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Predominance of MHC class II-restricted CD4$^+$ cytotoxic T cells against mouse hepatitis virus A59

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SUMMARY

Coronavirus-induced acute hepatitis is a complex event and the role of different components of the immune system with regard to defined viral proteins and the course of the infection is not yet clear. We have analysed the cytotoxic T-lymphocyte (CTL) response in mouse hepatitis virus (MHV-A59) infection. Surprisingly, we detected only a very clear virus-specific major histocompatibility complex (MHC) class II-restricted cytotoxicity in mice infected with MHV-A59. We found no evidence of activation of the classical CD8$^+$ MHC class I-restricted CTL. The virus-specific CD4$^+$ CTL derived from two different mouse strains having different MHC haplotypes recognized the same immunodominant epitope. This epitope, comprising the amino acid residues 329–343 of the viral S-glycoprotein, was recognized both at the polyclonal level and by virus-specific CTL clones. Transfer studies using a MHV-A59-specific CD4$^+$ CTL clone showed significant protection against a lethal challenge with MHV-A59, implicating that these CD4$^+$ CTL play a pivotal role in the protection against MHV-A59 infections.

INTRODUCTION

The development of a viral infection in a susceptible host involves a complex set of interactions between the virus and the ability of the host immune system to limit virus replication and to clear the infection to reduce excessive damage to critical tissues. Our studies were concerned with the cellular immune response of the mouse to a well-characterized coronavirus, mouse hepatitis virus (MHV-A59). MHV-A59 causes a variety of acute and chronic infections in mice and rats, ranging from acute hepatitis and encephalomyelitis to chronic demyelination.[1,2] In other viral infections, clearance of infected tissue is dominated by cytotoxic T lymphocytes (CTL) of the CD8$^+$ phenotype.[3–5] These cells recognize viral peptides carried to the cell surface by major histocompatibility complex (MHC) class I molecules.[6,7] Although several studies have focused particularly on the induction and specificity of CTL directed against the neurotropic JHM strain of MHV (MHV-JHM),[8,9] there are only a few reports describing the generation of specific CTL against this virus, but the role of these CD8$^+$ CTL as a defence mechanism has been controversial. Results obtained by some research groups suggest that cytotoxic CD8$^+$ cells are not only crucial for elimination of MHV infection from the target organs, but also have a pivotal role in the prevention of the chronic phase of central nervous system (CNS) disease.[8] In contrast, other studies have suggested that these cytotoxic T cells might play an important role in the development of MHV-induced demyelination.[9]

In order to elucidate the role of CTL in murine coronavirus-induced hepatitis, we tried to generate CTL specific for MHV strain A59, from primed BALB/c and C57BL/6 mice. We could not demonstrate MHV-A59-specific class I-restricted killing. Surprisingly, we were able to show a very clear MHV-A59-specific class II-restricted cytotoxicity in both strains of mice. Depletion of CD4$^+$ or CD8$^+$ T cells revealed the CD4$^+$ phenotype of the MHV-A59-specific CTL. Establishment of MHV-A59-specific CD4$^+$ CTL clones of both the H-2^b and H-2^d haplotype, and determination of the fine specificity of these clones, showed that both clones recognize amino acid residues 329–343 of the spike protein of MHV-A59. This 15-mer peptide is equally well recognized as MHV-A59 in polyclonal responses, indicating that this epitope is immunodominant. Furthermore, adoptive transfer studies using a MHV-A59-specific CD4$^+$ CTL clone showed significant protection against a lethal challenge with MHV-A59. The implications of these data for the understanding of the *in vivo* role of these CD4$^+$ CTL in virus elimination are discussed.

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; CTL, cytotoxic T cells; mAb, monoclonal antibody; MHV, mouse hepatitis virus; MOI, multiplicity of infection; VV, vaccinia virus.

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MATERIALS AND METHODS

Mice

Specific pathogen-free (which includes seronegative for MHV) BALB/c and C57BL/6 mice were purchased from the breeding facilities of the Central Animal Laboratory of Utrecht University (Utrecht, the Netherlands) at 6 weeks of age.

Viruses

MHV-A59, a virulent hepatotropic strain, and the less virulent temperature-sensitive mutant ts342 of MHV-A59, were propagated on Sac-cells and virus stocks were prepared as has been described previously.[10,11] Inactivated virus was prepared by UV-irradiation for 10 min (infectivity of virus stock reduced to zero). The generation of the vaccinia recombinant viruses (VV) expressing the structural proteins S and M of MHV-A59 (vMS and vMM) has been described elsewhere.[12,13] The VV recombinant expressing the N protein of MHV-A59 (vMN) was generated by isolation of the N-coding region from plasmid pRG68,[14] as a 1·5 kb *Bam*HI fragment. This fragment was filled with the Klenow fragment and ligated to *Sma*I-digested pSC11.[15] As a control VV recombinant, the pSC11 vector expressing the Lac Z gene (vSc) was used.[16] The individual MHV antigens for use in the proliferation assay were prepared by infecting confluent RK13 cells with the different VV recombinants at a multiplicity of infection (MOI) of 0·1 and harvesting the infected cells 24 hr later. For this purpose cells were washed twice and scraped into phosphate-buffered saline (PBS). Samples were freeze-thawed three times, clarified by centrifugation at 500 *g* for 10 min, and stored at −70°. The VV recombinant lysates were checked by Western blot analysis to determine if each MHV protein was present, and the VV recombinant lysates were inactivated for 10 min by UV-irradiation.

Cell lines

The cell lines used as target cells were LB15.13, an H-2^{bd}-expressing tumour cell line (ATCC, Rockville, MD), Sp2.0, an H-2^{d}-expressing myeloma (ATCC), A20, an H-2^{d}-expressing B-cell lymphoma (ATCC) and G4, a transfectant of the MHV non-permissive H-2^{b} T-cell lymphoma EL4 expressing the receptor for MHV-A59. The G4 transfectant cell line was obtained by lipofection with a pSV_2-neo-$MHVR_1$ construct, kindly provided by J. W. A. Rossen (Department of Virology, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, the Netherlands), containing the CMV promoter in front of the MHV receptor gene and the SV40 promoter in front of the neomycin resistance gene. The MHV receptor gene was kindly provided by Dr K. V. Holmes (Uniformed Services University of Health Sciences, Bethesda, MD).[17] One day after transfection the cells were transferred to medium containing 600 μg/ml G418, to select for neomycin-resistant transfectants. All established cell lines were tested for expression of the MHV receptor, by following the efficiency of MHV infection using immune fluorescence labelling. Briefly, cells infected for 6 hr with MHV-A59 (MOI = 50) were cytocentrifuged into microslides, air dried, fixed in acetone at −20° for 10 min, and dried again. Slides were rehydrated for 10 min in PBS containing 0·3% bovine serum albumin (BSA), and incubated with rabbit polyclonal anti-MHV-A59 (k134), or control antibody, for 30 min at room temperature. After two washes, the slides were incubated with fluorescence isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (GAR–FITC, Sigma, St Louis, MO) for 30 min at room temperature, and examined with a fluorescence microscope.

Antigens

Preparation of a MHV-A59 (S) expression library consisting of a set of overlapping fragments of the entire S gene fused to β-galactosidase has been described previously.[18]

Peptides spanning the sequence of MHV-A59 S protein amino acid residues 174–374 were prepared by automated simultaneous multiple peptide synthesis (SMPS).[19] A set of 39 peptides, each 15 residues in length and overlapping by 10 amino acids, was synthesized.

Generation of virus-specific CTL in bulk culture

BALB/c and C57BL/6 mice were injected intraperitoneally with 10^4 plaque-forming units (PFU) ts342 and boosted 10 days later with 5×10^4 PFU MHV-A59 wild-type. Three weeks after boosting, spleen cells (1×10^8) were isolated and stimulated in bulk culture with 5×10^7 irradiated (3000 rads) MHV-A59-infected syngeneic spleen cells (MOI of 0·3) in 50 ml Iscove's modified Dulbecco's medium (IMDM) (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, antibiotics and 2-mercaptoethanol (2-ME) (2×10^{-5} M) for 5 days.

Cytotoxicity assay

Varying numbers of *in vitro*-stimulated spleen cells or cloned cells were added to $2{\cdot}5 \times 10^3$ $Na^{51}CrO_4$ labelled target cells in 0·2 ml IMDM supplemented with 10% FCS in 96-well U-shaped plates and incubated for 5 hr at 37° and 5% CO_2. As target cells the transfected cell lines G4, Sp2.0, A20 and LB15.13 were used. A20 and LB15.13 cells express both MHC class I and class II glycoproteins of, respectively, H-2^{d} and H-2^{bd} haplotype, whereas G4 and Sp2.0 cells express, respectively, only MHC class I H-2^{b} and H-2^{d} molecules. Infected target cells were prepared by infecting the cells with MHV-A59 at a MOI of 50, and incubated for 3 hr at 37° prior to labelling. Incubation of target cells with inactivated MHV-A59 (at a concentration of 1×10^8 PFU/ml) or peptides (at a concentration of 20 μM) was performed after the ^{51}Cr labelling for 2 hr. The data are expressed as percentage specific lysis defined as [(experimental lysis) − (spontaneous lysis)]/[total (detergent lysis) − (spontaneous lysis)]. Maximum spontaneous release values were always < 20% of total lysis.

Complement-mediated depletion

In vitro-stimulated effector cells of MHV-A59-infected mice were resuspended in PBS containing 0·3% BSA at a concentration of 5×10^6 cells/ml, and incubated for 1 hr at 4° either with monoclonal antibody (mAb) specific for CD8 (53–6.7) or[20] CD4 (GK1.5), or without mAb. The cells were washed twice, resuspended in PBS containing 0·3% BSA and 10% Low-Tox Rabbit complement (Cedarlane Lab., Hornby, Canada) at a concentration of 5×10^6 cells/ml, and incubated for 1 hr at 37°. Viable cells were harvested and tested for the residual CTL activity.

Establishment of MHV-A59-specific $CD4^+$ T-cell clones

Spleen cells of primed mice (same immunization protocol as for the generation of virus-specific CTL) were isolated and cultured

for 3 days at a concentration of 2×10^6 cells/ml in medium supplemented with 10% FCS, 2 mM glutamine, antibiotics and 2-ME (2×10^{-5} M) in the presence of UV-irradiated MHV-A59 (10^7 PFU/ml). Subsequently, the cells were washed and resuspended in medium supplemented with 10% FCS and 5% rat concanavalin A (Con A) supernatant containing growth factors. Seven days later viable cells were harvested on a density gradient (Lympholyte M; Cedarlane Lab.), washed and stimulated at a concentration of 10^6 cells/ml with inactivated MHV-A59 (10^7 PFU/ml) and irradiated (3000 rads) syngeneic spleen cells as antigen-presenting cells (APC; 10^7 cells/ml) for another 3 days. After four stimulation cycles, viable cells were harvested and cloned by limiting dilution at 300, 30, 3, 1 and 0·3 cell/well in 96-well U-bottomed plates in the presence of 10^7 PFU/ml MHV-A59, 10% rat Con A supernatant and 10^5 APC. When clones were obtained and screened as positive, they were maintained with rat Con A supernatant-supplemented medium and stimulated every 10 days.

Lymphocyte proliferation assay

Proliferative responses of the T-cell clones were measured in flat-bottomed microtitre plates in triplicate cultures. Each well contained 1×10^4 T cells, irradiated (3000 rads) syngeneic spleen cells (2×10^5 cells/well) as APC, and various amounts of antigens in 0·2 ml IMDM supplemented with 2% mouse serum, glutamine, 2-ME and antibiotics. The cells were cultured for 2 days and pulsed for 18 hr with [^{3}H]thymidine. Cells were harvested on fibreglass filters, and [^{3}H]thymidine incorporation was measured. Data are expressed as stimulation indices (SI). SI = mean c.p.m. of triplicate samples in the presence of antigen/mean c.p.m. of triplicate samples in the absence of antigen.

Adoptive transfer of T-cell lines

BALB/c mice were injected intravenously with 5×10^6 cells of the MHV-A59-specific CD4$^+$ CTL clone HS1, in a volume of 100 μl PBS. One day later mice were inoculated intraperitoneally with 4000 PFU MHV-A59 ($100 \times LD_{50}$). Control mice received either 5×10^6 cells of the rabies-specific CD4$^+$ clone PM109 (kindly provided by Dr I. J. T. M. Claassen, RIVM, Bilthoven, the Netherlands), or PBS, before MHV-A59 infection. Infected mice were observed for disease and death for up to 20 days.

RESULTS

Anti-MHV-A59 cytotoxic activity of *in vitro*-stimulated cultures is class II-restricted

Intraperitoneal infection of BALB/c and C57BL/6 mice, even using a very low virus dose (100 PFU) of MHV-A59, resulted in acute hepatitis and death of almost all animals within 7 days. Previously, a less virulent temperature-sensitive mutant (ts342) of MHV-A59 was isolated,[10] and preimmunization of mice with ts342 resulted in complete protection against a subsequent challenge of MHV-A59. Spleen cells of BALB/c and C57BL/6 mice, preimmunized with ts342 and boostered with MHV-A59, were restimulated *in vitro* and tested for cytotoxic activity. Figure 1a shows the cytolytic response of *in vitro*-stimulated BALB/c effector cells to targets of the H-2^d haplotype. Two target cell lines were used: Ia$^-$ Sp2.0 and Ia$^+$ A20. The efficiency of MHV-A59 infection for A20, determined by immunofluorescence, was approximately 60–80%. It was not possible to infect the Sp2.0 cell line with MHV-A59. Figure 1a shows that the BALB/c effector cells lysed the MHV-A59-infected A20 cells. Moreover these same target cells sensitized with inactivated MHV-A59 were also lysed, in contrast to Ia$^-$ Sp2.0 cells sensitized with inactivated MHV-A59. The cytolytic responses of the *in vitro*-stimulated C57BL/6 effector cells are shown in Fig. 1b. The CTL bulk cultures lysed only the MHV-A59-infected MHC class II-expressing LB15·13 and not the Ia$^-$ transfectant cell line G4, although the efficiency of infection for both cell lines was the same (approximately 60–80%). Furthermore, sensitization of LB15.13 and G4 with inactivated MHV-A59 also demonstrated that only the MHC class II-positive cell line LB15.13 was recognized by the C57BL/6 bulk CTL. These experiments provided evidence for the presence of virus-specific class II-restricted CTL and not of class I-restricted cells. In addition, we investigated if preimmunization with ts342 contributed to the lack of MHV-A59-specific class I-restricted CTL responses, by measuring the CTL response of mice that survived a low dose MHV-A59 infection; however, with these mice similar results were obtained (data not shown). To confirm the identity of the MHV-A59-specific CTL, depletion studies were performed. Depletion of CD4$^+$ cells by anti-CD4 mAb and complement markedly reduced the cytotoxic activity of BALB/c effector cells against MHV-A59-infected A20. However, probably because of incomplete depletion of the CD4$^+$ T cells, some residual cytotoxicity was present. Depletion of CD8$^+$ cells had no effect on the cytotoxic

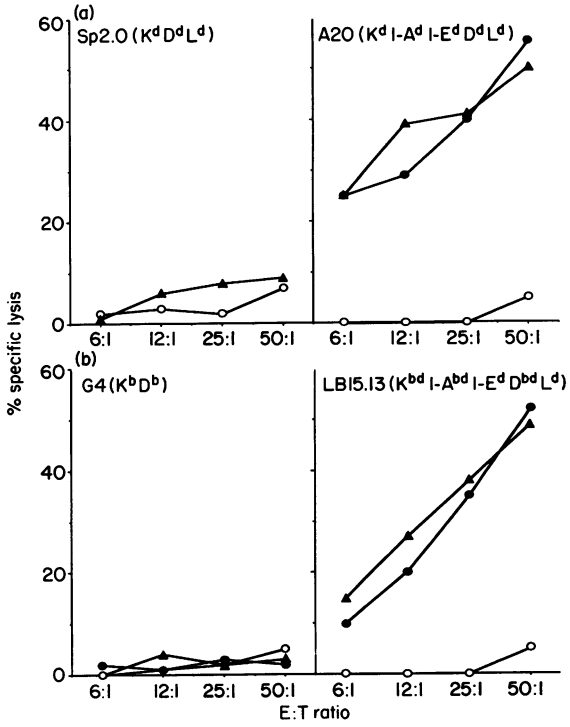

Figure 1. Anti-MHV cytotoxicity of BALB/c and C57BL/6 bulk cultures is MHC class II-restricted. BALB/c bulk CTL (a) and C57BL/6 bulk CTL (b) were assayed at different effector:target (E:T) ratios against Sp2.0 and A20 or G4 and LB15.13 target cells, respectively, uninfected (○), infected (●) or incubated with non-infectious virus (▲).

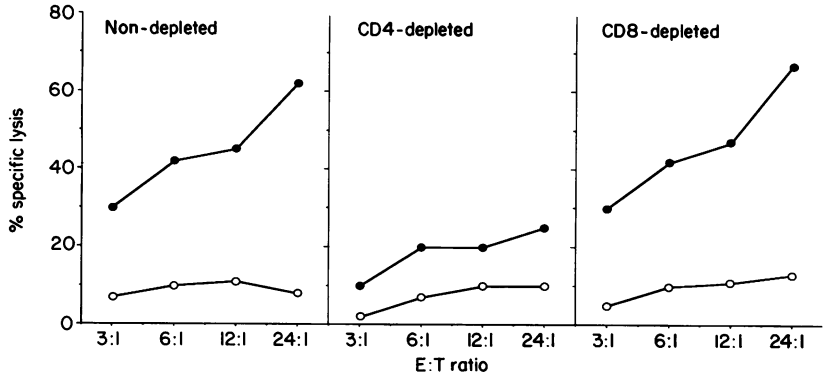

Figure 2. Depletion of $CD4^+$ cells reduced the MHV-A59-specific CTL activity. BALB/c bulk effector cells were depleted for CD4 or CD8 with, respectively, anti-CD4 or anti-CD8 mAb plus complement, and assayed at different E:T ratios against uninfected (○) or MHV-A59-infected (●) A20 cells.

activity of the effector cells (Fig. 2). A similar pattern of cytotoxicity was observed when C57BL/6 effector cells were depleted of $CD4^+$ or $CD8^+$ cells (data not shown). Depletion of B cells had no effect on the cytotoxic activity of the effector cells, and in addition no non-MHC-restricted lysis of MHV-A59-infected target cells was observed (data not shown); hence we have no evidence for a role of the previously described B-cell-mediated cytotoxicity.[23,24] In conclusion, the results of the experiments showed that the MHV-A59-specific CTL derived from virus-primed mice recognized MHV-A59 determinants only in the context of MHC class II molecules, and were of the $CD4^+$ phenotype.

$CD4^+$ CTL clones recognize peptide 329–343 of the S glycoprotein

To further characterize the cytotoxic activity of the $CD4^+$ T lymphocytes, we established two MHV-A59-specific $CD4^+$ clones, HS1 and B6.1, of, respectively, BALB/c and C57BL/6 origin. Both clones were shown to produce interleukin-2 (IL-2) and (interferon-γ) IFN-γ, but no IL-4, and were Thy-1.2^+, $CD4^+$, $CD8^-$, corresponding with a T-helper type 1 (Th1) phenotype (data not shown). To determine which structural protein was recognized, UV-inactivated VV recombinant lysates containing the structural proteins N, M and S were tested in a proliferation assay. Table 1 demonstrates a significant proliferative response of HS1 and B6.1 against the structural glycoprotein S.

In order to identify the MHC restriction of the BALB/c $CD4^+$ clone, different cells were used as APC. Table 2 shows that the virus response of clone HS1 was I-A^d restricted. Because C57BL/6 mice only express MHC class II I-A^b, clone B6.1 had to be I-A^b restricted.

Subsequently, an expression library consisting of a set of overlapping fragments spanning the entire S gene was used in the proliferation assay. Figure 3 shows that two overlapping fragments, 54–476 and 173–411, were both recognized by clones HS1 and B6.1, whereas the partly overlapping fragments at the N or C termini of this region (1–174 and 375–831) failed to elicit a response. The responses observed indicated that the epitope recognized by both clones lay within or overlapped the amino acid sequence 174–374.

Finally, to localize the exact epitope recognized by the $CD4^+$ clones, a set of 39 peptides spanning the sequence 174–374 was synthesized. The peptides, each 15 residues in length and overlapping by 10 amino acids, were tested at a

Table 1. Proliferative responses of the MHV-A59-specific $CD4^+$ clones are directed against the S glycoprotein

	Antigens				
Clone	MHV-A59	vSc	vMM	vMS	vMN
HS1	25·1 ± 3·0	1·4 ± 0·2	1·3 ± 0·5	29·0 ± 2·8	1·5 ± 0·2
B6.1	40·1 ± 4·0	1·3 ± 0·1	1·4 ± 0·4	8·3 ± 0·9	1·2 ± 0·3

T cells were cultured with syngeneic irradiated spleen cells as APC in the presence of 70 μg/ml inactivated VV recombinant lysates vSc, vMM, vMS and vMN or 5×10^6 PFU/ml MHV-A59 (UV irradiated). The results are expressed as SI values ± SEM.

Table 2. MHV-A59-specific proliferation of clone HS1 is MHC class II I-A^d-restricted

APC	MHV-A59	SI
BALB/c NSC* (H-2^d)	5	18·8
	50	25·1
C57BL/6 NSC* (H-2^b)	5	1·2
	50	1·3
RT 2.3.3H-D6† (I-A^d)	5	8·9
	50	19·8
RT 10.3B-C1† (I-E^d)	5	1·1
	50	1·7

Cloned T cells were cultured with different APC in the presence of 5 or 50×10^6 PFU/ml MHV-A59 (UV-inactivated).

As APC, *normal spleen cells (NSC) and †L cells expressing various MHC class II molecules[21,22] were used. The proliferative responses are expressed as SI values.

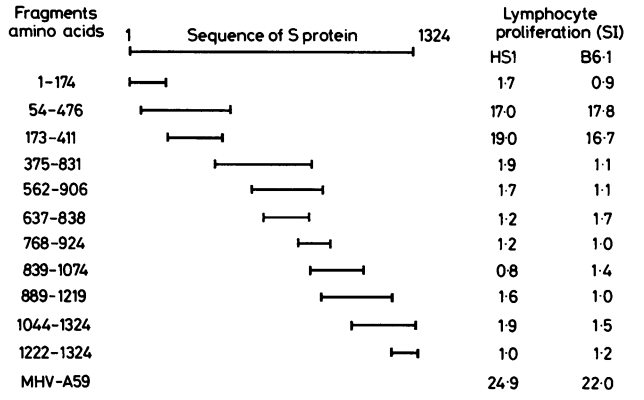

Figure 3. Both clones HS1 and B6·1 specifically recognize the amino acid sequence 174–374 of the S protein. T cells were cultured with syngeneic irradiated spleen cells as APC in the presence of 10 μg/ml pEX plasmid,[18] containing different overlapping fragments of the S protein, or 5×10^6 PFU/ml MHV-A59 (UV-irradiated). Data are expressed as SI values.

concentration of 10 μM in a proliferation assay. Figure 4 shows that only peptide 32, containing the amino acid residues 329–343 (ACNIEEWLTARSVPS), was able to induce a pronounced proliferative response of HS1 and B6.1; even at higher concentrations none of the other peptides was able to stimulate proliferation. To exclude the possibility that S 329–343 induced aspecific proliferation, a rabies-specific CD4$^+$ clone PM109 was tested against the set of synthetic peptides. No proliferation against any of the peptides was detected (data not shown). The fact that the two clones generated from two different mouse strains recognized the same epitope indicated that epitope S 329–343 was promiscuous.

Polyclonal CTL responses of primed BALB/c mice are directed against S 329–343

To test whether S 329–343 was important in polyclonal

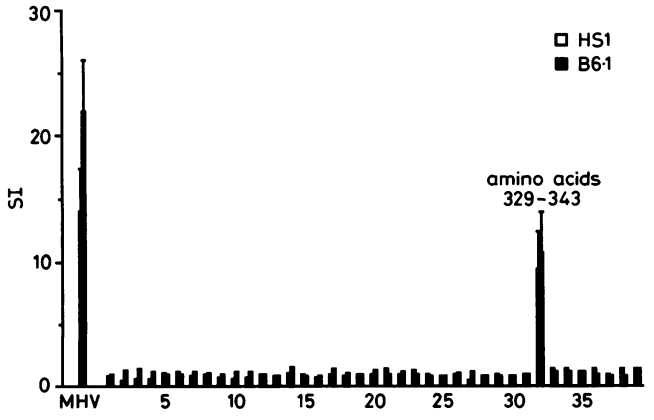

Figure 4. Both clones HS1 and B6.1 recognize peptide 329–343 of the S protein. T cells were cultured with syngeneic irradiated spleen cells as APC in the presence of 10 μg/ml overlapping peptides (represented at the x-axis), spanning the sequence 174–374 of the S protein, or 5×10^6 PFU/ml MHV-A59 (UV-irradiated). Data are expressed as SI values ± SEM of four experiments.

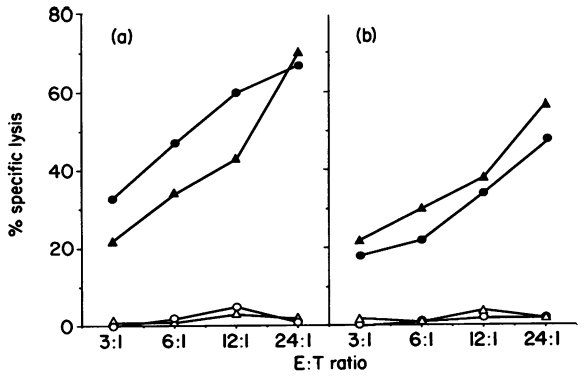

Figure 5. BALB/c bulk CTL and clone HS1 have the same cytotoxic specificity. Clone HS1 (a) and BALB/c bulk CTL (b) were assayed at different E:T ratios against A20 target cells untreated (○), incubated with non-infectious MHV-A59 (●), peptide 329–343 (▲) or control peptide 324–338 (△).

responses, we tested CTL bulk cultures of primed BALB/c mice and the CD4$^+$ clone HS1 against syngeneic target cells preincubated for 2 hr with inactivated MHV-A59, S 329–343 or the control peptide S 324–338. Figure 5 shows that BALB/c bulk and HS1 recognized A20 cells incubated with S 329–343 to the same extend as A20 cells incubated with inactivated MHV-A59, indicating that S 329–343 constituted an immunodominant epitope of MHV-A59 recognized by BALB/c CD4$^+$ CTL.

Adoptive transfer studies in BALB/c mice, using CD4$^+$ CTL clone HS1, showed significant protection against a lethal challenge of MHV-A59

To examine the role of MHV-A59-specific CD4$^+$ CTL, adoptive transfer experiments using the CD4$^+$ CTL clone

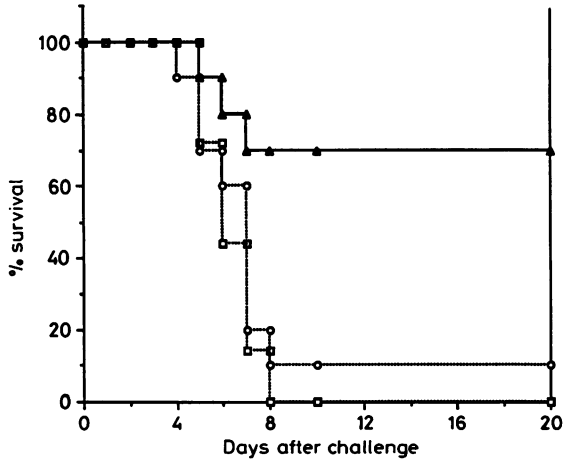

Figure 6. MHV-A59-specific CD4$^+$ CTL clone HS1 protects against a lethal challenge of MHV-A59. Three groups of BALB/c mice (7 weeks of age) were injected intravenously either with 5×10^6 cells of the MHV-A59-specific CD4$^+$ CTL clone HS1 (▲; $n = 10$), 5×10^6 cells of the rabies-specific CD4$^+$ clone PM109 (○; $n = 10$), or PBS alone (□; $n = 7$). All three groups were challenged after 1 day with 4000 PFU MHV-A59 ($100 \times LD_{50}$) intraperitoneally.

HS1, specific for S 329–343, were performed. As shown in Fig. 6 clone HS1 was able to protect mice significantly (70%) in several independent experiments against a lethal challenge of MHV-A59. In contrast, none of the PBS-injected mice recovered from acute hepatitis, and only one of the mice after transfer with control rabies-specific $CD4^+$ clone PM109 survived. Measurement of the MHV-specific antibody titres, 5 days after the MHV-A59 challenge showed no difference between the control mice (PBS and PM109 injected mice) and the HS1-injected mice (data not shown), indicating that the protective effect of HS1 $CD4^+$ CTL was not mediated by the induction of virus-specific antibodies.

DISCUSSION

Studies in several viral systems have shown that recovery from infection is associated with the development of cellular immunological responses, including $CD8^+$ CTL. Our results clearly demonstrate that no MHV-specific class I-restricted CTL activity was present in bulk cultures of spleen cells derived from immunized mice. On the contrary, we detected only a clear virus-specific class II-restricted cytotoxicity. Others groups have been able to demonstrate coronavirus-specific class I-restricted CTL using the neurotropic coronavirus strain MHV-JHM.[8,9] A possible explanation for the absence of a $CD8^+$ CTL response in MHV-A59-infected mice is that this predominant hepatotropic virus strain significantly decreases the cell-surface expression of MHC class I on different cell types (M. H. M. Heemskerk, manuscript in preparation). Bergmann *et al.*[25] have also suggested that different MHV strains can cause differential kinetics of MHC class I down-regulation. The down-regulation of the cell-surface expression of class I molecules, which is indispensible for recognition by $CD8^+$ CTL, may provide an escape mechanism for MHV-A59 as has been shown in other virus–host systems.[26–31]

Examination of the cytolytic T-cell response of C57BL/6 and BALB/c mice after immunization with MHV-A59 indicated that the host was able to compensate for the lack of class I-restricted CTL by utilizing virus-specific class II-restricted effectors. This phenomenon was described recently in two different systems, in which mice incapable of generating traditional $CD8^+$ CTL were used.[32,33] Experiments performed with A20, expressing both MHC class I and II, showed that MHV-A59 consistently and significantly reduced MHC class I, without any dramatic effect on MHC class II surface expression (M. H. M. Heemskerk, manuscript in preparation). These findings correlate with our functional data (Fig. 1), showing that after MHV-A59 infection a clear class II-restricted CTL response, but no MHC class I-restricted cytotoxicity, could be demonstrated.

Although the *ex vivo* proliferative capacity of spleen cells before the *in vitro* bulk expansion was directed against both the structural proteins S and N, cloned MHV-A59-specific $CD4^+$ cells recognized only the glycoprotein S. Earlier studies done with MHV-JHM in C57BL/6 mice and in rats showed both T- and B-cell reactivity against this glycoprotein.[34–37] This indicates that the S protein is both a good inducer of neutralizing antibodies and an effective stimulator of $CD4^+$ T cells. In addition, N- and S-specific $CD4^+$ T-cell lines derived from MHV-JHM-infected rats could confer protection against a lethal virus challenge, in the absence of $CD8^+$ T cells.[36] Residues 329–343 of the S protein was recognized by the $H\text{-}2^d$ clone HS1 and the $H\text{-}2^b$ clone B6.1. Since BALB/c bulk CTL lysed target cells labelled with S 329–343 to the same extent as target cells incubated with inactivated MHV-A59, we conclude that the epitope forms a dominant MHC class II-restricted cytotoxic epitope of MHV-A59. Moreover the fact that the epitope is recognized in both the $H\text{-}2^b$ and $H\text{-}2^d$ haplotype supports this.

There is a lot of scepticism about the feasibility of a major *in vivo* role for class II- plus virus-restricted effector cells because of the distribution of cells expressing MHC class II, and a wider range of cells that may be infected by viruses. However, it has been demonstrated that hepatocytes express class II MHC antigen during inflammation in the liver.[38,39] Additionally, many activated T cells, like the $CD4^+$ clone HS1 and B6.1, are able to secrete IFN-γ, a cytokine that has been shown to induce the expression of MHC class II antigen. Thus a physiological role for MHC class II-restricted effector cells is potentially not as limiting as may first have appeared. Recently, it was demonstrated that it is possible to obtain liver-derived virus-specific $CD4^+$ CTL clones from patients with chronic hepatitis B or C virus-induced liver disease.[40,41] Whether these intrahepatic T cells are the cause or the result of the disease is not known exactly yet, although these investigators claim a more immunopathological role for these virus-specific $CD4^+$ CTL.

We currently favour the notion that $CD4^+$ CTL play a pivotal role in protection against MHV-A59 infection, because transfer studies done with the MHV-specific $CD4^+$ CTL clone HS1 showed a significant protection of mice against a lethal challenge with MHV-A59. However, the mechanism of protection is not clear, and could be largely dependent on the time-point these $CD4^+$ CTL encounter the virus. If the cells are already present when the virus enters the host, they are protective. On the other hand, if their recruitment is slow and delayed during acute infection, they can cause considerable damage and disease in their attempt to eliminate the virus, because the virus is able to infect large areas of tissue during their recruitment. Therefore we cannot exclude at this moment an immunopathological role of the MHV-A59-specific $CD4^+$ CTL. In future experiments we want to focus more on the exact role of these cells, either in the protection against acute MHV-A59-induced liver disease or, in contrast, in the development of acute hepatitis.

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