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Murine CD7 Shares Antigenic Cross-Reactivity with HSP-60

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Abstract

Human (h) CD7 is a 40 kDa single chain Ig superfamily molecule that is expressed on thymocytes, a major subunit of peripheral T cells, and most natural killer cells. Ligands for hCD7 include the epithelial cell-produced molecule, K-12, and galectin. Mice deficient in CD7 have been shown to be resistant to LPS-induced endotoxic shock syndromes. However, monoclonal antibodies (MAb) to mouse (m) CD7 have yet to be produced, nor is the distribution of mCD7 protein in mice known. We have raised a panel of three rat MAbs to mCD7 by immunizing rats with recombinant mCD7 protein. However, using Western blot and immunoprecipitation of tissue extracts from mouse thymus, spleen, liver, brain, lymph node and skin, these anti-mouse CD7 MAbs bound only to murine heat shock protein 60 (HSP-60) present both in wild-type ($CD7^{+/+}$) and CD7-deficient ($CD7^{-/-}$) mice. Epitope mapping of the sites on HSP-60 and recombinant mCD7 recognized by mCD7 MAbs demonstrated non-homologous amino acid sequence epitopes recognized by anti-CD7 MAbs on both proteins. These data demonstrated molecular mimicry of mCD7 with HSP-60, and leave open the question of surface expression of mCD7.

INTRODUCTION

Human (h) CD7 is a 40 kDa single chain Ig superfamily molecule expressed on thymocytes, a major subset of peripheral T cells, and on natural killer cells (NK). CD7 is one of the earliest markers in human T cell development, and moreover, marks several populations of human progenitor cells capable of differentiating into T, B, and myeloid lineages.[1–9]

CD7 is co-mitogenic for human T cells and induces IL-2, TNF-α, TNF-β, and GM-CSF production.[10] On NK cells, cross-linking of CD7 stimulates proliferation, IFN-γ secretion, and upregulation of adhesion to fibronectin.[11] CD7 is differentially regulated during T cell

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ontogeny, with high CD7 expression during thymocyte maturation on naïve T cells followed by low or no CD7 expression on memory T cells.[12]

Mice deficient in CD7 are resistant to both high- and lowdose models of acute LPS-induced shock.[13] Recently, we have demonstrated that CD7/CD28 double-deficient mice have defects in thymic production of CD4+, CD25+ T regulatory phenotype cells and develop autoimmune thyroiditis.[14] CD7-negative T cells have been implicated in the pathogenesis of rheumatoid arthritis[15] and coeliac disease.[16]

Although CD7 deficient and CD7/CD28 double-deficient mice have proved interesting for studies of regulation of cytokine production and for the study of T regulatory cell ontogeny in mice, MAbs against murine CD7 that could be used as tools to explore murine CD7 biology are not available. Thus, to date there has been no study of CD7 protein expression in mice T lymphocytes.

To develop reagents to be able to explore CD7 protein expression in mice, we have produced three rat anti-mouse CD7 MAbs and tested them for reactivity against both wild-type and CD7-deficient T cells. While all MAbs reacted with recombinant mCD7, surprisingly none of the anti-mCD7 MAbs reacted with any identifiable T cell surface proteins. Rather, each of the three anti-mCD7 MAbs reacted with intracellular murine HSP-60.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Homozygous CD7 deficient ($CD7^{-/-}$) mice were generated as previously described.[17] CD7-deficient mice were backcrossed five generations onto C57BL/6. Detection of the disrupted genes was performed using specific primers to amplify genomic tail DNA.[17] Mouse handling and experimental procedures were conducted in accordance with Duke University IACUC and AAALAC guidelines.

Production of rat anti-mouse recombinant CD7 MAbs

Harlan-Sprague rats were immunized with recombinant mCD7-HIS-tag protein produced in *Escherichia coli*, and rat/mouse heterohybridomas were produced using P3X63/Ag8 murine plasmacytoma cells as described.[18] Three MAbs were made, 8F8, 9E7, and 35C8; all were rat IgG2a k paraproteins.

For production of purified MAbs, anti-mCD7 MAb hybridoma cells were cultured in serum-free tissue culture medium (Hybridoma-SFM, GibcoBRL, Grand Island, NY). Supernatant was harvested, equilibrated to 100 mM Tris (pH 8.0), passed through a 0.45 μm filter, and run on an anti-rat Ig affinity column (Sigma, St. Louis, MO). Purified antibody was then eluted with 100 mM glycine (pH 3.0) and quantified by Bio-Rad D_C protein assay (Bio-Rad Laboratories, Hercules, CA).

Immunoglobulin fusion proteins

Plasmids containing CD7-Ig fusion protein constructs (mCD7-hIgG1, hCD7-mIgG2b) were transfected into COS cells using DEAE dextran, as previously described.[19] Typically, ten 100 mm semi-confluent plates of COS cells were transfected with each construct. Twelve hours after transfection, cells were trypsinized, seeded onto fresh plates with media (10% FCS), and incubated overnight. Transfected cells were allowed to grow in serum-free medium for 4 days. On the fourth day, supernatants were harvested, centrifuged to remove

non-adherent cells and debris, pooled, and stored at 4°C. Purification of CD7-Ig fusion proteins was performed via affinity chromatography with anti-IgG-agarose (Sigma) chromatography columns (Bio-Rad). Elution of the proteins from the columns was performed using 100 mM glycine (pH 3.0). Once isolated, proteins were dialyzed against phosphate-buffered saline (PBS) and concentrated to 1 mg/mL.

ELISA

ELISA was used to determine reactivity of CD7 MAbs with recombinant mCD7-human IgG1 fusion protein as described.[3,20]

Western blot analysis

Western blot analysis was performed to determine reactivity of anti-mCD7 MAbs with recombinant CD7-Ig fusion proteins (mCD7-hIgG1, hCD7-mIgG2b), control immunoglobulins (hIgG1, mIgG2b), mouse cell/tissue extracts, and recombinant human and murine HSP-60 proteins (StressGen Biotechnologies, San Diego, CA). Total cellular protein was collected from wild-type (C57BL/6; $CD7^{+/+}$) and CD7-deficient ($CD7^{-/-}$) mice[17] by lysis of isolated splenocytes or tissues in lysis buffer (25 mM Tris [pH 7.4], 1 mM EDTA, 1% NP-40, 1 mM PMSF, 25 mM iodoacetamide). Samples were prepared and diluted in 1X PBS and loading dye (50% glycerol, 0.75% bromphenol blue, 0.75% xylene cyanol in running buffer), then loaded on 4–20% Tris-glycine gradient gel (Novex PreCast minigel, Invitrogen, Carlsbad, CA) and run at 200 V for 1 h in running buffer (25 mM Tris base, 192 mM glycine, 0.1% sodium dodecyl sulfate). Gels were transferred to nylon membrane at 25 V and 100 mA in transfer buffer (12 mM Tris base, 96 mM glycine, 20% methanol [pH 8.3]) for 2 h. Following incubation overnight at 4°C in blocking buffer (10% dry milk in 1X TBST; 50 mM Tris HCl, 150 mM NaCl, 0.1% Tween-20), nylon membranes were then incubated with MAbs for 2 h at 20°C. Following four washes in 1X TBST, membranes were incubated in horseradish peroxidase-conjugated goat anti-rat Ig (H+L) (Southern Biotechnology Associates, Birmingham, AL) and diluted 1:5000 in blocking buffer. After washing four times with 1X TBST, the blot was incubated with Renaissance Western blot chemiluminescence reagent (NEN Life Science Products, Boston, MA) and allowed to expose film (X-Omat Blue XB-1, Eastman Kodak, Rochester, NY), which was then developed. Human and murine HSP-60 protein was detected by rabbit polyclonal anti-HSP-60 versus control rabbit polyclonal Ig (Stress-Gen Biotechnologies), coupled with horseradish peroxidaseconjugated goat anti-rabbit Ig (H+L) (Southern Biotechnology Associates).

Immunoprecipitation

Immunoprecipitation was performed to isolate the antigen reactive with mCD7 MAb 35C8 in a variety of mouse tissues. CN-Br activated Sepharose 4B (Sigma) was prepared per the manufacturer's instructions and coupled with purified 35C8 MAb or Y13 (rat IgG2a isotype control) (1 mg/mL) at a ratio of 10 mg MAb to 1 mL swelled sepharose in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl [pH 8.4]). Unbound antibody was removed with coupling buffer, and un-reacted groups on the sepharose were quenched with 0.2 M glycine (pH 8.0) for 2 h at room temperature. Following five cycles of alternating washes with base (coupling buffer) and acid (0.1 M acetate/ 0.5 M NaCl [pH 4]), Y13-sepharose was incubated with tissue lysates (obtained per Western procedure) for 1 h at 4°C. Lysates precleared with Y13 control MAb were then incubated with 35C8-sepharose for 2 h at 4°C. Monoclonal antibody bound sepharose preparations were then washed twice with 0.1% Triton X-100 in TSA solution (0.01 M Tris-Cl [pH 8], 0.14 M NaCl, 0.025% NaN3), then once in TSA solution, then once in 0.05 M Tris-Cl [pH 6.8]. 35C8- and Y13-sepharose were then boiled in 50 μL loading dye (per Western procedure) for 5 min to elute antigen. Samples were

electrophoresed as described above. Gels were either transferred to nylon membrane and subjected to Western blot analysis (as above) or stained with Coomassie blue.

Protein sequencing and analysis

Mass spectroscopy was performed to determine the amino acid sequence of bands recognized by MAb 35C8 by cutting out the gel bands and subjecting them to proteolysis and sequencing of band fragments using MS-TOF technology. Briefly, the bands were washed in deionized water (3 times for 5 min each) and destained with several changes of 10% methanol/water (v/v.) The gel pieces were incubated with 50 mM bicarbonate buffer and digested overnight with trypsin (1 μg) at 37°C. The peptides were extracted in 50% acetonitrile/5% formic acid solution (3 × 50 μL). The extractions were combined and concentrated to 5 μL. The peptide mixture was analyzed in a ABI QSTAR electrospray mass spectrometer (Foster City, CA). Amino acid sequence was determined directly by interpretation of MS/MS spectra. The derived *de novo* sequence was used to search the NCBI data base using the FASTS algorithm.[21]

RESULTS

Anti-mCD7 MAbs bound to recombinant mCD7 and reacted with wild-type and CD7-deficient murine spleen

Three rat anti-mouse CD7 MAbs specifically bound recombinant mCD7-hIgG1 fusion protein in ELISA (Fig. 1). Next, we determined the reactivity of anti-mCD7 MAbs with $CD7^{+/+}$ and $CD7^{-/-}$ mouse tissue using lysates of splenocytes obtained from $CD7^{+/+}$ and $CD7^{-/-}$ mice and Western blot analysis (Fig. 2). We have previously documented the absence of mCD7 mRNA in thymus and spleen tissue from $CD7^{-/-}$ mice.[17] Surprisingly, Western blot analysis with all three anti-mCD7 MAbs (35C8, 8F8, 9E7) showed binding to 60 kDa and 55 kDa proteins in splenocyte lysates, with no ~40 kDa bands seen that were expected for mCD7 (Fig. 2).

Anti-mCD7 MAb 35C8 reacted with HSP-60 in mouse immunoprecipitates of splenocyte lysate

To rule out that the concentration of cellular mCD7 was perhaps too low for detection using Western blot analysis, immunoprecipitation with 35C8-sepharose versus control Y13-sepharose was performed to isolate all proteins reactive with mCD7 MAbs in $CD7^{+/+}$ tissue lysates. Immunoprecipitation of both $CD7^{+/+}$ and $CD7^{-/-}$ spleen lysates with MAb 35C8 showed the same two bands at 60 and 55 kDa that were seen in Western blot analysis, and no differences seen in proteins recognized by mCD7 MAbs in $CD7^{-/-}$ and $CD7^{+/+}$ tissues (Fig. 3A–D). Similar single or double bands at 60 kDa were seen in $CD7^{+/+}$ and $CD7^{-/-}$ thymus, liver, and brain (Fig. 3B–D). Interestingly, a 60 kDa band recognized by 35C8 was seen in small intestine only in $CD7^{+/+}$ but not in $CD7^{-/-}$ mice (Fig. 3E).

Amino acid sequencing by mass spectrometry was used to determine the identity of the 60 kDa band in splenocyte and small intestine lysates reactive with anti-mCD7 MAb 35C8. The sequence obtained from peptide fragments of the spleen 60 kDa band was IQEIFEQLDVTTSEYE, a complete match for HSP-60. The 55 kDa band in spleen recognized by MAb 35C8 also generated a similar MS-TOF spectrum, and was also identified as mouse HSP-60. Peptide fragments of the 60 kDa band in small intestine of $CD7^{+/+}$ mice was similarly sequenced and determined to be VPALELAN. This sequence was a match for HSP-60 with the exception of isoleucine in HSP-60 instead of leucine in position six of the peptide. However, 87.5% identity was the highest achievable matching score for this peptide since mass spectrometry does not distinguish between amino acids with identical mass.

MAbs against mouse CD7 reacted with both human and mouse HSP-60

The sequencing of the 60 and 55 kDa bands recognized by anti-murine CD7 MAbs strongly suggested that the tissue molecules immunoprecipitated by MAb 35C8 were HSP-60. To confirm this, we assayed MAbs 35C8, 8F8, and 9E7 for their reactivity with recombinant human and mouse HSP-60. We found that all three MAbs reacted with recombinant HSP-60 as well as with mouse CD7-human IgG1 fusion protein, but did not react with human IgG1 nor with human CD7-mouse IgG2b fusion protein or mouse IgG2b control protein (Fig. 4).

To determine the specificity of cross-reactivity of mCD7 MAbs with HSP-60, we next determined if the mouse CD7-hIgG1 fusion protein could inhibit the binding of MAb 35C8 to human and mouse HSP-60. Figure 5 shows that 10 μg/mL of mCD7-hIgG1 fusion protein completely inhibited the binding of 35C8 MAb to both human and mouse HSP-60. Next, the reciprocal experiment was performed in which we determined if 20 μg/mL heat shock protein could inhibit the binding of 35C8 to mouse CD7-hIgG1 fusion protein. As seen in Figure 6, HSP-60 completely blocked the binding of MAb 35C8 to mCD7. Next, we determined if rabbit polyclonal anti-HSP-60 could react with either murine or human CD7 protein. We found that polyclonal rabbit anti-HSP-60 reacted with both human and mouse CD7 fusion proteins in Western blot analysis (Fig. 7). However, in contrast to MAb 35C8 against mCD7, the anti-human CD7 MAb 3A1e did not react with HSP-60 (data not shown).

Epitope mapping of MAbs 35C8, 8F8, and 9E7 using overlapping peptides of both mouse CD7 and HSP-60 proteins

To determine the basis of cross-reactivity of the mCD7 MAbs with HSP-60, 15 mers overlapping by 11 amino acids were made of both mCD7 and mHSP-60. We found that the reactivity of all three MAbs was identical, and each mCD7 MAb bound to the sequence VIASEGDSVNITCST in mCD7 and also bound to two peptides that shared the common sequence TISANGDKDIG in murine HSP-60 (Table 1).

Inability of CD7 knockout mice to respond to mCD7-6 His protein

As a final strategy to raise MAbs against mCD7, mCD7-6 His protein was used to immunize three CD7-deficient mice[17] and the serum screened for reactivity with mCD7-huIg fusion protein in ELISA. After these immunizations with mCD7-6His protein in incomplete Freund's adjuvant, we found that none of the mice had antibodies that reacted with mCD7-huIg protein (data not shown).

Characterization of polyclonal rat anti-mCD7-6 His antisera

Next we produced polyclonal rat anti-CD7-6 His anti-serum that was strongly reactive with mCD7-huIg fusion protein. We showed that this serum did not react with BALB/c thymocytes using indirect immunofluorescence assays and flow cytometry (data not shown). Thus, neither the rat anti-mCD7 Mabs nor a polyclonal rat anti-mCD7 serum reacted with mouse thymocytes, strongly suggesting mCD7 is not a surface T cell protein.

DISCUSSION

In this study, we have shown that a panel of MAbs against recombinant mCD7 did not recognize native mCD7 in mouse spleen, thymus, or in a variety of other tissues, but rather cross-reacted with mouse HSP-60 in both $CD7^{-/-}$ and $CD7^{+/+}$ mice. The observed lack of reactivity of mCD7 protein in mouse spleen and thymus with our new mCD7 MAbs is at odds with the documented presence of mCD7 mRNA expression by Northern blot in those tissues.[17] Yoshikawa et al. demonstrated mCD7 message in thymus, spleen, bone marrow, and small intestine.[22] Our inability to identify mCD7 in lysates of mouse tissue could be due to short half-life mCD7 protein, lack of expression *in vivo* of the mCD7 epitope

recognized by the three mCD7 MAbs, or mCD7 could be under tight translational control. Nonetheless, until additional mCD7 MAbs are produced, our current hypothesis is that mCD7 protein, if expressed, is expressed at levels that are undetectable with the MAbs and detection assays used in this study.

In humans, CD7 is a signaling molecule on T cells and NK cells.[12] CD7-deficient mice have provided the only clues to functions of CD7 in mice. Mouse CD7 deficiency confers resistance to LPS-induced shock, with decreased TNF-α and IFN-γ production in response to LPS, as well as decreased number of liver NK-T cells.[13] In addition, from this study, CD7 may regulate HSP-60 expression in intestine (Fig. 3E). Addition of mCD7 to mCD28 deficiency prevented the production of $CD4^+$, $CD25^+$ T regulatory phenotype cells in thymus and decreased proliferation and survival of peripheral T regulatory phenotype cells, resulting in autoimmune thyroiditis.[14] This phenotype is also compatible with mCD7 serving as a key signaling molecule in thymus for T regulatory cell generation. Given these studies that suggest a role for mCD7 in immune cell signaling, it was surprising that mCD7 surface protein could not be found.

Other immunization strategies using mCD7 from additional expression strategies may still yield anti-mCD7 MAbs that can react specifically with murine CD7. Nonetheless, the molecular mimicry between mCD7 and HSP-60 has been clearly documented using the three MAbs reported in this study, and this molecular mimicry with an ubiquitous host antigen may play a significant role in the difficulty of raising anti-mCD7 antibodies with other strategies. That anti-HSP-60 polyclonal rabbit serum cross-reacted with human CD7 recombinant protein, suggested that human CD7 also shares HSP-60 mimicry, although hCD7 MAbs have readily been generated in mice.[1–9] Thus, lack of surface expression mCD7 and low levels of mCD7 expression in general may also play a role in lack of mCD7 MAb induction. The apparent lack of HSP-60 in small intestine of CD7-deficient mice is of interest and may imply that mCD7 may be involved in the regulation of HSP-60 expression in select tissues.

In summary, our study has demonstrated molecular mimicry of mCD7 with HSP-60 and raises questions regarding how mCD7 effects regulatory roles in murine cytokine production and T regulatory cell development and homeostasis.[14] Whether CD7 is a surface protein of T cells or other cell types remains an open question. Future studies of CD7 transcription, translation, and protein expression should prove useful to determine mCD7 protein expression status in lymphoid tissues.

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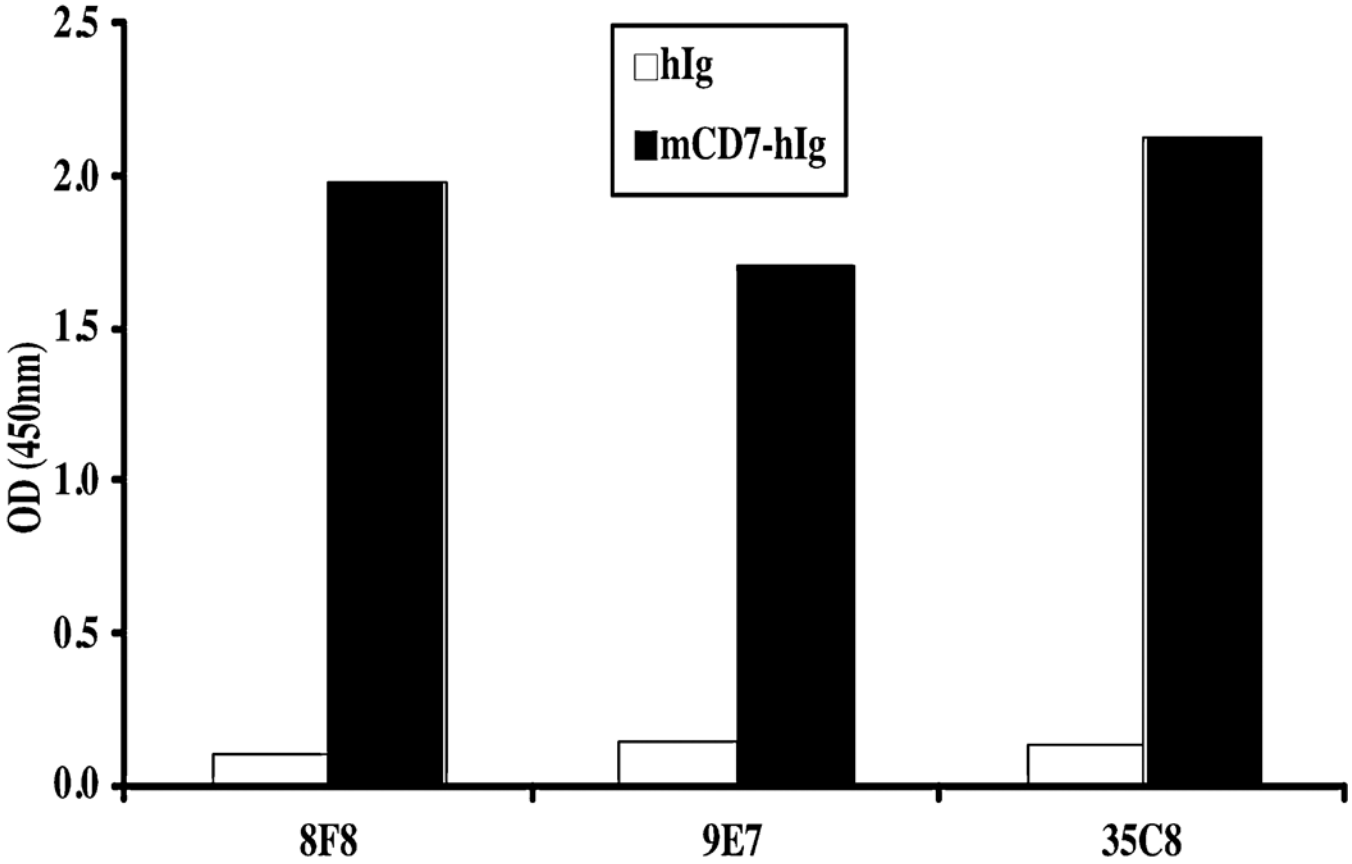

FIG. 1.

Anti-mouse CD7 antibodies bind mouse CD7-hIgG1 and not hIgG1 control. A 96-well plate was coated with either 50 ng per well of mCD7-hIgG1 or hIgG1. Anti-CD7 MAb supernatants (8F8, 9E7, 35C8) were added at 100 μL/well and allowed to react with the coated samples. Goat anti-rat Ig (H+L)-horseradish peroxidase (1:2000) was used as a detection reagent and developed with TMB. This graph is representative of two similar experiments.

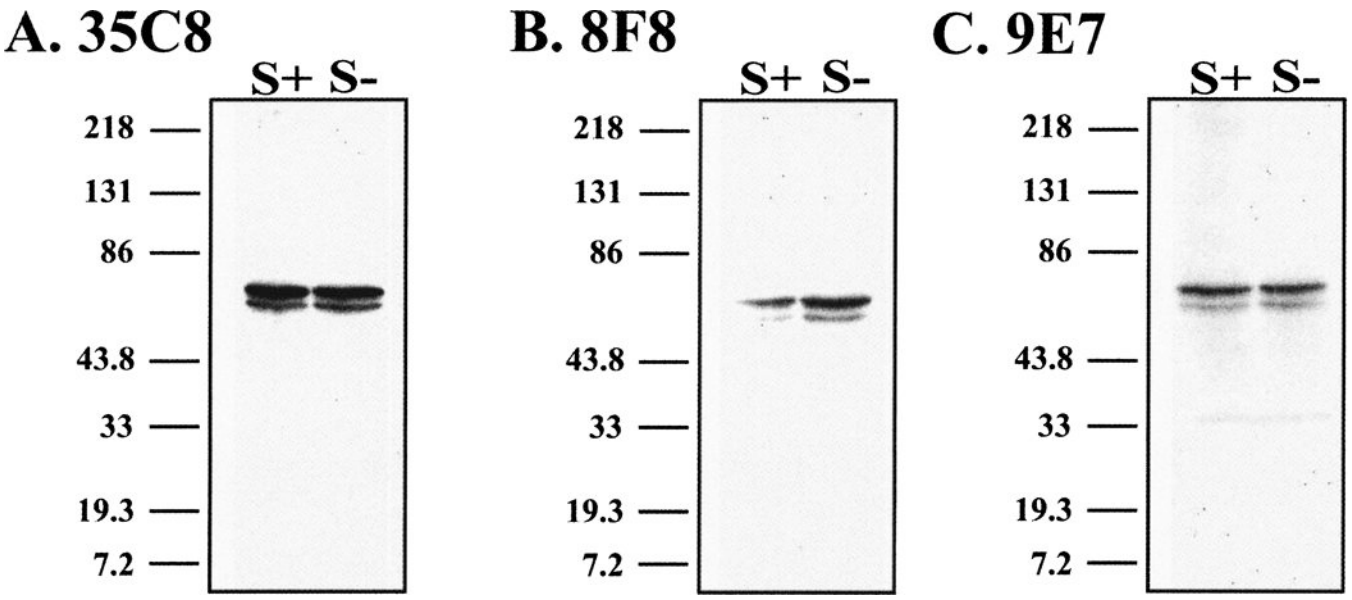

FIG. 2.

Anti-mCD7 MAbs 35C8, 8F8, and 9E7 do not bind native mCD7 in splenocyte lysates by Western blot analysis. $CD7^{+/+}$ (S+) or $CD7^{-/-}$ (S−) lysates were prepared from teased splenocytes as per Western protocol. Samples were electrophoresed under non-reducing conditions. Membranes were incubated with MAb supernatant for 2 h: (**A**) 35C8, (**B**) 8F8, (**C**) 9E7. Goat anti-rat IgG-HRP was used as a detection reagent.

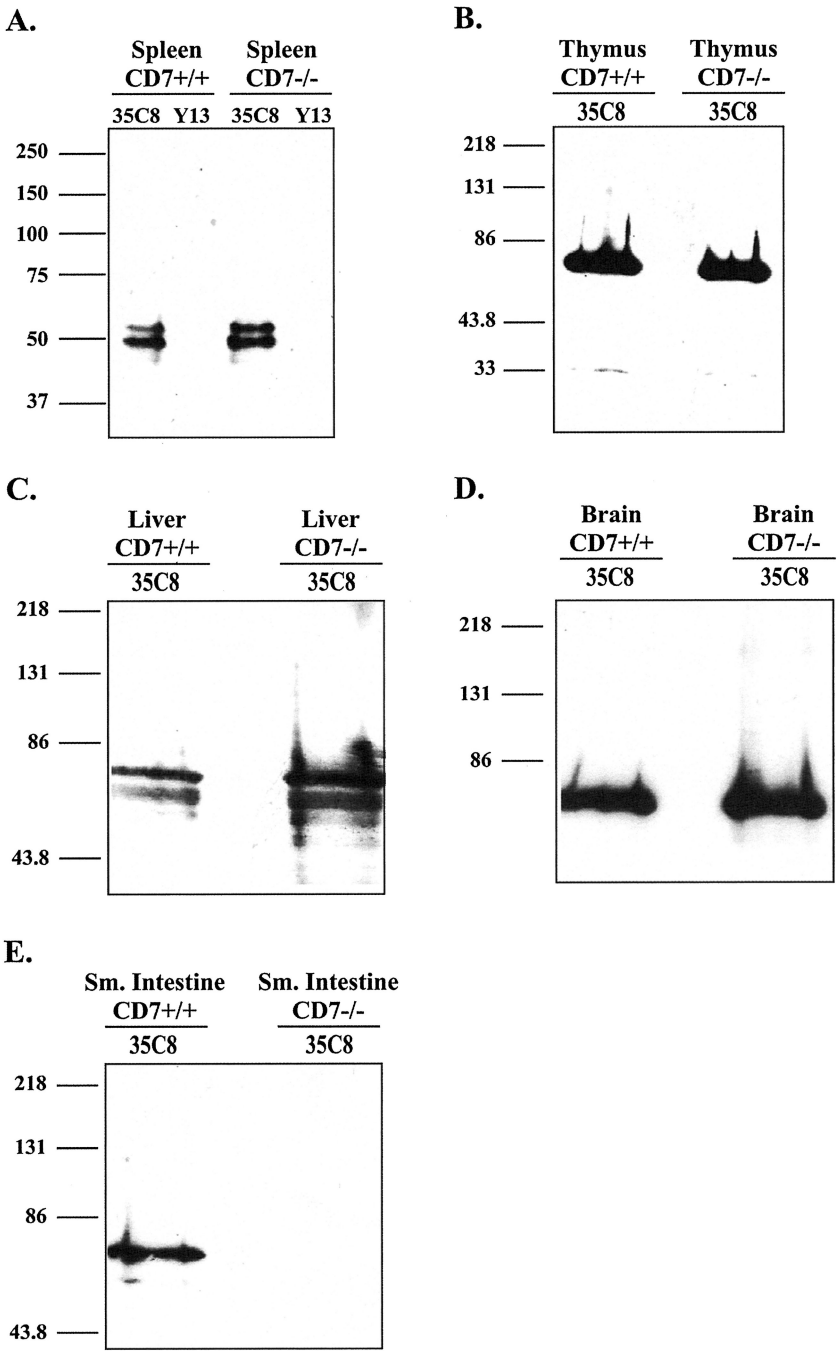

FIG. 3.

Tissue-screen Western analysis with anti-mCD7 MAb 35C8 following immunoprecipitation with anti-mCD7 MAb 35C8. $CD7^{+/+}$ or $CD7^{-/-}$ lysates were obtained from whole snap-frozen tissue as per Western protocol, immunoprecipitated with 35C8 or Y13 control MAbs, electrophoresed under non-reducing conditions, and probed with 35C8. Tissue lysates are as follows (with amount of lysate protein used in the immunoprecipitation in parenthesis): (**A**) whole spleen, $CD7^{+/+}$ (0.48 mg) and $CD7^{-/-}$ (0.6 mg); (**B**) whole thymus, $CD7^{+/+}$ (0.28 mg) and $CD7^{-/-}$ (0.35 mg); (**C**) liver, $CD7^{+/+}$ (5.1 mg) and $CD7^{-/-}$ (4.8 mg); (**D**) brain, $CD7^{+/+}$

(0.83 mg) and $CD7^{-/-}$ (1.2 mg); (**E**) small intestine, $CD7^{+/+}$ (0.22 mg) and $CD7^{-/-}$ (0.58 mg).

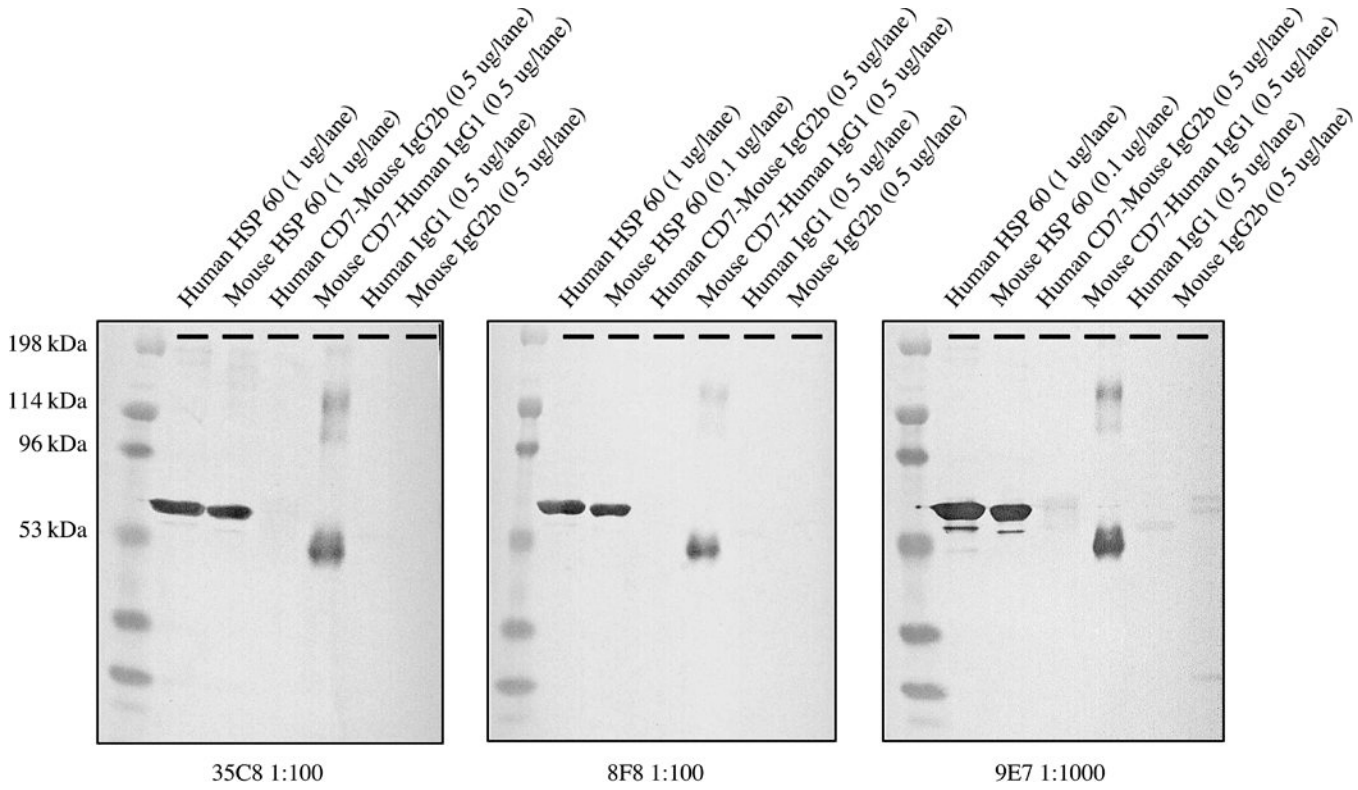

FIG. 4.

Anti-mCD7 MAbs 35C8, 8F8, and 9E7 react with recombinant mouse and human HSP-60 and mouse CD7, but not human CD7. Western blot analysis was performed on recombinant mouse and human HSP-60, mouse and human CD7-Ig fusion proteins, and human and mouse Ig fusion protein controls per Western protocol. The amount of each protein run per lane under non-reducing conditions is indicated.

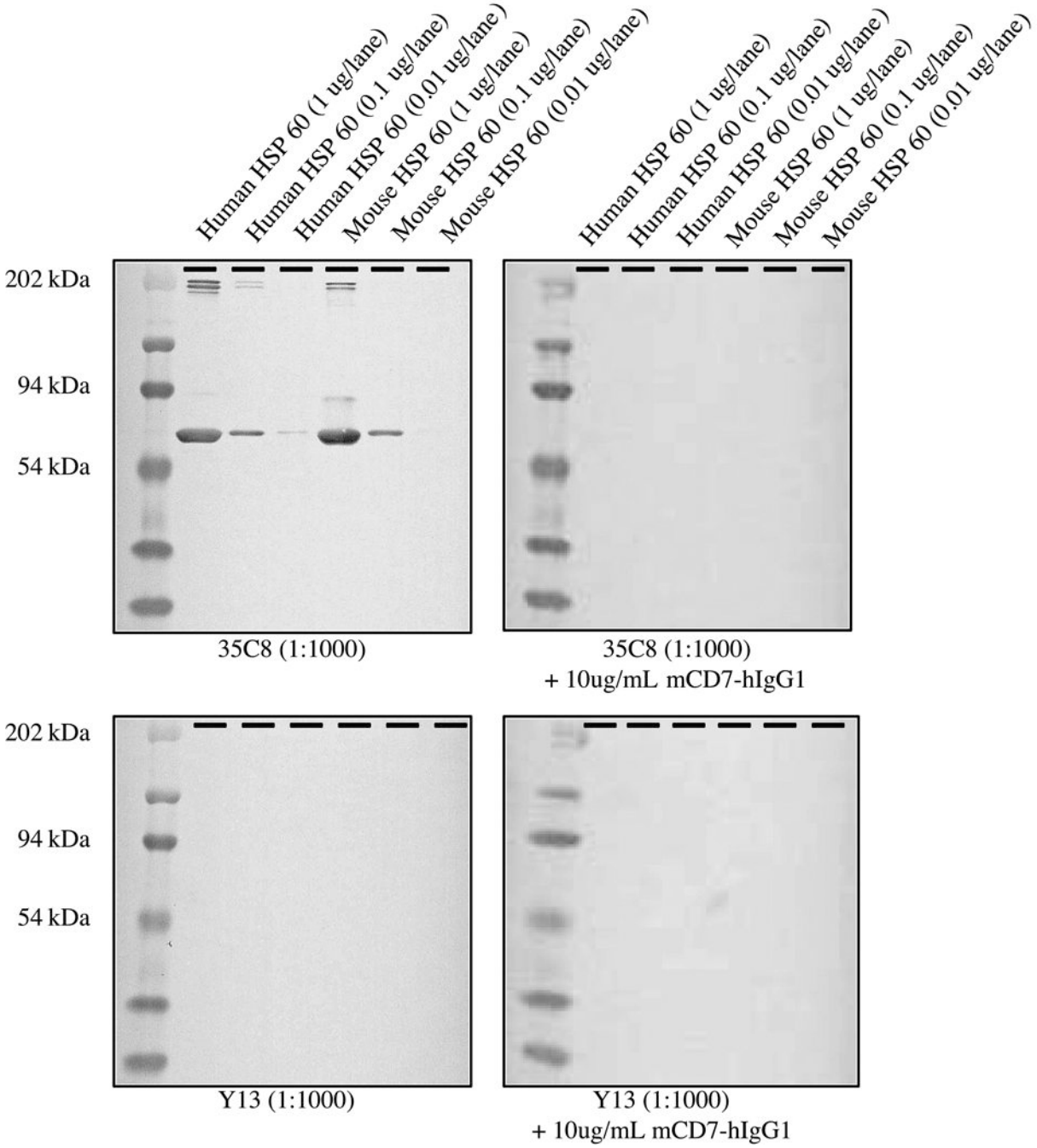

FIG. 5.

Reactivity of anti-mCD7 MAb 35C8 with recombinant mouse and human HSP-60 can be adsorbed by preincubation of the MAb with recombinant mouse CD7-hIgG1. Recombinant mouse and human HSP-60 (1, 0.1, and 0.01 μg/lane) were electrophoresed under non-reducing conditions per Western protocol. Western blot analysis was performed on replicate blots with 35C8 +/− preincubation with 10 μg/mL of mCD7-hIgG1 or Y13 +/− preincubation with 10 μg/mL of mCD7-hIgG1.

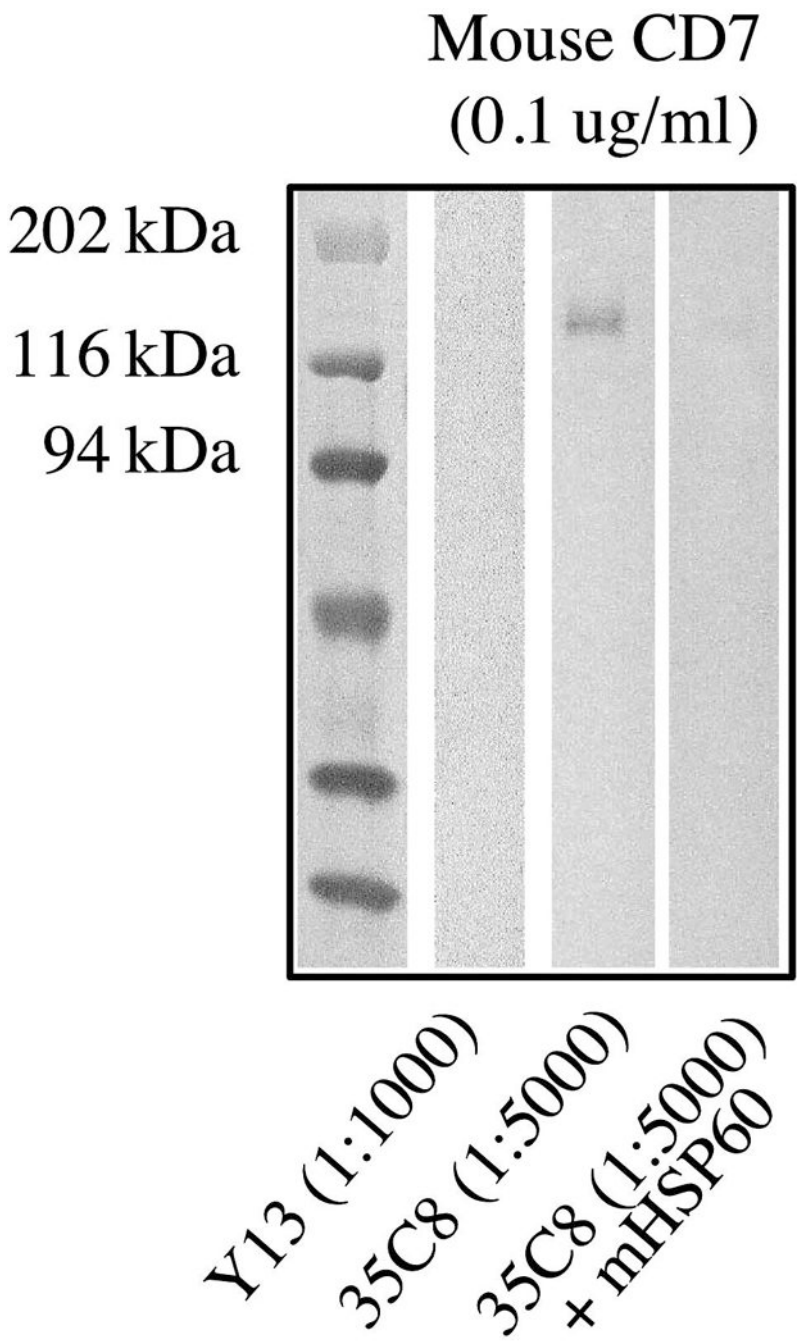

FIG. 6.

Reactivity of anti-mCD7 MAb 35C8 with mouse CD7-hIgG1 fusion protein can be adsorbed by preincubation of the MAb with recombinant mouse HSP-60. Recombinant mCD7-hIgG1 was electrophoresed under non-reducing conditions per Western protocol. Western blot analysis was performed on replicate blots with Y13, 35C8, or 35C8 + preincubation with 10 μg/mL of mCD7-hIgG1.

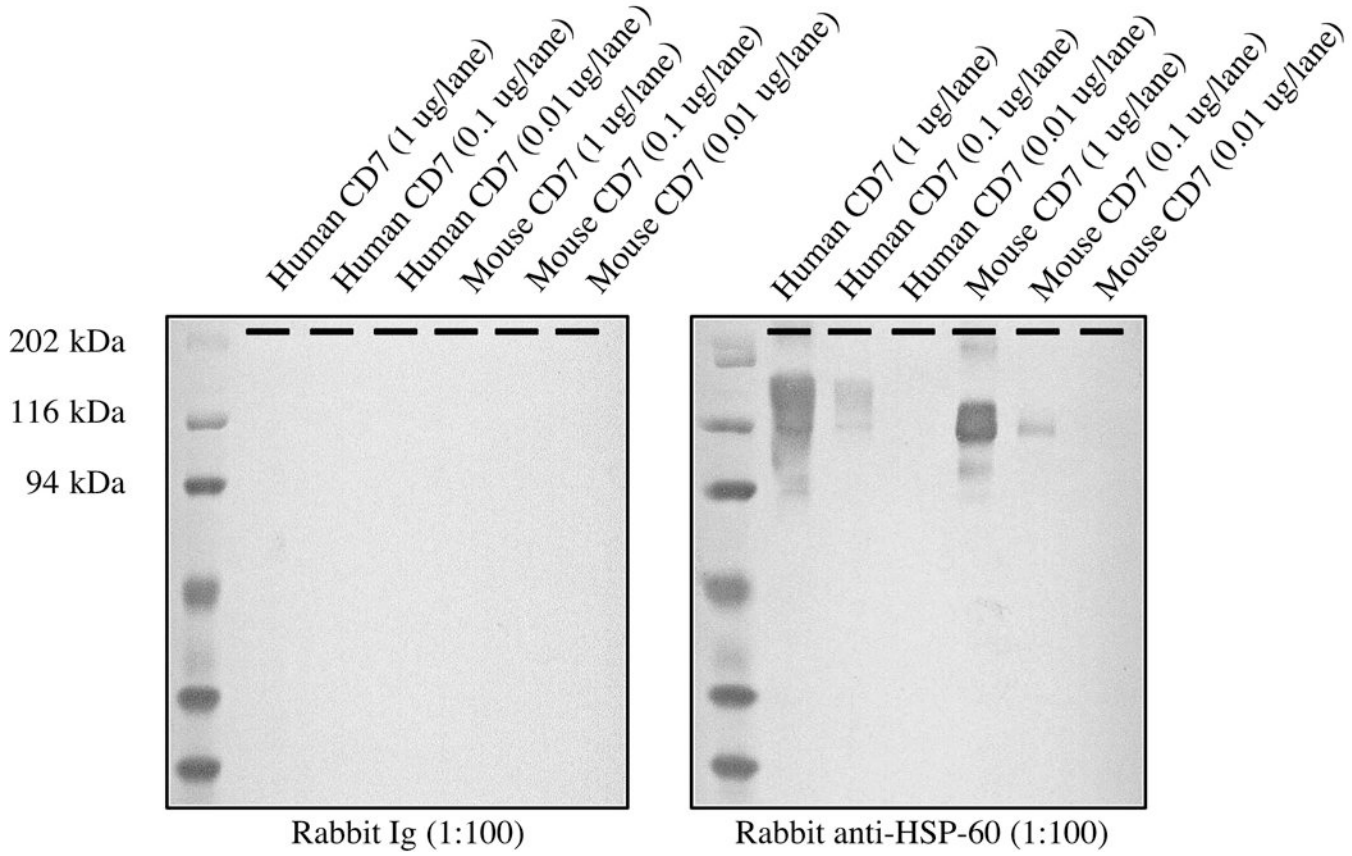

FIG. 7.

Rabbit anti-HSP-60 polyclonal antibody reacts with recombinant mouse and human CD7. Recombinant mouse and human CD7-Ig fusion proteins (1, 0.1, and 0.01 μg/lane) were electrophoresed under non-reducing conditions per Western protocol. Western blot analysis was performed on replicate blots with control rabbit Ig or rabbit anti-HSP-60 polyclonal antibody.

Table 1

Mouse CD7 and HSP-60 Peptides Recognized by Anti-mCD7 MAbs 35C8, 8F8, and 9E7

Coated on plate	Y13	35C8	8F8	9E7
Hu IgG	0.205[a]	0.513	0.238	0.156
mCD7-hIgG	0.381	**2.159**	**2.337**	**1.980**
mHSP-60	0.119	**2.114**	**2.389**	**2.059**
mCD7 peptide 9 VIASEGDSVNITCST	0.147	**1.642**	**0.911**	**0.375**
mHSP-60 peptide 43 AQVATISANGDKDIG	0.138	**1.590**	**1.482**	**0.800**
HSP-60 peptide 44 TISANGDKDIGNHS	0.091	**1.507**	**0.906**	**0.556**

[a]Optical density at 450 nm.

Boldface type indicates 3-fold over background.