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Immune Response to a Murine Coronavirus: Identification of a Homing Receptor-Negative $CD4^+$ T Cell Subset That Responds to Viral Glycoproteins

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The lymphocyte proliferative response to mouse hepatitis virus, strain JHM (MHV-JHM), a well-described cause of chronic and acute neurological infections, has been studied using vaccinia virus recombinants expressing individual MHV proteins. The surface (S) and transmembrane (M) glycoproteins were the most active proteins in causing proliferation of lymphocytes isolated from immunized adult mice, whereas lymphocytes from persistently infected mice proliferated only in response to the S protein. The cells from immunized mice which proliferated most actively in response to MHV were positive for the CD4 antigen and secreted interferon-γ. In addition, the most responsive subset of cells did not express $gp90^{MEL-14}$, the lymph node-specific homing receptor. The results identify a subpopulation of $CD4^+$ T cells that may be an important component of the cell-mediated immune response to this virus. The data also suggest that response to the M protein is important in preventing disease progression in C57BL/6 mice since cells which recognize this protein are absent from persistently infected mice. © 1992 Academic Press, Inc.

INTRODUCTION

Several strains of mouse hepatitis virus (MHV), a member of the coronavirus family, cause acute and chronic demyelinating diseases in mice and rats (Cheever *et al.*, 1949; Weiner, 1973; Lampert *et al.*, 1973; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980; Stohlman and Weiner, 1981; Siddell *et al.*, 1983). Demyelination most likely results from lytic destruction of oligodendrocytes, although immunopathological mechanisms may contribute to the process (Weiner, 1973; Lampert *et al.*, 1973; Watanabe *et al.*, 1983; Wang *et al.*, 1990).

Both humoral and cellular immunity protect mice from the acute, fatal encephalitis caused by the neurotropic JHM strain of MHV (MHV-JHM). The acute MHV-JHM infection can be prevented by infusion of monoclonal antibodies directed against the surface glycoprotein (S), the transmembrane glycoprotein (M), or the nucleocapsid protein (N) (Buchmeier *et al.*, 1984; Nakanaga *et al.*, 1986; Lecomte *et al.*, 1987; Fleming *et al.*, 1989). Suckling mice are protected from the acute disease if they are nursed by dams previously immunized against MHV-JHM (Pickel *et al.*, 1985; Perlman *et al.*, 1987a). Neutralizing antibodies, however, do not appear to protect mice or rats from persistent infections caused by MHV-JHM (Weiner *et al.*, 1973; Watanabe *et al.*, 1987; Jacobsen and Perlman, 1990).

Cell-mediated immunity is generally believed to be crucial for control of most viral infections, and published data suggest that cellular immunity is required for control of the infection caused by MHV-JHM. Adoptive transfer of cloned $CD4^+$ or $CD8^+$ cells prevents the acute encephalitis, and in some cases, eradicates MHV-JHM from the central nervous system (Stohlman *et al.*, 1986; Yamaguchi *et al.*, 1991; Korner *et al.*, 1991). In other studies, adoptive transfer of nylon wool-adherent $CD4^+$ T cells was shown to effect viral clearance in infected mice; elimination of $CD8^+$ cells from the recipient abrogated this effect, suggesting that both $CD4^+$ and $CD8^+$ cells were required for maximal effect (Sussman *et al.*, 1989). In addition, cytotoxic B cells and NK-like cells may have a role in the host immune response to MHV (Stohlman *et al.*, 1983; Welsh *et al.*, 1986; Carman *et al.*, 1986).

The cell-mediated response is also important in suppressing MHV infection in rats, since nude rats or rats whose T cell response was suppressed with cyclosporin developed acute encephalomyelitis at an age when control animals were resistant to the virus (Zimmer and Dales, 1989).

In a previous report, we showed that suckling C57BL/6 mice inoculated with MHV-JHM and nursed by immunized dams were protected from the acute encephalitis, but became persistently infected with MHV-JHM. At 3–8 weeks p.i., 40–90% developed a demyelinating encephalomyelitis characterized clinically by hindlimb paralysis (Perlman *et al.*, 1987a). With the goal of understanding the immune response in these persistently infected mice, we have compared the proliferative T cell response in these mice and that in immunized adult C57BL/6 mice. For these experi-

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ments, we have used recombinant vaccinia viruses (VV) which expressed the three structural proteins (surface glycoprotein (S), the transmembrane glycoprotein (M) and the nucleocapsid protein (N)) and one nonstructural protein, p28, a basic protein encoded by the putative polymerase gene (Denison and Perlman, 1986; Soe *et al.,* 1987). The results indicate that the cells from immunized adult mice which specifically proliferate in response to antigen are $CD4^+$ and do not express the lymphocyte surface homing receptor, recognized by the monoclonal antibody MEL-14 ($gp90^{MEL-14}$, gp90). Gp90 is a lymphocyte–endothelial adhesion molecule that mediates lymphocyte recirculation (Gallatin *et al.,* 1983). This $gp90^-$ phenotype defines a population of high-affinity, antigen-primed effector lymphocytes in other systems (Jung *et al.,* 1988; Bradley *et al.,* 1991).

MATERIALS AND METHODS

Animals

Male and female MHV-negative C57BL/6 mice, 8 to 12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, MA) or from Sasco Laboratories (Omaha, NE). To obtain persistently infected mice with hindlimb paralysis, suckling C57BL/6 mice were inoculated intranasally with live MHV-JHM and nursed by dams with high titers of anti-MHV-JHM antibody as previously described (Perlman *et al.,* 1987a).

Virus and cells

MHV-JHM, originally obtained from Dr. S. Weiss, University of Pennsylvania, was grown and titered as previously described (Perlman *et al.,* 1987a). Vaccinia virus, strain WR, was obtained from the ATCC. DBT cells, derived from a murine astrocytoma (Hirano *et al.,* 1974), were grown in Dulbecco's MEM supplemented with 10% fetal calf serum and antibiotics.

Construction of S, M, N, and p28 clones and of VV recombinants

A clone containing the entire MHV-JHM S gene was constructed from clones provided by Dr. S. Siddell, der Universitat Wurzburg, and inserted into VV as previously described (Oleszak *et al.,* 1992). The S protein encoded in this clone was 141 amino acids shorter than its homologue from wild-type MHV-JHM (Schmidt *et al.,* 1987; Parker *et al.,* 1989). Wild-type MHV-JHM was used in all of the animal studies.

Clones containing the N and M proteins were obtained from Dr. S. Siddell and flanking G-C tracts removed using polymerase chain reaction (PCR) technology (Skinner and Siddell, 1983; Pfleiderer *et al.,* 1986; Sambrook *et al.,* 1989). To modify the N clone, a 5′ primer (AGG<u>ATG</u>TCTTTTGTTCCTGG) and a 3′ primer (GAGTGCCGACATAGGATT) complementary to a sequence located 8 nucleotides downstream from the termination codon were used. The 5′ primer included the N initiation codon (underlined). The M clone was similarly modified using a 5′ primer containing the initiation codon (T<u>ATG</u>AGTAGTACTACTC) and a 3′ primer complementary to the sequence at the termination codon (GATTCTCAACAATACGGTG).

A clone containing p28, the 5′ terminal portion of the putative polymerase gene, was constructed using published sequences and PCR technology (Soe *et al.,* 1987). To construct the p28 clone, a 5′ primer (GCATA<u>ATG</u>GCAAAGATGG) and a 3′ primer (TAAGGTCGCC<u>T</u>TAGTCTTC) were synthesized. Since p28 is normally cleaved from a large precursor, a T nucleotide was substituted for an A present in the original sequence (underlined above) in the 3′ primer. This change introduced a termination codon into the construct at a position (amino acid 272) corresponding to a potential proteolytic cleavage site (Soe *et al.,* 1987). The 5′ primer includes the p28 initiation codon (underlined). Complementary DNA was synthesized from MHV-JHM-infected cell RNA, using the downstream primer and M-MLV reverse transcriptase (Bethesda Research Laboratories). Using standard PCR technology, the complete double-stranded DNA molecule was constructed.

All of the above clones were inserted into the polylinker region of a VV transfer plasmid, pTM3. This plasmid includes the T7 promoter, the untranslated region of encephalomyocarditis virus, and the gene for xanthine–guanine phosphoribosyl-transferase under the control of the VV P7.5 promoter (Elroy-Stein and Moss, 1990). These sequences are flanked by VV thymidine kinase sequences to facilitate homologous recombination with VV. The *Nco*I site was removed with T4 DNA polymerase so that the initiation codon of each construct was used to initiate translation. HeLa cells, infected with VV strain WR, were transfected with the appropriate constructs and recombinant virus was selected and propagated as described previously (Falkner and Moss, 1988).

Immunoprecipitation

DBT cells were either infected with MHV-JHM (m.o.i. = 1) or dually infected with VV expressing T7 RNA polymerase (pTF7.3—kindly provided by Dr. B. Moss, N.I.H.) and one of the MHV proteins (m.o.i. = 2.5 for each). Cells were labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) from 12 to 14 hr p.i. (MHV-JHM) or from 5 to 7 hr p.i. (VV recombinants) in

methionine-free media. Lysates were prepared by treating cells with a solution containing 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 150 m*M* NaCl, and 10 m*M* Tris, pH 7.4. Viral proteins were precipitated with anti-MHV-JHM antibody (S protein), anti-M or anti-N monoclonal antibody (provided by Dr. M. Buchmeier, Scripps Clinic and Research Foundation) (Talbot *et al.,* 1984), or anti-p28 polyclonal antibody (rabbit antibody directed against the p28 peptide containing amino acids 25–38). Antibody–antigen complexes were collected onto protein A–Sepharose (Pharmacia-LKB Biotechnology, Piscataway, NJ). Samples were analyzed by SDS–polyacrylamide gel electrophoresis (Maizel, 1971).

Proliferation assays

These assays were performed on immunized adult C57BL/6 mice and on persistently infected mice. First, using adult mice, we optimized conditions for splenocyte proliferation to MHV-JHM, based on published studies (Woodward *et al.,* 1984). Intravenous, intraperitoneal, and subcutaneous routes of inoculation, with varying amounts of virus or viral antigen in the presence or absence of Freund's adjuvant, were analyzed at different times after inoculation. Fetal calf serum (Hyclone Laboratories, Inc., Logan, UT) and mouse serum were compared for their ability to support lymphocyte proliferation *in vitro.* The most reproducible measurements were performed using cells cultured from adult mice inoculated intraperitoneally with $1-2 \times 10^5$ PFU of MHV-JHM without Freund's adjuvant. Specific proliferation was optimal with fetal calf serum.

C57BL/6 mice were inoculated intraperitoneally with MHV-JHM in 0.5 ml phosphate-buffered saline (PBS). Eight days later, a single-cell suspension of spleen cells was prepared in RPMI 1640 medium containing 5×10^{-5} *M* 2-mercaptoethanol, 2 m*M* glutamine, 10% fetal calf serum (FCS), and antibiotics. Cells (2×10^5) were cultured in 0.2 ml in the presence of 1:200 of antigen in 96-well round-bottom culture plates (Corning Inc., Corning, NY). Cells were also incubated *in vitro* with no antigen or with supernatant from concanavalin A-stimulated rat cells (positive control). After 4 days in culture at 37°, the cells were pulsed for 4 hr with 1 μC/well [*methyl*-^{3}H]thymidine (Amersham Corp.) and then harvested onto glass fiber filters (Skatron Instruments, Sterling, VA) with a cell harvester. Incorporated radioactivity was measured in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Each antigen was tested with six replicate wells.

To analyze the data, a mean proliferative response and standard error for each antigen were calculated for each group of mice (12 immunized adult mice and 6 persistently infected mice).

The proliferation index was calculated either as the ratio of stimulation by MHV-JHM-infected to uninfected cell lysate or as the ratio of stimulation by lysate from cells dually infected with vTF7.3 and VV expressing MHV protein to lysate from vTF7.3-infected cells. A proliferation index greater than 3 was considered significant (Wahren *et al.,* 1981).

MHV-JHM antigen was prepared for use in the proliferation assays by infecting confluent DBT cells with MHV-JHM (m.o.i. = 1) and harvesting plates 12–14 hr later. For this purpose, cells were washed three times with PBS and scraped into PBS. Samples were briefly sonicated, clarified by centrifugation at 500 g for 10 min, and stored frozen in small aliquots at −70° (Woodward *et al.,* 1984). Individual MHV antigens were prepared by dually infecting DBT cells with vTF7.3 and one of the VV recombinants expressing an MHV protein. After 7–14 hr, antigen was prepared as for MHV-JHM.

Immunofluorescence staining, analysis, and sorting

Preparation of cells for flow cytometric analysis was as previously described (Jung *et al.,* 1988), using phycoerythrin-conjugated mAb MEL-14 and fluorescein-conjugated anti-CD4 mAb GK1.5. Stained cells were analyzed and sorted on a FACS 440 (Becton–Dickinson Immunocytometry Sys., Mountain View, CA). Fluorescence and light scatter signals were collected on 20,000 cells and analyzed using the FACS/DESK computer program.

Separation of lymphocytes by panning

Cultured splenocytes were incubated with saturating amounts of anti-CD8 mAb 53-6.7 and anti-B220 mAb RA3-6B2 for 20 min at 4° in RPMI 1640 with 10% FCS. The cells were washed and resuspended to a concentration of 1×10^7 cells/ml, passed through a 30-gauge needle, and panned on goat anti-rat Ig-coated plastic petri dishes for 1 hr at 4°. Nonadherent, $CD4^+$ T cells were incubated with MEL-14 and applied to new anti-rat pans for separation of $gp90^+$ from $gp90^-$ cells.

Assays for gamma interferon (IFN-γ)

A sandwich ELISA was used to quantify IFN-γ in tissue culture supernatants of stimulated and unstimulated sorted $CD4^+$ T cells. Wells of an Immulon II plate were incubated with 100 μl of 10 μg/ml R4-6A2 (Spitalny and Havell, 1984) (rat IgG1 mAb to INF-γ in 0.1 *M* bicarbonate buffer, pH 9.6, for 1 hr at 37°. Wells were washed and the remaining protein binding sites were

blocked with 200 μl of RPMI with 10% horse serum for 2 hr at room temperature. Next, 50 μl of tissue culture supernatants was added in triplicate wells for 1 hr at room temperature. The wells were washed and 100 μl of a 1:1000 dilution of polyclonal rabbit anti-INF-γ (kindly provided by D. Hoft, University of Iowa) in RPMI 1640 supplemented with 10% FCS was added for 45 min. After washing the wells, 100 μl of a 1:3000 dilution of HRP-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Labs Inc., West Grove, PA) was added for 30 min. The wells were thoroughly washed and 100 μl of the HRP substrate ABTS (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the color change measured at 405 nm in a microplate ELISA reader (Bio-Tek Instruments Inc., Winooski, VT). A standard curve was generated using recombinant IFN-γ (Genentech, South San Francisco CA.)

Assays for IL-2 and IL-4 production

Bioassays of culture supernatants using the IL-2 indicator cell line CTLL-2 (ATCC) and IL-4 indicator cell line CT4S, kindly provided by Dr. William Paul, were performed as previously described (Hu-Li *et al.,* 1989).

RESULTS

Spleen cells from immunized and chronically infected mice proliferate in response to MHV-JHM antigen

Adult C57BL/6 mice were immunized with MHV-JHM intraperitoneally, and 8 days later spleen cells were stimulated *in vitro* for 4 days with lysates of MHV-JHM-infected or uninfected cells at a 1:200 dilution. A strong proliferative response to MHV-JHM antigen was observed in all 12 mice analyzed (Fig. 1), whereas no significant proliferation was observed *in vitro* in the absence of *in vivo* priming with MHV-JHM (data not shown). The mean proliferation index (calculated as described under Materials and Methods) was 4.6.

Suckling C57BL/6 inoculated intranasally with virus and nursed by immunized dams often develop a late onset demyelinating encephalomyelitis characterized clinically by hindlimb paralysis (Perlman *et al.,* 1987a). To determine if splenocytes from clinically ill, persistently infected mice also responded to MHV-JHM antigen, proliferation assays were performed on six mice with the onset of hindlimb paralysis 17–45 days p.i. A specific proliferative response similar to that observed in the immunized population was detected, with a mean proliferation index of 6.3 (Fig. 2).

Surface and transmembrane glycoproteins are stimulants for proliferation

To determine the antigen specificity of T cell proliferation *in vitro,* VV recombinants expressing the MHV

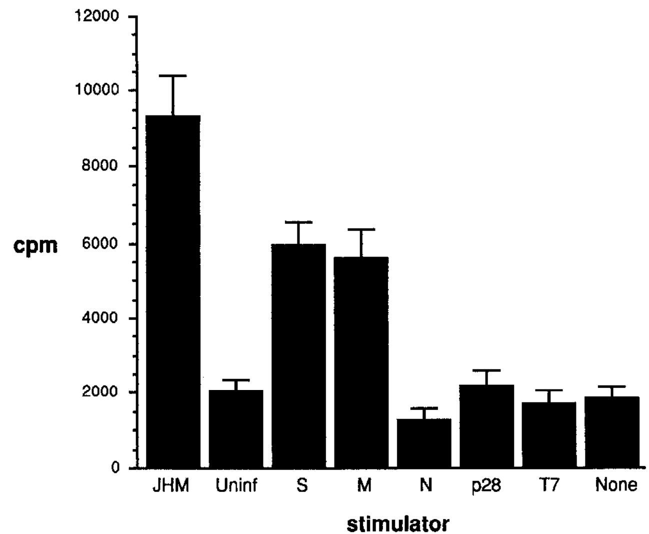

FIG. 1. Cell proliferative response in immunized mice. Twelve mice were infected i.p. with 1–2 × 10^5 PFU MHV-JHM. Splenocytes were analyzed 8 days p.i. as described under Materials and Methods. The mean number of counts incorporated and the standard error in response to each DBT lysate are shown. JHM, MHV-JHM-infected cells; uninf, uninfected cells; S, M, N, p28, cells dually infected with vTF7.3 and VV encoding the respective MHV protein; T7, cells infected with vTF7.3; none, no added antigen.

structural proteins, S, M, and N and the nonstructural protein, p28, were constructed as described under Materials and Methods. P28, a basic protein encoded by the putative MHV polymerase gene (Denison and Perlman, 1986; Soe *et al.,* 1987) was included in these

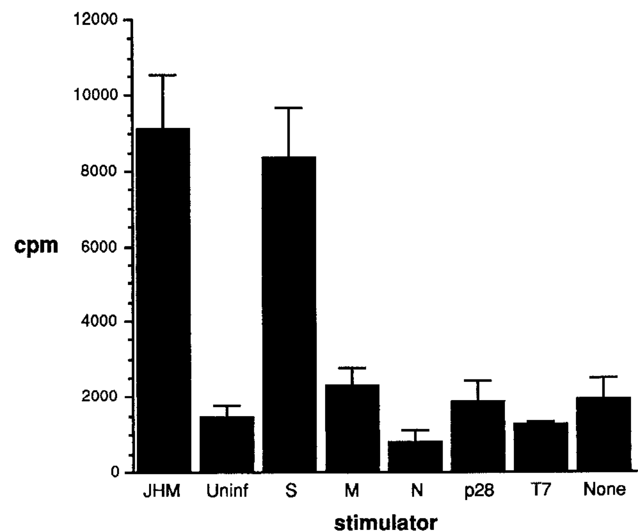

FIG. 2. Cell proliferation response in persistently infected mice. Splenocytes from six mice with hindlimb paralysis were analyzed as described under Materials and Methods. The mean number of counts incorporated and the standard error in response to each lysate are shown. The six mice developed hindlimb paralysis at 17, 19, 26, 29, 45, and 45 days p.i. Lysates are as those in Fig. 1.

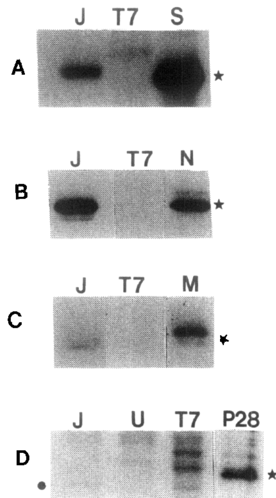

FIG. 3. MHV proteins produced by VV recombinants. MHV protein synthesis was assayed by infecting DBT cells with MHV-JHM or with vTF7.3 and VV recombinants expressing MHV proteins. Controls included uninfected DBT cells and cells infected with vTF7.3 alone. Lysates were prepared from [^{35}S]methionine-labeled cells as described under Materials and Methods and reacted with (A) rabbit anti-MHV-JHM antibody; (B) anti-N monoclonal antibody; (C) anti-M monoclonal antibody; (D) rabbit anti-p28 antibody. Proteins were analyzed by SDS–PAGE. J, MHV-JHM-infected lysate; T7, vTF7.3-infected lysate; S, N, M, p28, lysates infected with vTF7.3 and recombinants encoding the S, N, M, and p28 proteins. *, MHV-specific proteins encoded by VV recombinants; ●, in (D) marks p28 present in J and absent in U.

studies since analysis of its structure indicated the presence of several possible T cell epitopes (Margalit *et al.,* 1987).

To prove that each construct produced the MHV protein of interest, DBT cells were dually infected with vaccinia expressing T7 polymerase (vTF7.3) and one of the VV recombinants. Protein products were analyzed by radioimmunoprecipitation and SDS–polyacrylamide gel electrophoresis (Fig. 3). The S gene (Fig. 3A) coded for a protein which was 141 aa smaller than wild-type MHV-JHM S protein as described previously (Schmidt *et al.,* 1987; Parker *et al.,* 1989). The VV recombinant containing the N sequence expressed an appropriately sized nucleocapsid protein when similarly analyzed by immunoprecipitation and SDS–PAGE (Fig. 3B).

The VV recombinant containing the M sequence (Fig. 3C) expressed two proteins. The most abundant protein migrated more slowly than the M protein detected in MHV-infected cells, whereas the less abundant protein migrated identically to the M protein present in these cells. The two proteins expressed by the VV recombinants most likely differ in the degree of glycosylation and since the slightly larger protein was not observed when other cell lines were infected with the VV recombinant, its structure was not further investigated.

MHV-JHM p28 is normally cleaved from a larger precursor (Denison and Perlman, 1986), although the precise site of cleavage has not been identified. P28 was detected by immunoprecipitation in MHV-JHM-infected but not in uninfected cells (Fig. 3D). For the construction of the VV recombinant containing the p28 sequence, a termination codon was introduced at a possible cleavage site (Soe *et al.,* 1987) as described under Materials and Methods. When cells were infected with the VV recombinant containing the p28 sequence, a protein migrating at approximately 32 kDa was detected with anti-p28 antibody (Fig. 3D). The most likely explanation for the discrepancy in molecular weight between this protein and the 28-kDa product present in MHV-JHM-infected cells was that the termination codon introduced into the downstream primer was placed 3′ to the true cleavage site, resulting in a larger protein.

For use in cell proliferation assays, unlabeled cell lysates were prepared from DBT cells co-infected with vTF7.3 and the MHV VV recombinants. To determine the amount of each MHV protein synthesized relative to that observed in cells infected with MHV-JHM, equal volumes of the N, S, p28, and MHV-JHM-infected DBT lysates were compared by protein blot analysis. These results showed that equal or greater amounts of each MHV protein were present in the VV recombinant lysates when compared to MHV-infected cells (data not shown). Splenocytes from immunized and from persistently infected mice with hindlimb paralysis were incubated with VV-infected DBT lysates which expressed only a single MHV protein. Lysates from cells infected only with vTF7.3 were used as a negative control.

Cells from immunized mice showed significant proliferation in response to the surface and transmembrane glycoproteins with no response to the N or p28 proteins. The mean proliferation index was 3.5 for the S protein and 3.3 for the M protein. The sum of the proliferative responses to the M and S proteins was very similar to the response measured with the MHV-JHM lysate, suggesting that these two proteins accounted for most of the proliferative activity.

A different pattern of stimulation was measured in mice persistently infected with MHV-JHM. Splenocytes from these mice proliferated significantly only to lysate expressing the S protein, with a proliferation index of 6.7. The amount of S protein-specific proliferation was nearly equal to that observed with the MHV-JHM lysate, suggesting that the S protein was the major antigen recognized by these lymphocytes.

$CD4^+gp90^-$ cells proliferate in response to MHV-JHM

In the next set of experiments, we determined which type of lymphocyte proliferated in response to viral an-

TABLE 1

Proliferative Response of CD4$^+$ and CD4$^-$ Lymphocytes from Immunized Adult Mice

Mouse no.	CD4^{+a} cpm	CD4^{-a} cpm	CD4$^+$/CD4^{-b}	%CD4^{+c}	%CD4^{+d} proliferation
1	8882	1733	5.1	31.8	71
2	4276	902	4.7	32.6	70
3	4707	1984	2.4	40.8	62
4	50243	17483	2.9	39.0	66
5	69475	33733	2.1	41.7	60
6	28280	13190	2.1	53.6	71

[a] CD4$^+$ and CD4$^-$ cells were prepared from MHV-JHM-stimulated cultures. 30,000 cells/well were labeled with [^{3}H]thymidine. cpm represents means of triplicate samples.

[b] Relative proliferation of purified CD4$^+$/CD4$^-$ cells.

[c] Proportion of CD4$^+$ cells in the culture as determined by FACS analysis.

[d] Percentage of total proliferation in the culture which is from CD4$^+$ cells: [(CD4$^+$ cpm) × %CD4$^+$]/[(CD4$^+$ cpm) × %CD4$^+$ + (CD4$^-$ cpm) × (100 − %CD4$^+$)].

tigen. Spleen cells harvested from immunized adult mice were incubated with antigen for 4 days *in vitro* prior to fractionation into CD4$^+$, CD8$^+$, and B cell populations by panning or with the fluorescence-activated cell sorter (FACS). Preliminary results suggested that the majority of the active cells were CD4$^+$ and, for simplicity of analysis, further experiments were performed only on CD4$^+$ and CD4$^-$ populations.

Populations of CD4$^+$ and CD4$^-$ cells were isolated from Day 4 cultures either by negative selection panning with antibodies to CD8 and B220 or by using the FACS. Both methods gave greater than 90% pure CD4$^+$ populations, as measured by FACS analysis. In order to determine which cell subset was responsible for proliferation, triplicate cultures of 30,000 cells of each population were labeled with [^{3}H]thymidine as above. As shown in Table 1, CD4$^+$ cells were 2.1 to 5.1 times more active than the CD4$^-$ cells and the CD4$^+$ cells accounted for 60–71% of the total proliferation observed in the unfractionated cultures.

In previous studies, the subset of lymphocytes defined by the absence of the lymphocyte homing receptor, gp90, included most of the cells which showed high-affinity, antigen-specific activity (Dailey *et al.*, 1985; Jung *et al.*, 1988; Bradley *et al.*, 1991; Mobley and Dailey, manuscript submitted). To determine if the subset of cells proliferating in response to MHV-JHM antigen could also be delineated on the basis of gp90 expression, cells from immunized mice were stimulated with antigen *in vitro* and then analyzed with the FACS for both CD4 and gp90 expression. As shown in Fig. 4, a significant population of CD4$^+$gp90$^-$ cells (arrow) was present after *in vitro* stimulation with MHV-JHM lysate but was barely detected after exposure to the lysate of uninfected cells. Lymphocytes from 10 additional mice showed the same phenomenon after *in vitro* incubation with specific antigen.

To characterize further the phenotype of cells proliferating in response to antigen, CD4$^+$ cells were prepared from cultures which had been incubated for 4 days with MHV-JHM antigen. These cells were fractionated into gp90$^+$ and gp90$^-$ populations by panning, and [^{3}H]thymidine uptake was measured. As shown in Table 2, the CD4$^+$gp90$^-$ population was 10–15 times more active on a per cell basis than the CD4$^+$gp90$^+$ population.

IFN-γ production in response to MHV-JHM

Since activated lymphocytes secrete specific cytokines to enhance and modulate the antiviral response, we assayed cell supernatants after 3–5 days *in vitro* for IL-2, IL-4, and IFN-γ. We could not detect any antigen-specific release of IL-2 and IL-4 since both MHV-JHM- and control-stimulated cultures secreted equivalent, relatively low amounts (data not shown). In contrast, IFN-γ was produced in greater quantities by MHV-JHM-stimulated cells (6.54 units–S.E. 2.45) than by control cultures (0.97 units–S.E. 0.27) as shown in Fig. 5.

To determine the phenotype of the cells which actually produced IFN-γ, CD4$^+$ cells from three mice were prepared after 4 days *in vitro* and separated into gp90$^+$ and gp90$^-$ fractions by panning. Separated cells were then incubated *in vitro* for 24 hr and IFN-γ activity was measured in each supernatant. Anti-CD3 was added to replicate wells, a technique frequently used to enhance secretion in lymphokine assays.

Little difference was noted in interferon-γ production by the gp90$^-$ and gp90$^+$ cultures in the absence of anti-CD3 or specific antigen (Fig. 6), in agreement with previous observations in other systems (Fortier *et al.*, 1989). However, after stimulation with anti-CD3 antibody, large amounts of IFN-γ were produced by the gp90$^-$ cells, whereas secretion by gp90$^+$ lymphocytes was barely detectable (Fig. 6). These results suggest that the CD4$^+$gp90$^-$ T cells, shown previously to be the ones most actively proliferating in response to antigen, were also the source of IFN-γ produced in the MHV-stimulated cell cultures.

DISCUSSION

Adult C57BL/6 mice, like many other strains, are very susceptible to infection with MHV-JHM (Stohlman and Frelinger, 1978). In addition, suckling C57BL/6 mice, even if protected from acute encephalitis by ma-

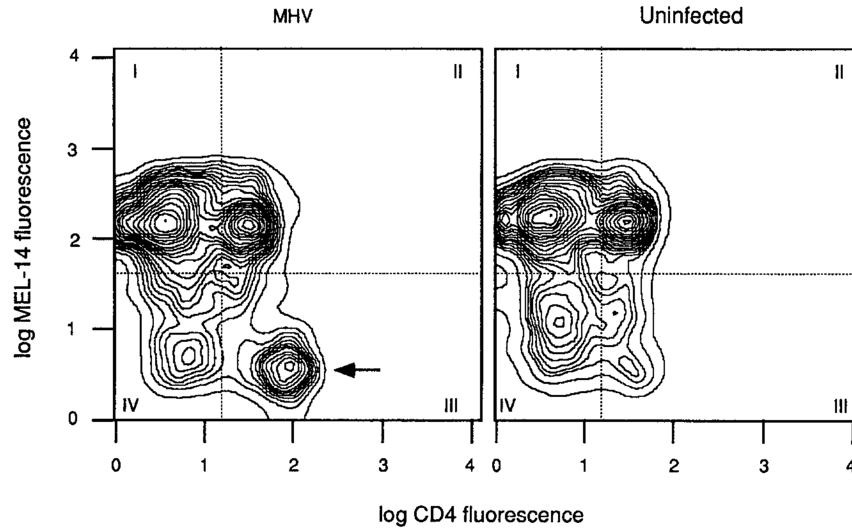

FIG. 4. Two-color flow cytometric analysis of CD4 and gp90 on cells proliferating in response to MHV-JHM-infected and uninfected lysates. After 4 days *in vitro,* cells were analyzed as described under Materials and Methods using the fluorescence-activated cell sorter. The arrow indicates the population of $gp90^-$ cells present predominantly in the MHV-JHM-stimulated cultures.

ternal antibody, develop a clinically apparent demyelinating encephalomyelitis 3–8 weeks after intranasal inoculation (Perlman *et al.,* 1987a). Suckling BALB/c mice, another susceptible strain, are also protected from acute encephalitis by nursing by immunized dams, but, unlike C57BL/6 mice, do not develop a late onset, demyelinating encephalomyelitis (Perlman *et al.,* 1987b). Thus, the immune response to MHV-JHM may be particularly suboptimal in C57BL/6 mice infected with MHV-JHM.

Cytotoxic $CD8^+$ cells are believed to be critical for resolution of viral infections, including the CNS infection caused by MHV-JHM (Sussman *et al.,* 1989; Williamson and Stohlman, 1990; Yamaguchi *et al.,* 1991). In ongoing studies, we have been unable to demonstrate cytotoxic T cell (CTL) activity against MHV-JHM in spleen cell preparations from immunized mice (S. Perlman, unpublished observations). Measurement of CTL activity from C57BL/6 mice is hindered by the lack of a convenient syngeneic target which can be infected by MHV. However, VV does infect syngeneic target cells and using our VV recombinants, we were unable to identify a significant CTL response to the N, M, S, or p28 proteins. While there are several explanations for this data, one possibility is that CTL activity against MHV-JHM is insubstantial in C57BL/6 mice and that this is a component of the ineffective immune response to the virus.

In contrast to the minimal CTL response observed in C57BL/6 mice, we easily detected a strong proliferative response to MHV antigen after *in vitro* culture of lymphocytes from immunized adult or persistently infected mice. The response was quantitatively the

TABLE 2

ACTIVATED LYMPHOCYTES EXPRESS gp90 ANTIGEN

Mouse no.	$CD4^+gp90^+$ [a] cpm	$CD4^+gp90^-$ [a] cpm	Ratio $CD4^+gp90^-/CD4^+gp90^+$
1	3157	42844	13.6
2	4345	68285	15.7
3	2301	23631	10.2
4	3519	38606	11.0

[a] Populations of $CD4^+gp90^+$ and $CD4^+gp90^-$ cells were prepared from Day 4 cultures by panning, and 30,000 cells/well were labeled with $[^3H]$thymidine. Counts represent means of triplicate samples.

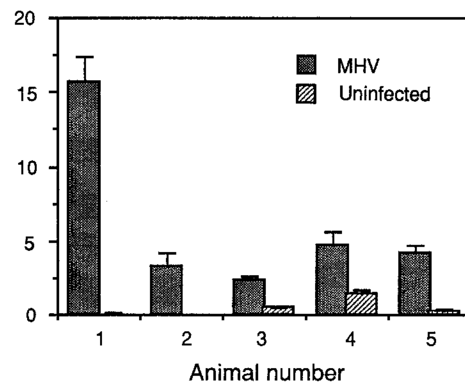

FIG. 5. IFN-γ production. IFN-γ was assayed from supernatants of unfractionated spleen cells incubated *in vitro* for 3 days (mouse No. 1 and 2) or 4 days (mouse No. 3, 4, and 5) with MHV-JHM-infected or uninfected DBT lysates as described under Materials and Methods. Measurements were made in triplicate and the standard error is indicated above each bar.

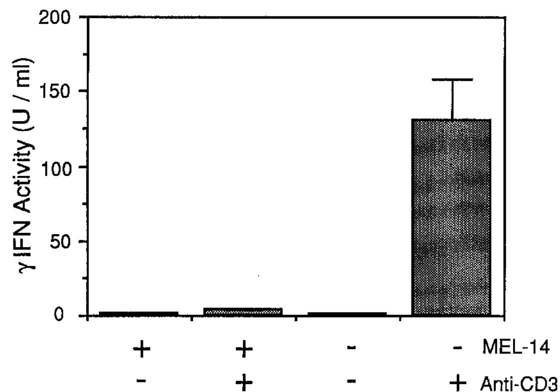

FIG. 6. IFN-γ production in $CD4^+gp90^+$ and $CD4^+gp90^-$ cells. After 4 days *in vitro,* cells were panned for $CD4^+$ cells which, in turn, were fractionated into $gp90^-$ and $gp90^+$ populations. 3×10^4 $gp90^+$ and $gp90^-$ cells were incubated *in vitro* for an additional 24 hr in the presence or absence of anti-CD3 antibody. IFN-γ activity was measured in the supernatants as described under Materials and Methods. Data from triplicate cultures of three separate mice are shown, with the standard error indicated above each bar.

same in the two groups of mice and in both cases, the S surface glycoprotein was an effective stimulator for proliferation. An important difference between the two groups was the absence of cells responsive to the M transmembrane protein solely in the persistently infected animals. Other studies have shown that monoclonal antibodies to the M protein protect mice from the acute encephalitis (Fleming *et al.,* 1989) and our results raise the possibility that cell-mediated recognition of this protein may be an important part of an effective immune response to MHV-JHM.

Recent studies have documented the importance of $CD4^+$ cells in suppressing neurological infections caused by MHV-JHM in both mice and rats (Williamson and Stohlman, 1990; Yamaguchi *et al.,* 1991; Korner *et al.,* 1991). In one of these studies, $CD4^+$ cells specific for the N and S proteins were shown to suppress viral replication in rats. The N protein elicited a stronger proliferative response than did the S protein in these animals (Korner *et al.,* 1991). The lack of recognition of the N protein after immunization or persistent infection may also contribute to the relatively ineffective immune response to MHV-JHM in C57BL/6 mice.

The cells which proliferate in response to MHV-JHM antigen secrete IFN-γ. Since IFN-γ is secreted by the TH1 subset of $CD4^+$ cells, it is likely that MHV-JHM antigen preferentially stimulates this subset of helper T cells in C57BL/6 mice. This stimulation of TH1-like cells may be strain-dependent as well as antigen-dependent, since C57BL/6 mice show a preferential TH1-like response under conditions in which other strains show a TH2-like response (Street and Mosmann, 1991). TH1 cells secrete IL-2 in addition to IFN-γ, but only small amounts of IL-2 were produced in the MHV-JHM-stimulated cultures, at a level the same as that found in cultures exposed to uninfected cell antigen.

The most actively proliferating cells and the ones with the greatest production of IFN-γ did not show surface expression of the lymphocyte homing receptor, gp90. This surface adhesion molecule mediates the binding of lymphocytes to the endothelium of lymph node high endothelial venules and is therefore required for normal lymphocyte recirculation (Gallatin *et al.,* 1983). Lymphocyte activation results in down-regulation of gp90, resulting in cells no longer able to home to lymph nodes. Effector cells responding to antigen *in vivo* become $gp90^-$ and the $gp90^-$ phenotype defines the subpopulation of T cells most active in secreting lymphokines (Bradley *et al.,* 1991; Mobley and Dailey, manuscript submitted). Gp90 is similarly down-regulated after stimulation with MHV-JHM antigen, and selection of cells lacking gp90 provides a source of lymphocytes highly enriched in effector cells which may be useful in future studies of T cells responding to MHV-JHM antigen.

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