CTGAATGAAGTGATGTGTTTTGGAAGAGAATCATCTATTGAGAACTGTAAAATCAACCAGTGGGGAGTAC

DBA/2       TAAGCTGTTCACATTCAGAAGATGCTGGGGTCACTTGTACTTCATAATGTATCATATTTTCAATGGCATT 1400
C57BL/6     TAAGCTGTTCACATTCAGAAGATGCTGGGGTCACTTGTACTTCATAATGTATCATATTTTCAATGGCATT

DBA/2       TATGAAATTACAATTATGAAACCAA                                              1425
C57BL/6     TATGAAATTACAATTATGAAACCAA
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Fig. 6. Nucleotide sequence of SR-A cDNA in DBA/2 and C57BL/6 mice. Nucleotide differences are represented in boldface.

spacer, transmembrane, and part of the cytoplasmic tail. A protected fragment for the collagen-like domain was demonstrated in approximately equivalent abundance in both strains. A protected fragment with the other probe was discernible for the DBA/2 strain, but not for C57BL/6 (**Fig. 5**).

Subsequently, we sequenced the SR-A cDNA from DBA/2 and C57BL/6. A sequence derived from DBA/2 was found to be identical to that published by other authors for a sequence from P388D1 cells, which are derived from DBA/2 mice (4, 6, 24). However, there were a small number of single base changes when compared with the sequences derived from C57BL/6 (**Fig. 6**). On the basis of the predicted amino acid sequence, these changes would result in six single-residue changes, one in the cytoplasmic domain, one in the spacer domain, and four in the α-helical coiled-coil domain (**Fig. 7**).

To determine which of these amino acid changes ac-

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DBA/2    MTKEMTENQRLCPHEREDADCSSESVKFDARSMTASLPHSTKNGPSVQEKLKSFK
C57BL/6  MTKEMTENQRLCPHEREDADCSSESVKFDARSMTASLPHSTKNGPSLQEKLKSFK
         <----------------cytoplasmic tail---------------------->

DBA/2    AALIALYLLVFAVLIPVVGIVTAQLL   NWEMKNCLVCSRNTSDTSQGPMEKEN
C57BL/6  AALIALYLLVFAVLIPVVGIVTAQLL   NWEMKNCLVCSRNTSDTSQGPMEKEN
         <-----transmembrane------>   <-------------spacer------

DBA/2    TSNVEMR  FTIIMAHMKDMEERIQSISNSKADLIDTGRFQNFSMATDQRLNDIL
C57BL/6  TSKVEMR  FTIIMEHMKDMEERIESISNSKADLIDTGRFQNFSMATDQRLNDIL
         ------>  <-----------------------------------------------

DBA/2    LQLNSLILSVQEHGNSLDAISKSLQSLNMTLLDVQLHTETLHVRVRESTAKQQED
C57BL/6  LQLNSLISSVQEHGNSLDAISKSLQSLNMTLLDVQLHTETLNVRVRESTAKQQED
         --------------alpha-helical coiled-coil----------------

DBA/2    ISKLEERVYKVSAEVQSVKEEQAHVEQEVKQEVRVLNNITNDLRLKDWEHSQTLK
C57BL/6  ISKLEERVYKVSAEVQSVKEEQAHVEQEVKQEVRVLNNITNDLRLKDWEHSQTLK
         ------------------------------------------------------

DBA/2    NITFIQ  GPPGPQGEKGDRGLTGQTGPPGAPGIRGIPGVKGDRGQIGFPGGRGN
C57BL/6  NITFIQ  GPPGPQGEKGDRGLTGQTGPPGAPGIRGIPGVKGDRGQIGFPGGRGN
         ------>  <------------------------------collagen-like----

DBA/2    PGAPGKPGRSGSPGPKGQKGEKGSV  GGSTPLKTVRLVGGSGAHEGRVEIFHQG
C57BL/6  PGAPGKPGRSGSPGPKGQKGEKGSV  GGSTPLKTVRLVGGSGAHEGRVEIFHQG
         ------------------------->  <----------------------------

DBA/2    QWGTICDDRWDIRAGQVVCRSLGYQEVLAVHKRAHFGQGTGPIWLNEVMCFGRES
C57BL/6  QWGTICDDRWDIRAGQVVCRSLGYQEVLAVHKRAHFGQGTGPIWLNEVMCFGRES
         ----------------------cysteine-rich----------------------

DBA/2    SIENCKINQWGVLSCSHSEDAGVTCTS
C57BL/6  SIENCKINQWGVLSCSHSEDAGVTCTS
         --------------------------->
```

Fig. 7. Predicted amino acid sequence of SR-A in DBA/2 and C57BL/6 mice. Amino acid differences are represented in boldface.

counted for the lack of immunoreactivity of 2F8 for SR-A protein from C57BL/6, SR-A fusion proteins were generated to specific regions, using the sequence determined for DBA/2. 2F8 did not react with a fusion protein of the cytoplasmic domain, but did against one that spanned the entire α-helical coiled-coil and collagen-like domains. Therefore, the inability of 2F8 to react with SR-A protein from C57BL/6 was due to one of the four single-residue differences in the α-helical coiled-coil domain. To identify the specific residue, site-directed mutagenesis was performed to convert the DBA/2 sequence to that of C57BL/6. Cell lysates of all four mutants reacted with the guinea pig polyclonal antisera. 2F8 immunoreacted with the A120E, Q130E, and H202N mutants. However, it failed to react with the L168S mutant, thus defining a region of the epitope for this monoclonal antibody (**Fig. 8**).

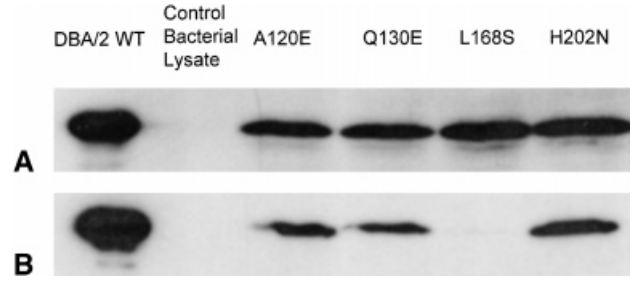

Fig. 8. 2F8 did not bind to SR-A protein containing mutation L168S. The indicated residues were mutated from the SR-A sequence of the DBA/2 strain to that of the C57BL/6 strain. Control bacterial lysate contained plasmid without SR-A cDNA. Mac5-2 recognized all mutations (A); while 2F8 did not recognize the L168S mutation (B).

To determine whether we were able to detect SR-A in atherosclerotic lesions from mice of mixed backgrounds, we cross-bred C57BL/6 apoE$^{-/-}$ mice with BALB/c mice. As can be seen in **Fig. 9**, immunostaining for SR-A was clearly distinguishable in macrophage-rich lesions of apoE$^{-/-}$ mice in a mixed C57BL/6 and BALB/c, but not in equivalent lesions of mice with this genotype in a C57BL/6 background.

DISCUSSION

SR-A appears to have an important role in the development of atherosclerotic lesions (2, 25). Both its function as a mediator of lipoprotein uptake and cellular adhesion could contribute to this role in atherogenesis, although the relative contributions of each of these properties to the disease process have not been defined. The identification of the domains that mediate these independent functions will permit the resolution of the contribution of each of these properties to the disease process.

In view of the proposed importance of SR-A in atherogenesis, we sought to determine whether expression of the protein was regulated locally during atherosclerotic lesion development, because expression is regulated by many factors (2). An extensive number of lesions were examined from two commonly used models of atherosclerosis, LDL receptor$^{-/-}$ and apoE$^{-/-}$ mice. Both these genetic deficiencies were bred back into a C57BL/6 strain, the most commonly used strain in atherosclerosis studies. Immunostaining of lesions from these mice with the monoclonal antibody 2F8 failed to detect the protein.

To understand the cause for the lack of 2F8 reactivity, immunostaining was performed on cells and tissues that

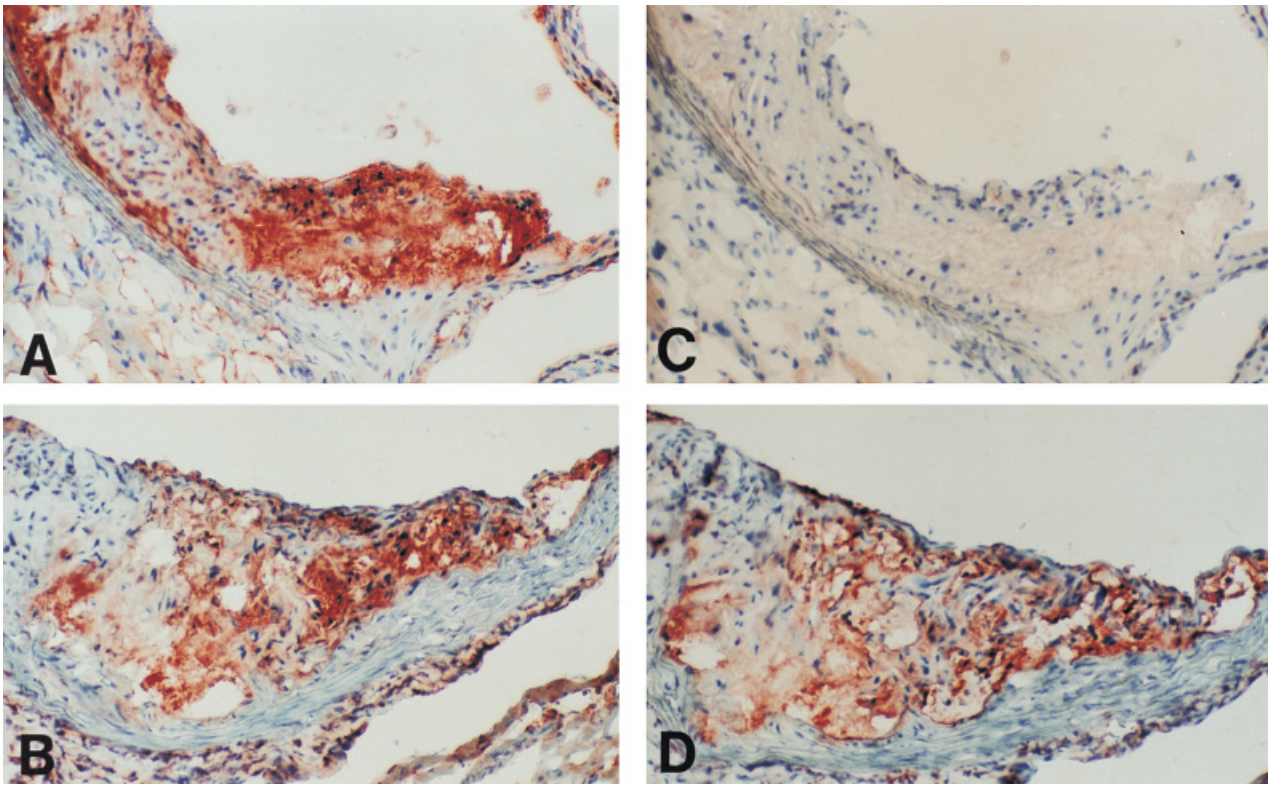

Fig. 9. Atherosclerotic lesions from apoE$^{-/-}$ mice of mixed C57BL/6 × BALB/c strains reacts with 2F8. Immunostaining was performed on atherosclerotic lesions from apoE$^{-/-}$ mice of a C57BL/6 (A and C) or mixed C57BL/6 × BALB/c (B and D) strain for macrophages (A and B) and SR-A (C and D). Immunoreactive cells were visualized with red chromagen, and nuclei were counterstained with hematoxylin. Sections were photographed at ×200 original magnification.

previously have been shown to express this protein abundantly. In addition to the well-known presence of SR-A in MPM, a survey of tissues demonstrated that SR-A was highly expressed in the thymic medulla, the outer marginal zone of the spleen, and lung alveolar macrophages (7). On the basis of these findings, the thymus and MPM were selected as tissues to compare SR-A expression among strains. Although the striking lack of immunostaining in tissue from C57BL/6 mice compared with other strains could have been attributable to lack of the protein, analysis using an SR-A polyclonal antisera demonstrated an approximately equal abundance of protein. This inability of 2F8 to interact with C57BL/6 SR-A was due to a single amino acid substitution in the α-helical coiled-coil region.

2F8 had a striking ability to totally ablate AcLDL-induced cholesterol esterification in specific strains. This ability of 2F8 to ablate SR-A-mediated metabolism of AcLDL has been noted previously (26). Others have demonstrated only a partial inhibition of AcLDL interactions with macrophages (7). Therefore, our finding of partial inhibition of AcLDL-induced cholesterol esterification in BALB/c mice, which is the strain from which RAW.264 cells are derived, is in agreement with Fraser, Hughes, and Gordon (7). In contrast to our complete ablation of AcLDL-induced cholesterol esterification in MPM from Swiss and DBA/2 mice, degradation of radioiodinated AcLDL was reduced by only ~66% in SR-A-deficient mice (26). The differing extent to which SR-A regulation affects modified LDL metabolism may be due to strain-specific differences in the relative expression of different classes of scavenger receptors leading to SR-A independent mechanisms mediating the metabolism of AcLDL (27).

Previous studies have demonstrated that AcLDL binds to a lysine cluster in the collagen-like domain. In the linear sequence, this region is distant from the site that we have identified to bind 2F8. It has been proposed that the region between the α-helical coiled-coil and the collagen-like region serves as a highly flexible hinge region (28). Furthermore, at physiological pH, the α-helical coiled-coil and collagen-like domains are juxtapositioned (28). In this conformation, it seems likely that binding of 2F8 to the α-helical coiled-coil domain could cause steric hindrance that would inhibit the binding of AcLDL to the collagen-like region.

2F8 was initially identified through its ability to inhibit SR-A-mediated adhesion (7). The data generated in the present study have demonstrated 2F8 interacts with the α-helical coiled-coil domain of SR-A. At present, we have not identified whether this region is responsible for the adhesion or whether 2F8 binding to this region causes steric hindrance of the binding process. Preliminary evidence would also suggest that the effect of 2F8 is the result of steric hindrance, because a peptide of a region of the

collagen-like domain inhibits the binding of SR-A-transfected U937 cells to glycated collagen (8). Another SR-A monoclonal antibody, D2, has been mapped to an approximate epitope by using a series of truncated mutants (6). Of interest, it appears that this antibody interacts with a region similar to the region on the mouse protein with which 2F8 interacts. Therefore, this region of the molecule appears to be highly immunogenic. It is unknown whether the D2 monoclonal antibody inhibits adhesion in the same manner as 2F8.

The role of SR-A in atherogenesis was initially demonstrated in SR-A$^{-/-}$ mice on an apoE$^{-/-}$ background, in which lesion size in the aortic root was decreased even in the face of an increase in plasma cholesterol concentrations (26). Subsequent studies have described effects of SR-A deficiency and overexpression on the development of atherosclerosis in other backgrounds including inbred C57BL/6 (29), LDL receptor$^{-/-}$ (29–31), and apoE3-Leiden (32). These studies have demonstrated that the presence of SR-A may either promote or decrease the development of atherosclerotic lesions depending on the accompanying genetic manipulation. Therefore, the effect of SR-A on the atherogenic process may depend on the local milieu within lesions.

In conclusion, this study has demonstrated that the commonly used monoclonal antibody, 2F8, interacts with a region within the α-helical coiled-coil domain of SR-A in all mouse strains tested, with the exception of C57BL/6. The inability of 2F8 to bind SR-A from C57BL/6 mice was subsequently determined to be the direct result of a newly identified polymorphism within this region. Although the polymorphism in SR-A from C57BL/6 resulted in decreased reactivity of 2F8, this did not affect the function of the receptor to interact with AcLDL. Given the common use of this strain in atherosclerosis studies, the inability of 2F8 to recognize SR-A means that this antibody will not be useful in describing local regulation of the protein as it relates to developing atherosclerotic lesions.

This work was supported by an NIH grant to A.D. (RO1 HL55487) and an American Heart Association (Ohio Valley Affiliate) Fellowship to S.C.W. We thank Dr. Steven Post for editorial assistance, Melissa Drawdy for maintaining the mouse colony, and Mike Russ and Jana Hawks of the University of Kentucky Macromolecular Structure Analysis Facility for primer synthesis and DNA sequencing.

Manuscript received 28 March 2000 and in revised form 5 June 2000.

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