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Monoclonal Antibody against Mouse CAR following Genetic Immunization

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ABSTRACT

To broaden our repertoire of monoclonal antibodies against CAR (coxsackievirus and adenovirus receptor), we inoculated mice with an expression vector containing the cDNA encoding human CAR extracellular and transmembrane sequence, and boosted the response by inoculation with soluble human CAR protein produced in *E. coli*. Of the hybridomas obtained following this immunization protocol, one secreted IgG with exceptional reactivity against mouse CAR. Since CAR has been shown to form dimers, expression of human CAR in cells that express mouse CAR may have stimulated the host immune system to recognize endogenous CAR in heterodimers.

INTRODUCTION

THE COXSACKIEVIRUS and adenovirus receptor (CAR) is an immunoglobulin superfamily protein with amino acid sequence similarity to A33 antigen and CTH, and is structurally and functionally related to junctional adhesion molecules (JAMS).[1–5] Human CAR (hCAR) and mouse CAR (mCAR) share 91% identical amino acid sequence. Even though mCAR and hCAR are highly conserved, monoclonal antibodies against human CAR have been generated from mice inoculated with hCAR enriched from cultured cells[6] or with recombinant hCAR expressed in *E. coli*.[7] The RmcB monoclonal antibody[6] binds hCAR on viable cells and has been used for virus receptor blocking and immunochemical applications,[4,6,8,9] but fails to detect hCAR on blots from SDS polyacrylamide gels. Our monoclonal antibodies (e.g., MAb.E1) raised against the soluble extracellular domain of hCAR produced in *E. coli* (called hECAR) work well for detecting hCAR on Western blots.[7,10]

In an effort to broaden our repertoire of monoclonal antibodies against hCAR, we employed biolistic inoculation of an expression vector[11] containing the cDNA for hCAR, with modified cytolasmic domain, to immunize mice. One of the hybridomas produced using this procedure secretes an antibody that recognizes both hCAR and mCAR.

MATERIALS AND METHODS

The cell lines used as sources of hCAR (full length and truncated) have been previously described.[10,12] Mouse fetal heart fibroblasts (MFHF) were used as a source of mCAR. Soluble mCAR (mECAR) was cloned and expressed in *E. coli* (Chapman et al., unpublished). The recombinant protein includes the extracellular domain of mouse CAR, with carboxyl-terminal FLAG and His_8 additions.

DNA encoding the extracellular and membrane spanning domains of hCAR (hCAR cDNA obtained from the I.M.A.G.E. Consortium) were cloned into pTracer (Invitrogen). The final construct (pTracer-pTACAR3) replaced the native cytoplasmic domain with a short cytoplasmic tail derived from the plasmid polylinker. This plasmid has been used to express CARt3 in human cells.[10] For inoculation, the cDNA was coated onto 5.5–9.0 micron gold particles (Alfa Aesar, Ward Hill, MA) as described by Williams et al.,[11] and subsequently modified as described by Sawant et al.[13] The gold particles were delivered into the pinna of the ear[14] of anesthetized mice.

Following a series of three biolistic cDNA inoculations (at 0, 2, and 4 weeks), administration of purified hECAR (soluble extracellular domain of hCAR expressed in *E. coli*[15]) by intraperitoneal injection, with adjuvant, was added to the protocol. At week 33, each mouse received 50 μg of hECAR in RIBI

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adjuvant (Corixa Corp., Hamilton, MT) intraperitoneally. The i.p. inoculation was repeated at week 38. At weeks 48, 51, and 59, the mice received both biolistic inoculation with cDNA and i.p. injection of soluble antigen in adjuvant. Three weeks prior to spleen harvest, a mouse with demonstrated serum antibodies against hCAR was boosted i.p. with hECAR in adjuvant. hECAR was injected i.p. without adjuvant 5 days before spleen harvest. Splenic lymphocytes were fused with NS-1 cells and cultured as previously described.[16]

The hybridomas that survived selection in HAT medium were screened for production of mouse IgG by ELISA, using goat anti-mouse Ig (heavy and light chain reactivity; Jackson ImmunoResearch) to capture mouse IgG from the media, and Fc-specific alkaline phosphatase-conjugated goat anti-mouse reagent to detect the mouse IgG. Wells that showed production of IgG were then screened for reactivity against hECAR by ELISA, using microtiter wells coated with rabbit Fc-specific anti-mouse IgG (Jackson ImmunoResearch) to capture the mouse IgG, and HRP-conjugated hECAR[17] to detect anti-CAR reactivity. The cultures with greatest reactivity in the ELISA were subsequently screened on Western blots of hECAR and hCAR expressed in cultured cells. Western blots were developed with ECL+Plus (Amersham) and visualized with RX-G film (www.clinicalfilms.com).

RESULTS

The monoclonal antibodies from this experiment worked well for detecting hCAR on Western blots. Figure 1 shows that the antibody designated MAb.E(mh)1 detects native hCAR expressed by HeLa cells and the CARt3 expressed by RDt3, but does not react with proteins present in lysates of RD cells, a cell line that does not produce CAR detectable on Western blots. MAb.E(mh)1, at 1/500 dilution of conditioned medium, reacts as well as the MAb.E1 that has been used for several years.[7]

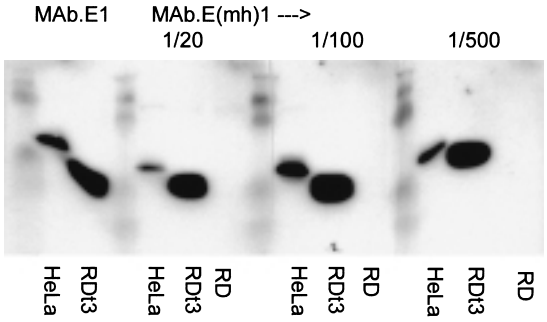

FIG. 1. MAb.E(mh)1 detects hCAR on Western blots. Western blots of human cell extracts were probed with the anti-hCAR monoclonal MAb.E1 and dilutions of the the new monoclonal antibody, MAb.E(mh)1. HeLa cells express native hCAR (Mr~46,000), RDt3 express the truncated CARt3 (Mr~39,000). RD cells express no detectable hCAR and serve as a negative control. MAb.E1 was used as a 1/10 dilution of conditioned medium. MAb.E(mh)1 was also present in conditioned medium and was tested at dilutions of 1/20, 1/100, and 1/500. Prestained marker proteins were visualized on the figures by photographing the exposed films on top of the blot membrane.

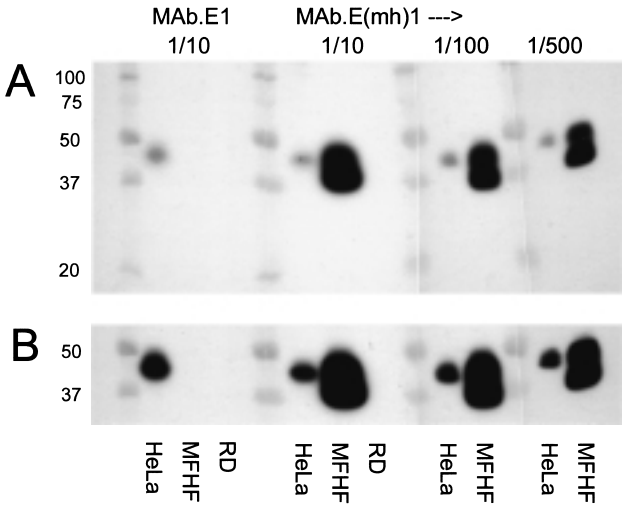

FIG. 2. MAb.E(mh)1 reacts with both hCAR and mCAR. Western blots of proteins from acetone precipitated octyl glucoside lysates of cultured HeLa, MFHF, and RD cells were probed with MAb.E1 (1/10) or dilutions of MAb.E(mh)1 (1/10; 1/100; 1/500). Films were exposed for 30 sec (**A**) and 2 min (**B**). Prestained marker proteins were visualized on the figures by photographing the exposed films on top of the blot membrane.

No additional reactivities are apparent even when MAb.E(mh)1 was tested at 1/20. Clearly, MAb.E(mh)1 is reactive with hCAR on Western blots, and appears to be specific.

We screened antibodies for cross reactivity with mCAR. MAb.E(mh)1 showed exceptional reactivity against mCAR on

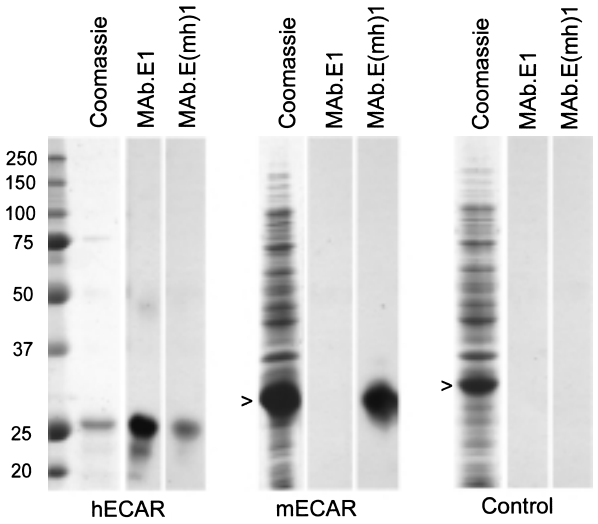

FIG. 3. Western blots of cloned extracellular domain of hCAR (hECAR), mCAR(mECAR), and an unrelated protein (Control; contains both FLAG and His_8 additions), were probed with MAb.E1 at 1/10 or MAb.E(mh)1 at 1/500. Coomassie stained gels of the blotted proteins (10-fold more loaded for staining) are also shown. hECAR had been purified while the mECAR and Control proteins were present in lysates of IPTG-induced *E. coli* (> indicates induced proteins). Marker proteins shown were run on the coomassie stained gel and used to align blot segments with markers transferred to the blot membrane.

Western blots of MFHF (Fig. 2). The MAb.E(mh)1 antibody detects two bands in the MFHF, one of which is slightly smaller than the hCAR and corresponding mCAR bands. A similar pattern has been previously reported for hCAR[10] and is presumably due to alternative transcripts or post-translational modifications.[18,19] This result indicates that MAb.E(mh)1 has strong reactivity against mCAR as well as hCAR.

To further establish the specificity of MAb.E(mh)1, it was compared with MAb.E1 on Western blots of hECAR, mECAR, and an unrelated control protein that also included FLAG (present in the mECAR protein) and His_8 (present in mECAR; hECAR contains His_6) (Fig. 3). Both MAb.E1 and MoAb.E (mh)1 detected purified hECAR (Fig. 3, left series), but only MAb.E(mh)1 detected the IPTG-induced mECAR in the *E. coli* lysate (Fig. 3, center series). Neither antibody bound other proteins in the mECAR sample or in the control (Fig. 3, right series).

DISCUSSION

Within the panel of monoclonal antibodies that we have raised against hCAR, MAb.E(mh)1 is unique in its strong reactivity against mCAR. While this reactivity may be serendipitous or the consequence of inoculation with highly conserved antigen,[20] it has occurred to us that the immunization protocol may have facilitated development of an antibody with reactivity against the native mouse counterpart of the immunogen. CAR naturally forms dimers in solution,[21] and it is probable that cellular CAR also forms homodimers in cell junctions. Inoculation of cDNA likely results in hCAR expression in some cells that also express mCAR. The co-expressed proteins may form heterodimers (hCAR-mCAR). However, the expressed hCAR appears to have limited antigenicity as no antibody was detectable until the animals were boosted with soluble antigen in adjuvant. This suggests a series of events in which hECAR with adjuvant initiates an immune response against hCAR, after which responding cells recognize the hCAR expressed by transfected cells and subsequently mount an immune response against the mCAR complexed with the hCAR. This hypothetical scenario suggests that vaccinating the host with a foreign antigen that forms complexes with the target host protein may induce an immune response against self- antigens. Inoculation with cDNA for expression of the foreign antigen should facilitate formation of hetero-complexes by cell–surface proteins.

The mechanisms that resulted in development of the MAb.E(mh)1 antibody are speculative at this point. The unexpected occurrence of this anti-mCAR antibody following the unconventional inoculation protocol, however, raises questions that can be tested. Irrespective of mechanisms by which it arose, MAb.E(mh)1 will be a useful reagent for studies of CAR expression and function in mice.

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