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B lymphocyte and macrophage expression of carcinoembryonic antigen-related adhesion molecules that serve as receptors for murine coronavirus*

The expression of carcinoembryonic antigen (CEA)-related glycoproteins that have been associated with intercellular adhesion and that serve as receptors for mouse hepatitis virus (MHV) was analyzed in cells from the immune system of BALB/c mice using immunolabeling and RNA polymerase chain reaction amplification of receptor transcripts. These glycoproteins, which are called biliary glycoproteins, were highly expressed in B lymphocytes, including cells of the B-1a (CD5⁺) lineage, and in macrophages, but were not detectable in resting T lymphocytes. Similarly, murine cell lines of B cell and macrophage origin expressed messenger RNA encoding CEA-related molecules, while the corresponding mRNA was only slightly detectable in a T cell line. These CEA-related cell adhesion glycoproteins were also expressed in endothelial cells. Therefore, their specific interaction with their so far unknown ligand may be of functional importance in cellular interactions in the immune response.

Monoclonal antibody directed against these glycoproteins blocked MHV-A59 infection of the B cell-derived SP20 cell line. Thus, the functional receptors for MHV on B lymphocytes, like those on murine fibroblasts, are isoforms of CEA-related glycoproteins. Treatment of B cells with anti-receptor antibody also blocked B cell-mediated cytotoxicity against MHV-A59-infected fibroblasts, indicating that this phenomenon is mediated by interaction of viral attachment protein on the infected target cells with specific CEA-related receptor glycoproteins on the effector B cells.

1 Introduction

Efficiency of the immune system relies on the development of an appropriate communication network between various cell types. Such a communication system involves both soluble lymphokines that can reach their target cells at some distance and molecules that allow direct recognition between different cell types. It has indeed been demonstrated that adhesion molecules belonging to three distinct families, namely the Ig superfamily, the integrin family and the selectins, play a crucial role in various immune func-

tions [1]. In the Ig superfamily, different mouse isoforms of glycoproteins related to the biliary glycoprotein (BGP) group in the carcinoembryonic antigen (CEA) family have been shown to promote adhesion between transfected fibroblasts [2, 3]. The homologous rat molecule, cell-CAM 105, mediates adhesion between hepatocytes [4], and human CEA is a cell adhesion molecule for colon cells and for transfected cells [5, 6]. Receptors for mouse hepatitis virus (MHV)-A59, a virus with known lymphotropism, have been identified as glycoproteins in the CEA-related family of BGP [7–11], and clones, oligonucleotides and antibodies specific for these molecules are now available. BALB/c and SJL/J mice differ markedly in susceptibility to MHV infection [12], and their receptor glycoproteins, called MHVR and mmCGM2, respectively, differ significantly in the N-terminal Ig-like domain that is the site of virus binding [13]. Splicing of transcripts occurs in various murine tissues, resulting in isoforms with either two or four Ig-like domains, and with either long or short cytoplasmic tails.

Using anti-MHVR antibodies and PCR to analyze expression of the isoforms of this molecule in BALB/c immune cells, we found that virus receptors were highly expressed in B lymphocytes and macrophages, but that little or no receptor was expressed in T lymphocytes. Expression of these BGP glycoproteins on B cells correlated with functional virus receptor activity and with the MHV-specific, B cell-mediated cytotoxicity previously described [14, 15]. This is the first report of the expression and function of CEA-related molecules on murine lymphocytes and macrophages.

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Abbreviations: MHV: Mouse hepatitis virus CEA: Carcinoembryonic antigen BGP: Biliary glycoprotein VK: Virus-killer activity

Key words: Mouse hepatitis virus receptor / Adhesion molecule / B lymphocyte / Carcinoembryonic antigen

2 Materials and methods

2.1 Mice and virus

Specific pathogen-free female BALB/c mice were bred at the Ludwig Institute for Cancer Research by Dr. G. Warrier or purchased from Iffa Credo (L'Arbresle, France) or Jackson Labs (Bar Harbor, ME) and were used for experiments at the age of 6–8 weeks. Animals were infected by i.p. injection of approximately 10^4 tissue culture infectious doses (TCID₅₀) of the A59 strain of MHV (MHV-A59) grown in NCTC 1469 cells. Cell cultures were infected with MHV-A59 grown in 17 CI 1 cells [10].

2.2 Antibodies

Mouse anti-MHV antibody was obtained 34 days after infection of BALB/c mice with MHV-A59. Polyclonal mouse anti-receptor antibody was raised by immunization of SJL/J mice with deoxycholate-solubilized intestinal brush border membranes from adult BALB/c mice [16]. This serum recognized the 100–120-kDa receptor for MHV-A59 in Western blots, immunoprecipitated this and the 58 kDa isoform, recognized the MHVR glycoprotein expressed in hamster cells from cloned MHVR1 cDNA, and protected mouse fibroblasts from infection with MHV-A59. Polyclonal rabbit anti-receptor antibody (#655) was obtained after immunization of New Zealand White rabbits with immunoaffinity-purified receptor from Swiss Webster mouse liver [9]. Anti-receptor mAb (MAb-CC1) was derived from SJL/J mice immunized with protein extracted from BALB/c intestinal brush border membranes [7]. MAb-CC1 reacts with the N-terminal domain of MHVR and blocks binding of MHV-A59 to MHVR glycoproteins [13, 16]. MAb-CC1 will be made available for research purposes upon request to K. V. Holmes.

2.3 Cells

WEHI-3, a macrophage-like cell line of BALB/c origin, [17] (ATCC, Rockville, MD) was grown in RPMI medium with 10 % FCS. SP207 (gift from J. Van Snick), a cloned cell line from the BALB/c myeloma SP2/0-Ag14 [18], was maintained in Iscove's medium containing 10 % FCS. TB6D2 (gift from A. Vink) was derived from a BALB/c plasmacytoma [19]. TS1C3 is a subclone of TS1, that was derived from TUC2.15, a T helper cell line of C57BL/6 origin [20] (gift from C. Uyttenhove). It was cultured in Dulbecco's modified Eagle's medium supplemented with 10 % FCS, 50 μ M 2-mercaptoethanol, 0.55 mM L-arginine, 0.24 mM L-asparagine and 1.25 mM L-glutamine. The 17 CI 1 line of spontaneously transformed BALB/c 3T3 cells was used to propagate MHV-A59 and as a target for cytotoxicity studies [14] and the L2 cell line was used for plaque assays.

Total B lymphocytes were purified either by panning on plates coated with anti-mouse Ig antibody, as described previously [21] or by magnetic cell sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany). After depletion of macrophages by two sequential incubations in tissue culture dishes, 10^8 spleen cells were incubated for 10 min at 4 °C with biotinylated anti-B220 rat mAb, followed with fluor-

esceinated streptavidin for 10 min at 4 °C and MACS biotin microbeads (5 μ l in 500 μ l PBS containing 5 mM EDTA and 1 % BSA), for 5 min at 4 °C. Cells were then loaded in a magnetic cell sorter on a cooled B2 column through a 22-gauge needle, washed with 20 ml PBS containing 1 % BSA using a 19-gauge needle and eluted outside the magnetic sorter with 40 ml PBS with BSA. For T cell purification, an additional treatment with anti-Mac-1, anti-IA^d and anti-B220 antibodies, followed by complement was performed before the MACS separation. The purity of the cell preparations was checked after labeling with biotinylated anti-B220 or anti-Thy-1 mAb, followed by alkaline phosphatase streptavidine and naphthol-AS-MX-phosphate. B cell preparations contained 87–97 % B and approximately 2 % T lymphocytes and T cell preparations contained 90–94 % T and 2–3 % B lymphocytes.

2.4 Tissue preparation and immunolabeling

Tissues were prepared for the ultracryosection technique of Tokuyasu [22]. After fixation by transcardiac perfusion of 4 % paraformaldehyde solution in 0.1 M PBS (pH 7.4), dissection, mincing and overnight post-fixation in the same solution at 4 °C, organs were sequentially incubated at 4 °C for 12 h in 5 % and 15 % and for 24 to 48 h in 50 % and 2.3 M sucrose in PBS, then stored in liquid nitrogen. Sections were cut with a Reichert-Jung Ultracut E microtome with ultracryotomy system FC4D and transferred onto polylysine-coated slides on 2.3 M sucrose drops. Slides were then post-fixed for 5 min in 4 % paraformaldehyde solution in 0.1 M PBS and rinsed in 0.1 M PBS. Before immunolabeling, endogenous peroxidase was inhibited with 3 % H₂O₂ for 30 min. After incubation for 30 min at room temperature in 0.05 M Tris buffer (pH 7.4) containing 10 % BSA, tissues were incubated overnight at 4 °C with rabbit anti-receptor polyclonal antibody #655, or with control rabbit serum diluted in Tris buffer with 1 % BSA, followed by biotinylated anti-rabbit Fab fraction (Boehringer Mannheim) and streptavidin peroxidase, both for 30 min at room temperature. After incubation with diaminobenzidine, sections were counterstained in a Mayer Hemalun solution.

2.5 Flow cytometry analysis

After lysis of erythrocytes in 0.83 % NH₄Cl, spleen cells were incubated for 60 min at 4 °C in HAFA buffer (137 mM NaCl, 5 mM KCl, 0.4 mM MgSO₄, 0.3 mM MgCl₂, 5 mM glucose, 4 mM NaHCO₃, 1 mM EDTA, 1 mM phosphate, 20 mM NaN₃, 100 U/ml penicillin, 100 μ g/ml streptomycin, pH 7.4, supplemented with 3 % FCS) with control rabbit serum or rabbit anti-receptor antiserum #655, followed by fluoresceinated swine anti-rabbit Ig antibody (Dakopatts, Copenhagen, Denmark). After fixation in 0.62 % paraformaldehyde, fluorescence was analyzed with an ATC3000 flow cytometer (ODAM, Wissembourg, France). Cell subpopulations were analyzed by simultaneous labeling with biotinylated mAb specific for surface markers followed by streptavidin-conjugated RED613 (Gibco BRL, Gaithersburg, MD). For triple labeling, cells were incubated with fluoresceinated, biotinylated and phycoerythrin-conjugated antibodies, followed by Cy-chrome-labeled streptavidin (PharMingen, San Diego, CA).

2.6 PCR analysis of MHVR transcripts

Primers from conserved regions that recognize either the MHVR isoforms or mmCGM2 isoforms of murine BGP were used to determine which splice variants were transcribed in the cells of the BALB/c immune system. The antisense primer (5'-CTGAATAAACTGTTTCTGTGGC-3') maps to the 3' untranslated region and the sense primer (5'-AATTCGAAGAAGCTAGCAGGCAGG-3') maps to the 5' untranslated region of both isoforms. Using 2 µg of RNA isolated from cells by the guanidine isothiocyanate method [23] with the antisense primer and 0.5 mM of each of the four nucleotide triphosphates, cDNA was synthesized with 200 U of Moloney murine leukemia virus RNase H reverse transcriptase (Life Technologies) at 42°C for 1 h, after which the reaction was incubated for an additional 10 min with 2 U of *E. coli* RNase H. The reaction was terminated by heating at 95°C for 10 min. Controls without template RNA were included. The cDNAs were amplified for 30 cycles with AmpliTaq (Perkin-Elmer Cetus) in a 100-µl reaction with the two primers. The PCR products were analyzed by Southern blot hybridization with ³²P-labeled oligonucleotides (5'-ACACTACGGCTATAGACAAAG-3' and 5'-AGACTACAACAGGGCCTG-3') that hybridize to the first domain of MHVR or mmCGM2 isoforms, respectively [10]. The sizes of the PCR products indicated whether the transcripts encoded BGP with two or four Ig-like domains. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) expression was used as an internal control to test the quality of the cDNA [24].

2.7 Receptor blockade with anti-receptor antibody

SP20 cells were incubated for 60 min at 37°C with 2 ml of a 1:2 dilution of MAb-CC1 culture supernatant or with a control IgG1 mAb, the challenged with MHV-A59. After virus adsorption for 1 h at 37°C, cells were washed twice with 5 ml of medium. At 1 h after virus addition (p. i.), control and infected cells were attached to coverslips with poly-L-lysine. At 8 h p.i., cells were fixed with acetone at -25°C, and immunolabeled with mouse anti-MHV Ab, followed by rhodamine-conjugated goat anti-mouse Ab. To determine the effect of anti-receptor Ab upon the yield of MHV-A59, aliquots of 5 × 10⁶ SP20 cells were incubated in 2 ml of a 1:2 dilution of MAb-CC1 or control mAb for 1 h at 37°C, then pelleted and resuspended in 2 ml of medium with 2 × 10⁷ PFU of MHV-A59 and incubated at 37°C for 1 h. Cells were then washed three times and resuspended in a final volume of 5 ml of medium and plated in 60-mm tissue culture dishes. Supernatant medium was harvested at 1, 18, 25 and 50 h p.i., and the infectivity of released virus was analyzed by plaque assay in L2 cells.

2.8 Assay for MHV-specific, B cell-mediated cytotoxicity

Plasmocytoma Sp20 or spleen cells from 4–12-week-old BALB/c or SJL mice were washed in saline and incubated for 30 min at 4°C with various dilutions of polyclonal mouse anti-receptor antibody, normal SJL/J mouse serum, anti-receptor MAb-CC1, or control mAb directed against

an irrelevant antigen. Cells were then washed and used as effector cells to lyse MHV-A59-infected 17 CL 1 cells prelabeled with ⁵¹Cr, followed by incubation for 4 h at 37°C in a CO₂ incubator as described previously [14, 15, 25]. The specific ⁵¹Cr release was calculated as follows:

$$\% \text{ specific } ^{51}\text{Cr release} = \left[\frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{total cpm} - \text{spontaneous cpm})} \right] \times 100.$$

Spontaneous release and total release were determined from target cells incubated with medium alone or with 5% triton detergent, respectively.

3 Results

3.1 Detection of CEA-related glycoproteins in BALB/c spleen

To determine whether CEA-related glycoproteins were expressed in the immune system, 1-µm sections of BALB/c spleen were analyzed after immunolabeling with anti-MHVR antibody #655. Large clusters of lymphoid cells that may correspond to follicles, as well as vascular endothelial cells were strongly stained by this antibody (Fig. 1A). Higher magnification showed that the receptor was distributed in small dots on the membranes (Fig. 1C). Lymphocytes in adjacent areas of the spleen were not labeled by anti-receptor antibody, suggesting that the receptor glycoproteins were only expressed on particular subsets of spleen cells. No reaction with spleen cells occurred with control normal rabbit serum (Fig. 1B).

CEA-related glycoproteins were also detected on spleen cells by flow cytometry analysis. As shown in Fig. 2, approximately 44% of these cells were recognized by anti-MHVR MAb-CC1, confirming the expression of these glycoproteins in cells of the immune system. Similar results were obtained with polyclonal antibody #655 (data not shown).

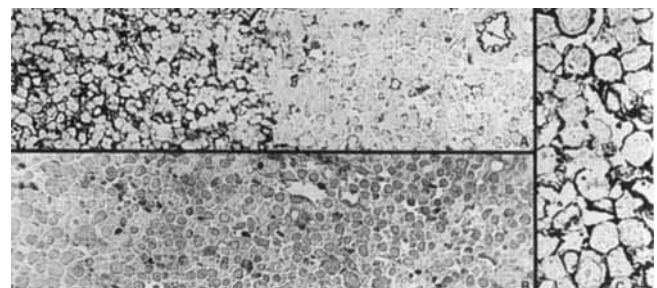


Figure 1. Immunolabeling of BALB/c spleen sections with anti-MHVR antibody. Spleen sections of 1 µm thickness were immunolabeled with control rabbit serum (B) or with anti-MHVR serum #655 (A, C). The virus receptor glycoproteins were detected on the membranes of a fraction of lymphoid cells (A, C) and on the luminal face of vascular endothelial cells (A, arrow). A, B × 500; C × 1250.

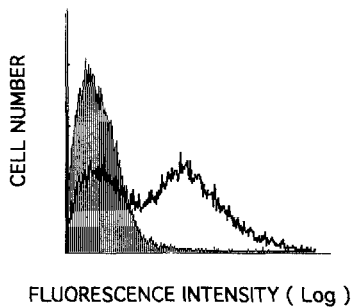


Figure 2. FACS analysis of expression on spleen cells of CEA-related glycoproteins. Expression of MHVR was analyzed with FACS on pooled spleen cells from three BALB/c mice, after incubation with biotinylated control IgG1 mAb (hatched zone) or MAb-CC1 (bold line), followed with streptavidin-RED613.

3.2 Expression of CEA-related glycoproteins on B lymphocytes and macrophages

Spleen cell subpopulations that expressed CEA-related glycoproteins were identified by flow cytometry analysis by

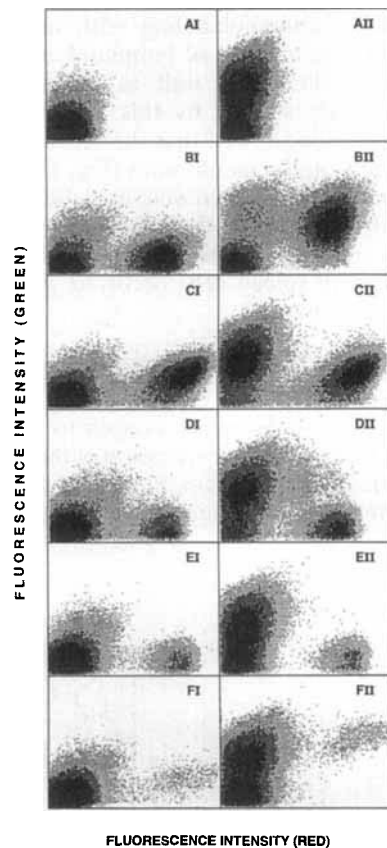


Figure 3. Analysis of spleen cell subsets expressing CEA-related glycoproteins. Pooled spleen cells from three BALB/c mice were labeled with biotinylated monoclonal antibodies reacting with different cell markers, followed by streptavidin-RED613 (red fluorescence) and with normal rabbit serum (I) or anti-MHVR rabbit serum #655 (II), followed by fluoresceinated anti-rabbit Ig antibody (green fluorescence). Results are shown without cell marker (A) and after labeling with markers specific for B lymphocytes (anti-B220, B), T lymphocytes (anti-Thy-1, C), T helper lymphocytes (anti-CD4, D), cytolytic/suppressor T lymphocytes (anti-CD8, E) and macrophages (anti-Mac-1, F).

double labeling with antibody #655 and mAb to different cell markers. B lymphocytes and macrophages bound anti-MHVR antibody (Fig. 3B and F), while most T lymphocytes, identified either by anti-Thy-1 mAb or by CD4 and CD8 subset markers, were not significantly labeled with antibody #655 (Fig. 3C–E). Similar results were obtained when cells were incubated with anti-receptor MAb-CC1 (data not shown).

Expression of CEA-related glycoproteins on macrophage- and B lineage-derived cell lines correlated with expression on B cells and macrophages isolated from the spleen. Anti-MHVR antibody #655 reacted strongly with WEHI-3, a macrophage-like cell line of BALB/c origin, and SP207, a BALB/c myeloma (Fig. 4A and B), but only minimally with TS1C3 cells derived from a C57Bl/6 T helper line (Fig. 4C). Because antibody #655 labeled spleen cells from C57Bl/6 and BALB/c mice similarly (data not shown), the differences in staining of the cell lines could not be attributed to the strain from which the lines originated and probably reflected the types of cells studied.

RNA PCR of cell populations isolated from BALB/c spleen and of lymphocyte and macrophage cell lines also showed that CEA-related glycoproteins were expressed in B cells and macrophages, but barely in T cells (Fig. 5). In some experiments, with an increased number of amplification cycles, MHVR expression was detected in T cells but at much lower levels than in B cells or in macrophages (data

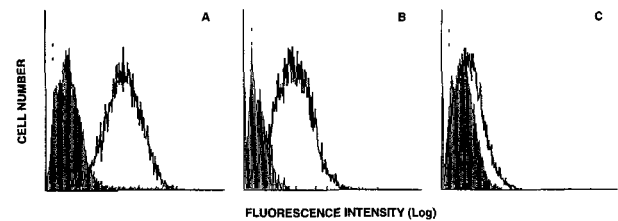


Figure 4. FACS analysis of CEA-related glycoprotein expression on B, T and macrophage cell lines. WEHI-3 (A), SP207 (B) and TS1C3 (C) cells were incubated with control rabbit serum (hatched zone) or anti-MHVR rabbit serum #655 (bold line), followed by fluoresceinated anti-rabbit antibody and analyzed with FACS after exclusion of dead cells on the basis of their light scatter values and of their labeling with propidium iodide.

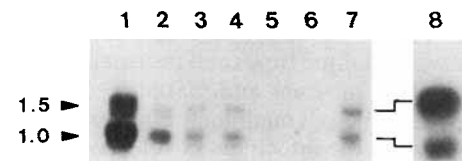


Figure 5. Characterization of transcripts encoding the two- and four-domain isoforms of the CEA-related glycoprotein in BALB/c spleen and thymus cells and B and T cell lines and macrophages. RNA PCR of SP207 and TB6D2 B lineage cell lines (lanes 1 and 2), B cells (lanes 3 and 4) and T cells (lane 5) purified from BALB/c spleen, TS1C3 line of T cells (lane 6), WEHI-3 macrophage cell line (lane 7) and BALB/c macrophages (lane 8) was done using primers common to both the MHVR and mmCGM2 isoforms. Hybridization of the PCR products with an oligonucleotide specific for the N-terminal domain of the MHVR isoforms is shown. The PCR products obtained are 1525 bp and 985 bp long and correspond to the four- and two-domain MHVR isoforms, respectively.

not shown). However, it is not clear whether this observation was due to very low expression of the CEA-related glycoprotein message in T lymphocytes or to minor contamination of the samples with a few B lymphocytes or macrophages. The levels of CEA-related glycoprotein expressed in TB6D2 cells varied from one experiment to another, possibly reflecting effects on expression of CEA-related proteins or modifications in cell conditions (data not shown). The sizes of the PCR products indicated that the B cells, macrophages, WEHI-3 and SP207 cells all produced transcripts that would encode proteins with two and four Ig-like domains. Blotting with oligonucleotides specific for either the MHVR or mmCGM2 alleles showed that these BALB/c or C57Bl/6 cells and cell lines expressed only transcripts of MHVR (Fig. 5), and not mmCGM2 (data not shown).

3.3 Expression of CEA-related glycoproteins on cells of the B-1a lineage

CD5⁺ B lymphocytes constitute a separate lineage of B cells, called B-1a, that is particularly abundant in neonatal mice and in the NZB strain and may play an important role in autoimmune responses [26, 27]. Expression of MHVR glycoproteins was analyzed in this B-1a cell subpopulation by flow cytometry after triple labeling of spleen cells from 1-week-old BALB/c mice with anti-B220, anti-CD5 and anti-MHVR antibodies. As shown in Fig. 6, expression of CEA-related glycoproteins was as strong on CD5⁺ B lymphocytes as on CD5⁻ B cells (Fig. 6C–D), while CEA-related glycoproteins were not detected on

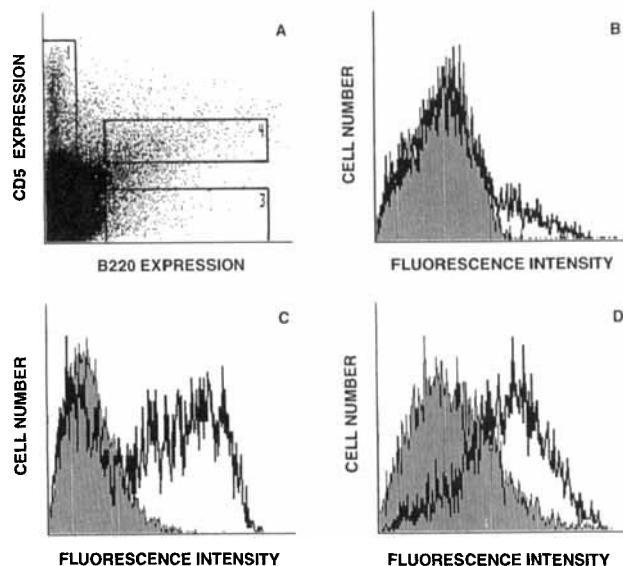


Figure 6. Expression of CEA-related glycoproteins on B-1a lymphocytes. Pooled spleen cells from five 1-week-old BALB/c mice were incubated with fluoresceinated anti-B220 mAb (PharMingen), phycoerythrin-conjugated anti-CD5 mAb (Boehringer-Mannheim) and either biotinylated control IgG1 mAb or biotinylated MAb-CC1, followed by Cy-chrome labeled streptavidin. Cell subpopulations, identified (panel A) as B220⁻, CD5⁺ (1); B220⁺, CD5⁻ (3); and B220⁺, CD5⁺ (4) were analyzed as controls (hatched zone) or for MHVR (bold line) expression (panels B, C and D, corresponding to areas 1, 3 and 4 in panel A, respectively). For technical reasons, CD5 and MHVR expression are shown on a log scale, whereas B220 expression is on a linear scale.

B220⁻ CD5⁺ T lymphocytes (Fig. 6B). Similar results were obtained with NZB mice and on B-1a cells identified with anti-IgM and anti-CD5 antibodies (data not shown).

3.4 Inhibition of MHV-A59 infection of B lymphocytes by anti-MHVR antibodies

One of the functions of the murine CEA-related BGP proteins is as a receptor for mouse coronavirus MHV. We therefore tested whether that CEA-related glycoprotein expressed in mouse B cells could serve as a receptor for MHV. Pretreatment of SP20 cells with anti-MHVR MAb-CC1 blocked infection of the cells by MHV-A59 (Fig. 7). At 8 h p.i., viral antigens developed in the cytoplasm of cells pretreated with medium alone or with irrelevant antibody (Fig. 7b and c), but no infected cells were observed in cultures pretreated with anti-receptor MAb-CC1 (Fig. 7d). Table 1 shows that pretreatment of cells with MAb-CC1 inhibited the replication of virus in SP20 cells. Thus, anti-MHVR MAb-CC1 blocked virus receptor activity on SP20 cells of the B cell lineage, showing that the CEA-related glycoproteins expressed on these cells serve as functional receptors for MHV-A59.

To determine whether this ability of MHV-A59 to infect B lymphocytes would cause lysis of large numbers of B cells *in vivo*, spleen cell subpopulations were isolated from

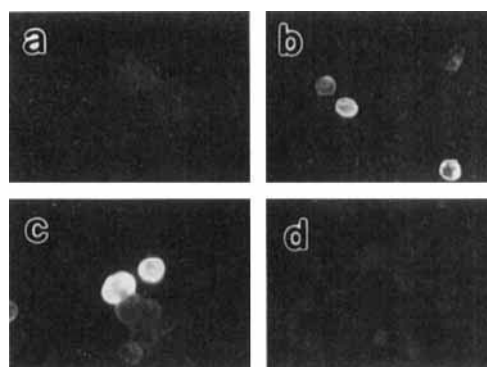


Figure 7. Blockade of MHV receptor with anti-receptor antibody prevents infection of SP20 cells by MHV-A59. SP20 cells were mock-infected (a), or infected with MHV-A59 (b–d). Panels d and c, respectively, show SP20 cells pre-incubated with anti-receptor MAb-CC1 or an irrelevant mAb of the same isotype prior to inoculation with the virus. At 8 h after inoculation, cells were fixed and labeled with convalescent antibody from mice infected with MHV-A59, showing viral antigens in the cytoplasm.

Table 1. Inhibition of MHV-A59 infection of SP20 cells by anti-MHVR MAb-CC1

Time p.i. (h)	Virus yield after pre-treatment with ^{a)}		
	nothing	control mAb	anti-receptor MAb-CC1
1	< 10 ⁴	< 10 ⁴	< 10 ⁴
18	1.3 × 10 ⁸	9.5 × 10 ⁷	2.4 × 10 ⁵
25	1.0 × 10 ⁸	1.2 × 10 ⁸	2.6 × 10 ⁵
50	2.9 × 10 ⁷	3.0 × 10 ⁷	2.7 × 10 ⁵

a) Yield of virus from supernatant medium (PFU/ml).

Table 2. Spleen cell populations after MHV infection

Time ^{a)}	Total ^{b)}	Cell subpopulations (%)				
		B220 ^{c)}	Thy-1 ^{c)}	CD4 ^{c)}	CD8 ^{c)}	Mac-1 ^{c)}
0	83 ± 6	33.5	28.2	22.8	10.1	3.9
3	56 ± 7	40.4	31.4	21.8	10.5	4.8
7	82 ± 7	36.1	28.1	19.7	11.7	4.4
12	113 ± 9	31.0	34.8	21.4	14.9	7.2

- a) Time (days) after MHV infection of groups of four BALB/c mice.
 b) Total spleen cell number ($\times 10^6$, mean \pm SE) was determined after teasing with forceps and passage through nylon filters.
 c) Cell subpopulations measured by flow cytometry analysis after labeling of pooled cells with mAb specific for indicated markers (% of total cells).

BALB/c mice at different times after virus inoculation and analyzed by flow cytometry. As shown in Table 2, only minor changes in total number of spleen cells and no change in the relative proportions of the various cell subpopulations were observed.

3.5 Inhibition of B cell-mediated cytotoxicity by anti-MHVR antibodies

Mouse fibroblasts infected with MHV-A59 can serve as target cells for an unusual, rapid, MHV-specific, non-MHC restricted cytotoxicity mediated by B cells from naive BALB/c mice which have not been exposed to MHV [14, 15, 25]. We previously showed that treatment of the target cells with antibody directed against the viral spike glycoprotein prior to addition of the effector cells could block this cytotoxic reaction and postulated that the effect was mediated by interaction of virus glycoproteins on the target cell with receptors on the effector cell [14, 15]. We pretreated the effector B cells from BALB/c mice with polyclonal mouse anti-receptor antibody or normal SJL/J mouse serum or with anti-MHVR mAb-CC1 or control mAb to determine whether blocking of virus receptors on

the B cells could prevent the B cell-mediated cytotoxicity or "virus-killer" (VK) activity. Table 3 shows that both polyclonal mouse anti-receptor antibody and MAb-CC1 prevented MHV-specific cytotoxicity mediated by spleen cells of BALB/c mice, while neither control antibody had any effect. SJL/J spleen cells did not induce B cell-mediated cytotoxicity [14]. The values of specific ^{51}Cr release in this assay when antibody-treated, serum-treated or untreated SJL/J spleen cells were used as effectors varied between 0 and 3% (data not shown). Thus, as previously demonstrated, spleen cells from SJL/J mice could not serve as effectors for this cytotoxicity assay. Interestingly, SP20 cells could also serve as effectors of B cell-mediated, MHV-specific cytotoxicity, and this function was also blocked by polyclonal anti-receptor antibody (Table 3).

4 Discussion

Using immunohistochemistry, flow cytometry, RNA PCR and immunoblotting (data not shown), we observed expression of CEA-related glycoproteins in mouse macrophages and B lymphocytes, including cells of the B-1a lineage. These molecules were also detected in endothelial cells (Fig. 1), as well as in thymic epithelial cells (data not shown), but not in T cells. The splenic B lymphocytes and macrophages, as well as the WEHI-3 BALB/c macrophage-like cell line and the TB6D2 and SP207 BALB/c myeloma cell lines, expressed transcripts that would encode both isoforms with two or four Ig-like domains. As expected, the transcripts were of the MHVR allele of the murine BGP, and not the mmCGM2 allele which has only been detected in SJL/J and outbred mice [7, 10, 28]. Because in other systems, mouse CEA-related molecules, as well as rat cell-CAM 105 and human CEA, have been shown to participate in intercellular adhesion [2, 3, 5, 29], their expression on the membrane of cells which all require close contact with T lymphocytes for physiological functions, suggests that these glycoproteins play a role in recognition and binding to T cells. If so, then a ligand for these molecules might be found on T lymphocytes.

Table 3. Effects of anti-MHVR antibody treatment of effector cells on B cell-mediated, MHV-specific cytotoxicity

Effector cells	Antibody	% of specific ^{51}Cr release at E:T ratio				
		50:1	25:1	12.5:1	6:1	
BALB/c spleen cells	–	37	26	14	8	
	Polyclonal mouse anti-receptor	1/5	13	8	5	3
		1/10	21	14	9	4
		1/20	27	17	10	6
	Normal mouse serum	1/5	38	23	12	9
		1/10	42	26	12	9
		1/20	36	22	13	8
	MAb-CC1	1/4	19	11	6	5
		1/8	29	20	13	8
		1/16	32	17	11	8
	Control mAb	1/4	41	30	20	17
		1/8	43	32	21	12
1/16		44	33	23	13	
SP20 cells	–	35	29	24	21	
	Polyclonal	1/5	11	9	6	4
	Normal serum	1/5	32	27	21	16

Our data add to the increasing evidence of the expression of a variety of CEA-related glycoproteins in immune cells from many species. CEA expression has been observed in human B cell lymphomas and in Epstein-Barr immortalized B cell lines [30] and a related protein, CD66, is expressed in human neutrophils and epithelial cells, but not in lymphocytes or monocytes [31]. A cell adhesion molecule homologous to human CD22 that was recently identified in murine B lymphocytes shows limited homology to MHV receptors and to CEA [32]. CD22 has seven Ig-like domains and is considerably larger than the MHVR-related glycoproteins which have either two or four domains. CD22 mediates binding of B lymphocytes to monocytes, T cells and B cells [33–35]. It may facilitate antigen recognition [33], regulate T cell receptor/CD3-induced signaling [36] and promote B cell activation and proliferation in association with tyrosine phosphorylation [37–39]. CD31, a CEA-related glycoprotein with six extracellular Ig-like domains, is expressed on platelets, endothelial cells and monocytes, as well as on B lymphocytes and subsets of CD4⁺ and CD8⁺ T lymphocytes [40–45]. It mediates cell-cell adhesion and may be involved in lymphocyte recruitment and transmigration through the vascular endothelium [45–48]. Clearly the complex regulation of the expression of these CEA-related glycoproteins in the immune system indicates their importance in many aspects of the immune response. The natural ligands for these glycoproteins have not yet been identified.

The observation that CEA-related glycoproteins of the Ig superfamily found on immune cells serve as virus receptors is not unexpected. Indeed, other proteins of the Ig superfamily expressed on lymphocytes are also receptors for viruses [49], including CD4 for human immunodeficiency virus [50], ICAM-1 for rhinovirus [51, 52], and PVR for poliovirus [53]. One might therefore expect that cells bearing these adhesion molecules can be infected, or that their function can be altered, when exposed to the viruses which recognize these molecules as receptors. Thus, it has been known for many years that MHV strains can infect macrophages [54], so it was not surprising to find that these cells express the CEA-related glycoproteins that serve *in vivo* as receptors for MHV in fibroblasts [55], intestinal epithelial cells, hepatocytes and other cell types. Whether MHV infection modifies the *in vivo* expression of the receptor on macrophages, resulting in their altered function [56–58] remains to be determined.

Our results indicate that the CEA-related glycoprotein isoforms are expressed on B lymphocytes. We previously reported that MHV-A59 can cause agglutination of spleen cells *in vitro* [15] and can replicate in hybridomas. MAb-CC1, which binds to the N-terminal domain of MHVR glycoprotein, blocks binding of MHV-A59 to MHVR on murine fibroblasts, prevents infection of these cells *in vitro*, and inhibits replication of the virus in infant mice, was found to block infection of SP20 myeloma cells. This shows that isoforms of MHVR serve on cells of the B lineage as receptors for MHV-A59. Because the mAb completely blocked infection of the SP20 cells, we conclude that the MHVR isoforms are the only functional virus receptors expressed on these BALB/c cells. Although MHVR isoforms on cultured cell lines of the B lineage can function as MHV receptors, it is not yet clear whether they are used similarly during infection *in vivo*. In adult BALB/c animals

infected with MHV-A59, we found no loss of B cells from the spleen, which contrasts with the splenolysis resulting from MHV-3 infection of C57Bl/6 B lymphocytes [59, 60]. Additional studies will therefore be needed to show whether MHVR glycoproteins are functional virus receptors on B cells *in vivo* as well as in cell lines.

A second biological function of the CEA-related MHVR glycoproteins on the B cell membrane, that of B cell-mediated, MHV-specific cytotoxicity, is also associated with the virus receptor activity of murine BGP. MHV-A59-infected murine fibroblasts are killed rapidly and with high efficiency by incubation with splenocytes from BALB/c mice that have never been infected with MHV. This VK activity is not restricted by MHC and, surprisingly, the effector cells were shown to be B lymphocytes [15]. We previously showed that SJL/J B cells did not bind MHV-A59 virus or serve as effectors for VK activity and that treatment of MHV-A59-infected fibroblasts that serve as target cells with antibody directed against the viral spike glycoprotein blocked the VK activity [15]. This paper shows that treatment of the effector BALB/c spleen cells with anti-receptor antibody also blocked this VK activity, and that SP20 cells, which express MHVR, can serve as effector cells for VK activity. VK activity is associated with fusion of the virus-infected target cell with the effector B lymphocyte [25], and MHV-induced fusion depends upon specific interaction of the viral spike glycoprotein with glycoprotein receptors related to MHVR. Thus, VK activity depends upon specific interaction of the virus glycoproteins with specific CEA-related glycoprotein receptors on B cell membranes. It is not yet clear whether VK activity plays any role in MHV infections *in vivo*.

Finally, MHV infection of mice can induce IgG2a-restricted hypergammaglobulinemia and enhancement of concomitant antibody responses [61–64]. Whereas Ig production is most probably a T-dependent phenomenon, binding of virions to receptor glycoproteins in the membrane of B lymphocytes could possibly be required to trigger proliferation of these cells. Similarly, *in vitro* activation of B lymphocytes results from influenza virus binding to the membrane of these cells [65, 66].

Expression of CEA-related glycoproteins in the BGP subfamily in macrophages and B lymphocytes has been demonstrated using specific antibodies and RNA PCR. The roles of these glycoproteins in the numerous cell adherence, activation and proliferation processes in the normal immune system can now be explored. The expression of the virus receptor glycoproteins in immune cells can explain some, but probably not all, of the changes of the immune system that are induced by MHV infection. Virus strain differences and differential expression of various receptor isoforms may explain the variable effects of MHV infection on the immune system of different inbred mouse strains.

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