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Dissecting the host response to a γ-herpesvirus

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The murine γ-herpesvirus 68 (MHV-68) provides a unique experimental model for dissecting immunity to large DNA viruses that persist in B lymphocytes. The analysis is greatly facilitated by the availability of genetically disrupted (−/−) mice that lack key host-response elements, and by the fact that MHV-68 is a lytic virus that can readily be manipulated for mutational analysis. The mutant virus strategy is being used, for example, to characterize the part played *in vivo* by an MHV-68-encoded chemokine-binding protein that may ultimately find an application in human therapeutics. Experiments with various −/− mice and monoclonal antibody depletion protocols have shown very clearly that type I interferons (IFNs) are essential for the early control of MHV-68 replication, while $CD4^+$ T cells producing IFN-γ function to limit the consequences of viral persistence. Virus-specific $CD8^+$ effectors acting in the absence of the $CD4^+$ subset seem initially to control the lytic phase in the lung following respiratory challenge, but are then unable to prevent the reactivation of replicative infection in epithelia and the eventual death of $CD4^+$ T-cell-deficient mice. This could reflect the fact that the interaction between the $CD8^+$ T cells and the virus-infected targets is partially compromised by the MHV-68 K3 protein, which inhibits antigen presentation by MHC class I glycoproteins. Immunization strategies focusing on the $CD8^+$ T-cell response to epitopes expressed during the lytic phase of MHV-68 infection can limit virus replication, but are unable to prevent the establishment of latency. Other experiments with mutant viruses also suggest that there is a disconnection between lytic MHV-68 infection and latency. The massive non-specific immunoglobulin response and the dramatic expansion of $V\beta4^+$ $CD8^+$ T cells, which is apparently MHC independent, could represent some sort of 'smoke screen' used by MHV-68 to subvert immunity. Although MHV-68 is neither Epstein–Barr virus nor human herpesvirus-8, the results generated from this system suggest possibilities that may usefully be addressed with these human pathogens. Perhaps the main lesson learned to date is that all the components of immunity are likely to be important for the control of these complex viruses.

Keywords: vaccination; cell-mediated immunity; immunodeficiency; autoimmunity; chemokines; homeostasis

1. INTRODUCTION

The rhadinoviruses or γ_2-herpesviruses include a spectrum of related pathogens known to infect voles (Blaskovic *et al.* 1980; Efstathiou *et al.* 1990), mice (MHV-68), horses, pigs, a number of free-living and domestic ruminants and a variety of primates (Gompels *et al.* 1988; Greensill *et al.* 2000; Li *et al.* 2000*a*,*b*; Ensser *et al.* 1997; Ehlers *et al.* 1999; Holloway *et al.* 1999; Alexander *et al.* 2000). Some of these viruses have also been transmitted experimentally to hamsters, rats, rabbits and guinea-pigs (Jacoby *et al.* 1988). By contrast the lymphocryptoviruses or γ_1-herpesviruses have, to date, been found only in primates. Most attention is, for obvious reasons, focused on the two prototype γ_1- and γ_2-herpesviruses that infect humans, Epstein–Barr virus (EBV) and human herpesvirus 8 (HHV-8) (Rickinson & Keiff 1996; Chang *et al.* 2000, respectively). In addition, the γ_2-herpesviruses that cause malignant catarrhal fevers in ruminants can constitute substantial problems for wildlife, zoo populations and animal agriculture (Gulland *et al.* 1989; Reid & Rowe 1973).

Two practical questions come to mind when we consider immunity to the γ-herpesviruses. The first is whether it is possible to make effective vaccines against this class of pathogens, a possibility that has received some attention for EBV in cotton-top tamarins and humans (Epstein 1986; Morgan *et al.* 1988; Thomson *et al.* 1996, 1998; Wilson *et al.* 1999). The second addresses the much more speculative idea that appropriately engineered γ-herpesviruses might be used as vaccine-delivery vehicles. Although it may well be true that antigen persistence is not essential for maintaining (at least) $CD8^+$ T-cell memory (Murali-Krishna *et al.* 1999), it is likely that

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regular boosting enhances the quality of such memory (Christensen *et al.* 2000). Can we make safe γ-herpesvirus mutants that persist and regularly reactivate from latency? Could such a variant that carries, for example, immunogenic peptides derived from the human immunodeficiency virus (HIV) function as a permanent, self-boosting immunogen? Obviously, the testing of such a live vaccine would need to be very stringent.

The MHV-68 model offers a unique experimental system (Nash & Sunil-Chandra 1994; Simas & Efstathiou 1998; Virgin & Speck 1999; Speck & Virgin 1999), both for dissecting the basis of immunity and for the development of vaccine strategies at the level of 'proof of principle'. Unlike EBV and HHV-8, MHV-68 causes rapid, lytic infection in a spectrum of cell lines and primary cultures. This means that it is relatively easy to generate deletion mutants, a strategy that has been pursued with great effectiveness over the past three years. The fact that MHV-68 is so lytic may explain why it is much less likely to cause transformation than either EBV or HHV-8. A cell line persistently infected with MHV-68 has been isolated from murine lymphoma (Sunil-Chandra *et al.* 1994), but the virus does not 'immortalize' lymphocytes in culture, although it carries (Van Dyk *et al.* 1999) at least one potential oncogene (a cyclin D homologue). Even so, the fact that MHV-68 establishes latency in B lymphocytes (Sunil-Chandra *et al.* 1992) and macrophages (Pollock *et al.* 1997) *in vivo* means that the effectiveness of any vaccine strategy for controlling both the replicative and persistent phases of γ-herpesvirus infections can readily be evaluated.

The capacity to do *in vivo* experiments, together with the spectrum of reagents that are unique to the laboratory mouse, has allowed the rapid dissection of immunity to MHV-68 (Nash & Sunil-Chandra 1994; Virgin & Speck 1999). Even so, although progress to date has been considerable, there is much to be done and new experiments (especially with mutant viruses) continue to open out novel possibilities. We now know that both $CD4^+$ and $CD8^+$ T-cell effectors play major roles in the control of MHV-68 infection (Ehtisham *et al.* 1993; Christensen *et al.* 1999). Kinetic studies have characterized the $CD4^+$ T-cell, $CD8^+$ T-cell, B-cell and antibody responses through the initial phase of replicative infection into long-term memory (Christensen & Doherty 1999; Stevenson *et al.* 1999*a*,*b*; Sangster *et al.* 2000), if 'memory' is a term that can be used legitimately when discussing a persistent virus. A few vaccine strategies have been tried (Stewart *et al.* 1999; Belz *et al.* 2000; Stevenson *et al.* 1999*b*; Liu *et al.* 1999*b*), although we still know relatively little about antigen specificity in (particularly) the $CD4^+$ T- and B-cell compartments. The aim of this review is both to summarize what we have learned to date about the host response to MHV-68, and to persuade others interested in the γ-herpesviruses that it is worth thinking about the findings generated in this readily manipulated model system.

2. PATHOGENESIS OF MHV-68 INFECTION

Several modes of virus challenge have been used to analyse the disease process caused by MHV-68. The group of Nash, Efstathiou and colleagues (University of Cambridge, UK) used intranasal (i.n.) challenge of anaesthetized mice when they initiated both the molecular and immunological dissection of MHV-68 pathogenesis (Nash & Sunil-Chandra 1994). This protocol has been followed by most of those working directly on γ-herpesvirus-specific immunity (Cardin *et al.* 1996; Coppola *et al.* 1999; Lee *et al.* 2000). The approach favoured by Virgin and Speck (Washington University, St Louis, MI, USA) is to inject a large dose of MHV-68 intraperitoneally (i.p.) (Virgin & Speck 1999; Speck & Virgin 1999), a method that has merit for evaluating the pathogenesis of replication-defective mutant viruses. Oral exposure has also been shown to result in virus replication in the intestinal epithelium, establishing that MHV-68 can transit the acid environment of the stomach (Peacock & Bost 2000). The virus is probably transmitted in nature via the gastrointestinal or respiratory route but, although no contemporary comparison has been made, the longer-term profiles of persistence and latency are likely to be broadly comparable for these three modes of infection. Any mention of samples taken from the lung, cervical lymph node (CLN), mediastinal lymph node (MLN) or bronchoalveolar lavage (BAL) populations throughout this review obviously refers to mice that had been given MHV-68 by the i.n. route.

The virus first establishes itself as a productive infection of the respiratory epithelium. Within a few days, evidence of MHV-68 replication can also be detected in distal epithelial sites, including the adrenals. No evidence has been found for plasma viraemia, and it is thought that infected B cells are important for the blood-borne dissemination of this pathogen (Weck *et al.* 1996). In normal mice, the lytic phase is essentially controlled within 10–12 days, although evidence for low-level virus persistence in the lung can still be demonstrated in the long term by polymerase chain reaction (PCR) and *in situ* hybridization analysis (Stewart *et al.* 1998).

Latent infection of the B-lymphocyte compartment can also be detected soon after i.n. or i.p. challenge (Sunil-Chandra *et al.* 1992; Cardin *et al.* 1996). Somewhat surprisingly, the extent of latency seems relatively independent of virus seeding from the infected epithelium (Stevenson *et al.* 1999*b*; Clambey *et al.* 2000), supporting the idea of Thorley-Lawson (developed for EBV) that persistence is first established as a consequence of virus entry into naive B cells (Babcock *et al.* 1998). These latently infected B cells are then considered to assume a 'memory' phenotype, and are regulated by the same homeostatic processes that control long-term B-cell memory (Thorley-Lawson *et al.* 1996). Studies of μMT mice that have a disrupted immunoglobulin (Ig) μ-chain ($Ig^{-/-}$), and thus lack B cells, established that MHV-68 can also persist in macrophages (Weck *et al.* 1996). This was missed when the extent of latency was measured by a classical infectious-centre assay (Usherwood *et al.* 1996*b*), but can be demonstrated by a more sensitive limiting-dilution analysis (LDA) and, of course, by appropriate PCR protocols (Weck *et al.* 1996).

The overall impression is thus that the pathogenesis of both the acute and persistent phases of EBV and MHV-68 infection share many features, although the molecular differences between these two viruses are such that each will obviously have unique features. Little is known about

the early phases of HHV-8 infection, so it is not clear how closely the MHV-68 mouse model mimics this disease process. We shall no doubt understand the likely pathogenesis of HHV-8 infection better as the issue is pursued with other γ_2-herpesviruses in non-human primates (Greensill *et al.* 2000). Hopefully the findings from the cheap, and readily manipulated, MHV-68 model will have some influence on the design of those experiments.

3. THE CD8$^+$ T-CELL RESPONSE

Prior to the development of the MHV-68 mouse model, everything that was known about the nature of cell-mediated immunity (CMI) to the γ-herpesviruses was derived from studies of EBV infection in normal and immunologically compromised individuals (Rickinson & Moss 1997). This extraordinarily effective research effort on the part of a relatively small group of investigators has led to an understanding of CD8$^+$ T-cell specificity profiles that is unparalleled for any other virus disease of humans. Both the cost and the absence of a convenient, well-characterized experimental system have, however, inhibited *in vivo* analysis of the functional significance of EBV-specific CD8$^+$ T-cell-mediated immunity. Those working with HIV have been somewhat more fortunate in this regard. The availability of the simian immunodeficiency virus model, together with the enormous financial resources that have been dedicated to this problem, is leading to rapid clarification of the strengths and limitations of the CD8$^+$ T-cell response to primate lentiviruses (Egan *et al.* 2000; Kuroda *et al.* 1999). Our understanding of CD8$^+$ T-cell function with the γ-herpesviruses has been greatly enhanced by recent experiments with MHV-68. Would the analysis of immunity to the HIVs have moved much more rapidly if there had been a naturally occurring mouse lentivirus?

(a) *Measuring the MHV-68-specific CD8$^+$ T-cell response*

One property that MHV-68 shares with HHV-8 is that the homologous K3 genes of these two viruses can function to reduce the level of cell-surface expression for MHC class I glycoproteins (Stevenson *et al.* 2000; Ishido *et al.* 2000). The HHV-8 K3 gene product has been shown to target class I MHC molecules for rapid degradation in lysosomal vesicles (Coscoy & Ganem 2000). This may explain why the demonstration of MHV-68-specific cytotoxic T-lymphocyte (CTL) activity initially proved to be difficult. The problem was, however, overcome and kinetic analysis of the virus-specific CD8$^+$ T-cell response in BALB/c (H-2^d) mice that were challenged i.n. with MHV-68 showed profiles (Stevenson & Doherty 1998) that were much like those that might have been expected from studies with other viruses (Doherty *et al.* 1992).

Evidence of MHV-68-specific CTL activity was found during the acute phase of the infectious process in the regional MLN and the spleen, then declined to background levels in the longer term (Stevenson & Doherty 1998). As with primary respiratory pathogens like influenza virus and Sendai virus (Doherty *et al.* 1997*b*), peak levels of effector function were detected in the inflammatory cell population that was recovered by BAL of the infected lung. Again, both CD8$^+$ T-cell numbers and the level of MHV-68-specific CTL activity declined subsequent to the reduction of virus titres and as a consequence, antigen load. LDA of spleen and MLN populations extending for four months beyond virus challenge indicated that the frequency of MHV-68-specific 'memory' CTL precursors for this persistent virus was no higher than for influenza virus or Sendai virus (Doherty *et al.* 1996; Stevenson & Doherty 1998), negative-strand RNA viruses that are not known to be maintained in any form after the initial phase of infection.

The initial LDA and CTL studies thus left us with some understanding of the scope and duration of the CTL response, but with no information about the fine specificity of these CD8$^+$ T cells. This situation changed very quickly with the publication of the full sequence of MHV-68 (Virgin *et al.* 1997), allowing the identification of viral gene products that would probably be expressed in the lytic phase of the infectious process. This in turn facilitated the selection of peptides (Falk *et al.* 1991) that were likely to be bound by the H-2K^b and H-2D^b MHC class I glycoproteins of the H-2^b haplotype in C57BL/6J (B6) mice (table 1). Two of these epitopes were identified by classical protocols using CTL effectors or hybridoma cell lines (Liu *et al.* 1999*b*; Stevenson *et al.* 1999*a*), while the majority were found by a new flow-cytometric approach that measures cytoplasmic interferon-γ (IFN-γ) subsequent to *in vitro* stimulation with peptide (Pepγ assay).

The Pepγ assay is one of two analytical systems that have revolutionized our understanding of CD8$^+$ T-cell-mediated immunity over the past three years (McMichael & O'Callaghan 1998; Doherty 1998). Flow-cytometric analysis of acute lymphocytic choriomeningitis virus (LCMV) infection with fluorochrome-labelled, tetrameric complexes of MHC class I glycoprotein; peptide (tetramers) demonstrated unequivocally that the numbers of LCMV-specific CD8$^+$ T cells present during the acute and convalescent stages of this disease process were at least tenfold in excess of the values previously inferred from LDA experiments (Murali-Krishna *et al.* 1998). Comparable results were found for T-cell populations that were stimulated *in vitro* with peptide in the presence of brefeldin A (to stop protein secretion), then stained for cytoplasmic IFN-γ. We quickly used the Pepγ assay (Stevenson *et al.* 1999*a*) to measure the magnitude of the MHV-68-specific response (figure 1), while Altman at Emory University, Atlanta, GA, USA made tetrameric complexes (Altman *et al.* 1996) for the two most prominent MHV-68 peptides.

The MHV-68 peptides identified in table 1 account for more than 70% of the CD8$^+$ T cells detected in the BAL of acutely infected B6 mice (Stevenson *et al.* 1999*a*), suggesting that the experiments are measuring the great majority of the CD8$^+$ T-cell response to lytic phase epitopes. Extensive screening of overlapping 'mimeotopes' with the Pepγ assay has not led to the detection of other peptides (table 1) presented during the acute phase of MHV-68 infection in H-2^b mice. Also, despite intensive effort, no evidence has been found for epitopes that might be presented by H-2K^b or H-2D^b during latency. This search for a latency-associated CMI response has been a

Table 1. *The MHV-68 peptides recognized by CD8$^+$ T cells in H-2^b mice*

(The 'p' refers to the pin number in the original mimeotope screen. Most of these peptides were originally described by Stevenson *et al.* (1999*a*) and Liu *et al.* (1999*a*).)

identifier	MHC restriction	amino-acid sequence	gene/amino-acid number	putative protein homology
p56	D^b	AGPHNDMEI	ORF $6_{487–495}$	ssDNA-binding protein
p79	K^b	TSINFVKI	ORF $61_{524–531}$	ribonuclease reductase large
p11	not tested	DCTQFTKL	ORF $9_{395–402}$	DNA polymerase
p77	not tested	PYILNKKAI	ORF $61_{370–378}$	ribonuclease reductase large
p119	not tested	IKYVYNFL	ORF $44_{438–490}$	helicase primase
p214	not tested	IVCVNFEKI	ORF $11_{131–139}$	unknown
gB	K^b	KNYIFEEKL	ORF 8-$gB_{604–612}$	glycoprotein B

little more successful with H-2^d mice (Husain *et al.* 1999; Usherwood *et al.* 2000), where CD8$^+$ T cells specific for an H-2K^d-restricted peptide derived from the viral M2 gene product have been shown to modulate the initial load of infected B cells, although there was no effect on numbers in the long term. This latency-associated epitope may, it seems, be expressed for only a relatively short interval.

Kinetic analysis of the CD8$^+$ T-cell response to MHV-68 showed the general profile established previously for other respiratory virus infections (Stevenson *et al.* 1998, 1999*a*; Doherty & Christensen 2000). The numbers of CD8$^+$ T cells specific for the p56, p79 and p11 peptides in B6 mice increase rapidly from about day 10 after i.n. challenge (figure 1*a–d*), then decline after termination of the lytic phase of the infection. The MHV-68-specific CD8$^+$ set concentrates in the BAL population recovered from the site of virus-induced pathology in the lung, with three peptides (p56, p79 and p11) accounting for most of the CD8$^+$ T cells that localize to the respiratory tract at the time that the virus is being controlled (figure 1*a*). The response to D^b-restricted p56 peptide peaks a little earlier, while the K^b-restricted p59-specific population remains at higher prevalence for longer (figure 1*a–d*). Probing stimulator cell populations from the MLN and spleen with a T-cell hybridoma assay has shown that the difference in CD8$^+$ T-cell response profiles for these two epitopes reflects distinct phases of antigen presentation in the lymphoid tissue (Liu *et al.* 1999*a*). Also, dendritic cells, macrophages and B cells isolated directly from infected mice were all found to be presenting MHV-68 peptides. Other, biological evidence suggests that B cells are particularly important for the p79-specific response (Stevenson *et al.* 1998, 1999*a*).

(b) *Extent of CD8$^+$ T-cell-mediated control*

Early experiments completed prior to the development of a capacity for measuring either CTL activity or virus-specific T-cell numbers indicated that the replicative phase of MHV-68 infection is dealt with very effectively by virus-specific CD8$^+$ effectors (Ehtisham *et al.* 1993). Mice (BALB/c, H-2^d) that were depleted of the CD4$^+$ T-cell subset by treatment with an appropriate monoclonal antibody (mAb) reduced virus titres in the lung, and seemed to be recovering when the experiment was terminated by three weeks after virus challenge. The same result was found for the acute phase of MHV-68 infection in B6 mice that lack CD4$^+$ T cells due to homozygous disruption of the H2IA^b MHC class II gene (II$^{-/-}$). This apparent CD8$^+$ T-cell-mediated control of productive MHV-68 infection in the MHC II$^{-/-}$ mice proved, however, to be transient (Cardin *et al.* 1996). The lytic phase in the lung soon re-emerged, although virus production stabilized at titres that were generally at least 100 times lower than those detected prior to the onset of immunity. Even so, the MHC II$^{-/-}$ group developed a uniformly lethal wasting syndrome after 90–120 days. The CD8$^+$ T-cell response acting alone can thus limit MHV-68 infection, but is ultimately unable to protect against the development of fatal disease.

The initial interpretation of these results was that, as argued for persistent LCMV infection, there was a requirement for CD4$^+$ T-cell help to promote the CD8$^+$ T-cell response (Christensen *et al.* 1994). However, when we looked closely at the long-term prevalence of MHV-68-specific CD8$^+$ T cells in the MHC II$^{-/-}$ mice (Grusby *et al.* 1991), we found that they were generally present at higher frequency than in the conventional, MHC II$^{+/+}$ B6 controls, reflecting the greater antigen load resulting from virus reactivation to lytic phase (Stevenson *et al.* 1998). Furthermore, unlike the situation for the mice with persistent LCMV infection (Zajac *et al.* 1998), the MHV-68-specific CD8$^+$ effectors produced IFN-γ when pulsed with the viral p56 or p79 peptides (Stevenson *et al.* 1998). Thus, at least by the criterion of IFN-γ production following *in vitro* exposure to a large dose of peptide, they did not seem to be functionally defective. We are currently looking more closely to see if we can find evidence of some other impairment in these T cells.

(c) *Expansion of a Vβ4$^+$CD8$^+$ population: infectious mononucleosis in the mouse?*

A characteristic of MHV-68 infection is the massive expansion of a diverse CD8$^+$ T-cell population expressing the Vβ4 T-cell receptor (TCR) chain in conjunction with a variety of TCR-α chains (Tripp *et al.* 1997; Hardy *et al.* 2000). Greatly increased numbers of these lymphocytes are first observed subsequent to the resolution of the acute phase of the infectious process, and persist for a month or more. The continued presence of this large, activated (CD62L^{lo}) CD8$^+$ T-cell population in both the peripheral blood and lymphoid tissue suggested initially that the Vβ4$^+$CD8$^+$ T-cell expansion might be comparable with the infectious mononucleosis (IM) syndrome detected (particularly) in adolescents suffering primary EBV infection (Henle *et al.* 1968).

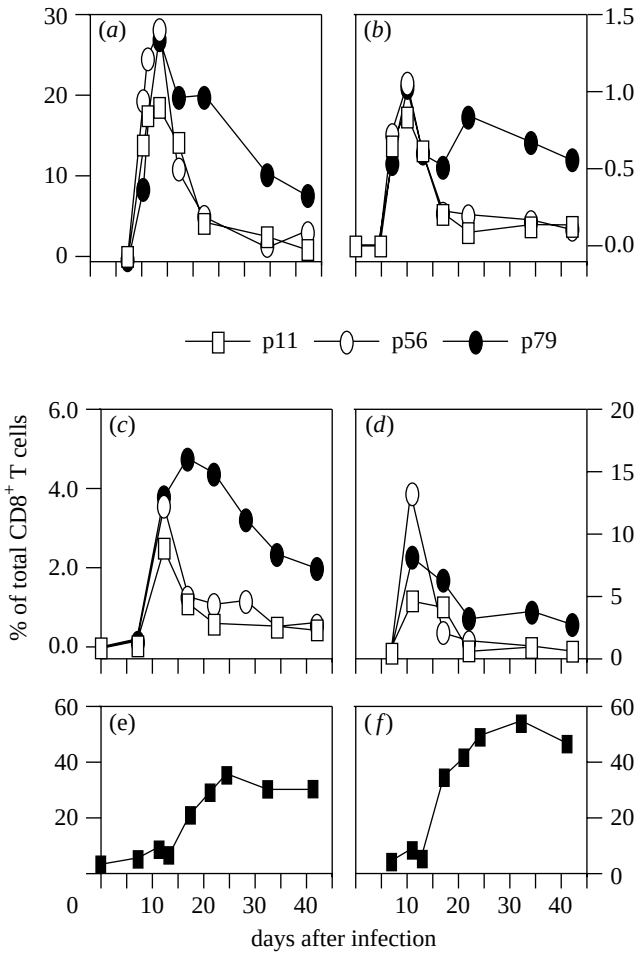

Figure 1. The $CD8^+$ T-cell response. Quantification of the peptide-specific $CD8^+$ T-cell response by intracellular IFN-γ staining (*a–d*) and comparison with $V\beta4^+CD8^+$ T-cell numbers (*e*,*f*) following i.n. infection with 600 plaque-forming units of MHV-68. Cells were pooled from six mice per time-point, and incubated for 6 h in the presence of brefeldin A plus 1 μM (p56, p79) or 10 μM (p11) peptide before staining for intracellular IFN-γ (Pepγ assay). Spleen and MLN samples were first enriched for the $CD8^+$ T-cell subset by *in vitro* depletion of $CD4^+$, MHC class II^+, and Ig^+ cells. (*a*) BAL, (*b*) MLN, (*c*) spleen, (*d*) blood, (*e*) spleen $V\beta4^+$ and (*f*) blood $V\beta4^+$. Reproduced from Stevenson *et al*. (1999*a*).

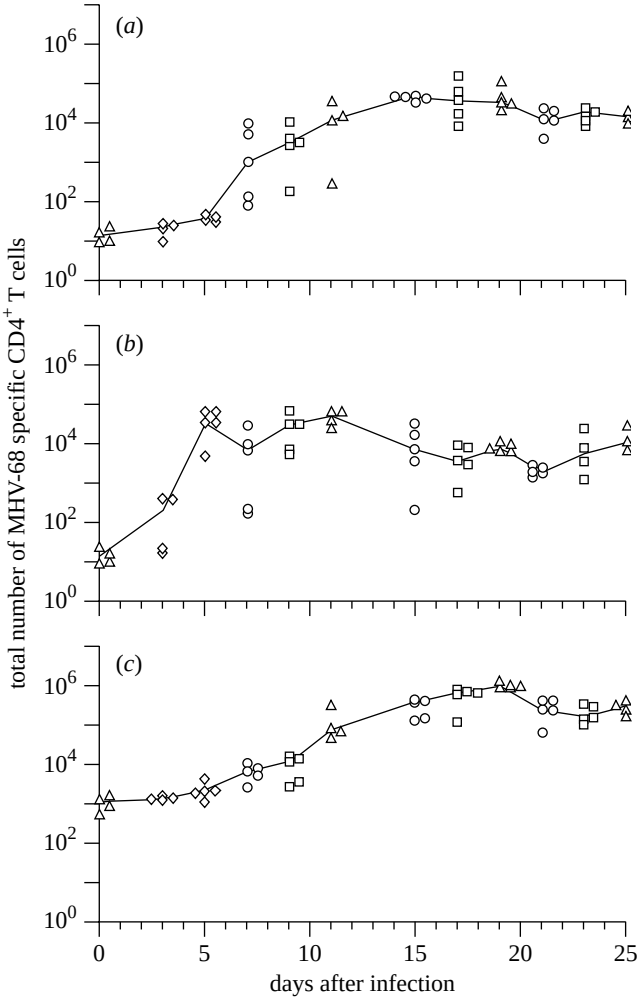

Figure 2. The $CD4^+$ T-cell response. The B6 mice were infected i.n. with 600 plaque-forming units of MHV-68 and CLN, MLN and spleen cells from individual animals were incubated for 48 h in the IFN-γ ELISpot assay. The numbers of MHV-68-specific $CD4^+$ T cells in each organ were determined from the frequency, the percentage of $CD4^+$ T cells, and total cell count. Each data point represents an individual mouse. The results were compiled from four experiments, indicated by the different symbols. (*a*) CLN, (*b*) MLN and (*c*) spleen. Reproduced from Christensen & Doherty (1999).

It now seems unlikely, however, that the dramatic and prolonged increase in $V\beta4^+CD8^+$ T-cell numbers following MHV-68 infection is directly comparable with human IM. Tetramer studies of EBV infection have shown that the activated $CD8^+$ set in blood consists largely of conventional, EBV-specific, MHC class I-restricted T cells (Callan *et al.* 1998). Increased numbers of $V\beta4^+CD8^+$ T cells are found in a variety of MHC-different mouse strains following infection with MHV-68 and, at least for the $H\text{-}2^b$ haplotype, are not associated with known antigenic peptide epitopes (Coppola *et al.* 1999; Stevenson *et al.* 1999*a*). The proportion of MHV-68-specific $CD8^+$ T cells in the spleen and peripheral blood lymphocyte (PBL) pool (figure 1*c*,*d*) in fact drops, concurrently with the $V\beta4^+CD8^+$ T-cell expansion (figure 1*e*, *f*).

Another possibility was that the Vβ4 TCR chain might bind to some 'superantigen' encoded by MHV-68 (Tripp *et al.* 1997). One difference from classical superantigen-driven responses (Scherer *et al.* 1993) is that the expansion of this $V\beta4^+CD8^+$ set tends to be oligoclonal, at least within individual mice (Hardy *et al.* 2000). Also, if there is some direct interaction between the Vβ4 TCR and a viral protein, this does not seem to be mediated via binding to MHC glycoproteins, the defining characteristic of the superantigens (Coppola *et al.* 1999). The $V\beta4^+$ expansion is also seen in the small set of $CD8^+$ T cells found in MHC class I-deficient $\beta2m^{-/-}$ mice (Stevenson *et al.* 1999*a*). Spleen populations that are completely MHC-negative can stimulate $V\beta4^+$ hybridoma cell lines generated from MHV-68-infected mice, with the optimal effect being observed at the peak of viral latency.

The *in vivo* $V\beta4^+CD8^+$ T-cell expansion in mice recovering from the acute phase of MHV-68 infection is, however, dependent both on CD40 ligand (CD40L)-mediated $CD4^+$ T-cell help and on the presence of

B lymphocytes (Brooks *et al.* 1999; Flano *et al.* 1999). The effect is not seen in lymphotoxin-α-deficient (LT-$\alpha^{-/-}$) mice (Lee *et al.* 2000), but this could be more a function of the disruption of lymphoid tissue architecture resulting from the absence of LT-α throughout development than a direct consequence of the absence of this cytokine. The $V\beta4^+CD8^+$ T-cell response in MHV-68 infection thus represents an intriguing phenomenon that is not clearly understood and does not, at least at this stage, seem comparable with any other known biological system.

4. THE CD4+ T-CELL RESPONSE

Both $CD4^+$ and $CD8^+$ T cells are important for the control of MHV-68 infection. Sorting out the role of $CD4^+$ T cells in disease processes has, in the past, often been confounded by the need to distinguish between the part played by T-cell help in the promotion of a high-quality B-cell response and the possibility that the $CD4^+$ T cells themselves are acting directly as effectors of CMI. This difficulty was overcome with the development of $Ig^{-/-}$ μMT mice, which lack both B lymphocytes and antibody (Kitamura *et al.* 1991).

(a) *IFN-γ and CD4+ T-cell effector function*

Respiratory challenge of μMT mice with MHV-68 leads to the development of lytic infection in the lung which is essentially comparable with that in $Ig^{+/+}$ controls, although the extent of latency in the lymphoid tissue is less because of the lack of B lymphocytes (Weck *et al.* 1996; Usherwood *et al.* 1996*b*). Depleting MHV-68-infected μMT mice with mAbs to the $CD4^+$ and $CD8^+$ subsets resulted in the prolongation of MHV-68 replication in both cases, while the consequence of removing both lymphocyte populations simultaneously was (as might be expected) death (Christensen *et al.* 1999).

Further depletion of the CD8-depleted group with a mAb to IFN-γ greatly compromised the extent of $CD4^+$ T-cell-mediated control, indicating that IFN-γ production was central to the function of the $CD4^+$ subset (Christensen *et al.* 1999). Just what the IFN-γ is doing has not been worked out, although substantial levels of this cytokine are detected in the fluid obtained from the infected lung by BAL. Also, experiments with irradiated, bone marrow (BM)-grafted chimeric mice lacking the MHC class II gene expression in radiation-resistant lung epithelium but not in radiation-sensitive macrophages and other haemopoietic cells indicated that it is important for the $CD4^+$ effectors to make direct contact with the cells that are supporting virus growth (Christensen *et al.* 1999). This is totally different from the situation for the influenza-A viruses, where IFN-γ production does not seem to be very important (Price *et al.* 2000*a*) and the role of the virus-specific $CD4^+$ population seems mainly to provide T-cell help for both the B-cell and the $CD8^+$ T-cell responses (Topham *et al.* 1996; Topham & Doherty 1998*a*).

Both $CD4^+$ T cells and IFN-γ seem also to be important for the control of persistent MHV-68 infection. This prominent role for IFN-γ production by $CD4^+$ T cells in limiting both the lytic and latent phases of MHV-68 infection in μMT mice should not be too surprising, although it has received comparatively little attention from those studying immunity to EBV. The same CD4–IFN-γ role has also been identified in the control of murine cytomegalovirus infection, and in the mouse herpes simplex ocular inflammation model (Presti *et al.* 1998; Smith *et al.* 1994; Lucin *et al.* 1992; Polic *et al.* 1998; Yu *et al.* 1996).

(b) *CD4+ T-cell help*

The development of a high-quality antibody response to MHV-68 infection is totally dependent on the concurrent presence of $CD4^+$ T cells (Sangster *et al.* 2000). This is covered below (§5) in the discussion of the B-cell response. On the other hand, the experiments with CD4-deficient, MHC class $II^{-/-}$ mice (see §3(b)) did not provide any clear indication that $CD4^+$ T-cell help is particularly important for either the generation or maintenance of the MHV-68-specific $CD8^+$ T-cell response (Stevenson *et al.* 1998). The fact that the MHC $II^{-/-}$ mice eventually die from MHV-68 infection could equally be explained by the absence of $CD4^+$ effector T cells producing IFN-γ (see §4(a)). We are, however, still looking to see if we can find any evidence of defective functional capacity for the virus-specific $CD8^+$ T cells in MHV-68-infected MHC $II^{-/-}$ mice that have developed the late-onset wasting disease. This is of some interest because of the obvious parallel with EBV and HHV-8 reactivations and virus-associated oncogenesis in CD4-deficient, HIV-infected individuals with advanced acquired immune deficiency syndrome (AIDS).

As discussed above (§3(c)), CD40–CD40L-mediated T-cell help is essential to drive the proliferation of the unusual $V\beta4^+CD8^+$ population that dominates the IM-like phase of MHV-68 infection (Brooks *et al.* 1999; Flano *et al.* 1999). The experiments that have been done so far suggest that the $V\beta4^+CD8^+$ T-cell expansion also depends on the presence of B cells. A highly speculative scenario is that the MHV-68-specific $CD4^+$ T cells interact with α-MHC class II glycoprotein-presenting MHV-68 peptide(s) on an infected B cell. This then results in the production of cytokines that promote the expansion of $V\beta4^+CD8^+$ T cells that bind directly (via the TCR Vβ4 chain) to an MHV-68 protein (or some abnormally expressed host component) on the surface membrane of the infected B lymphocyte, or some other cell type. This process does not seem to depend on IFN-γ production by the $CD4^+$ T cells.

(c) *Specificity and quantification of the MHV-68-specific CD4+ T-cell response*

The search for MHV-68 peptides presented by MHC class II glycoproteins to $CD4^+$ T cells has not been particularly productive, despite a fair amount of effort. The only published epitope to date is the $gp150_{139–155}$ peptide, which binds to $H2IA^b$ and is derived from one of the viral surface glycoproteins (Liu *et al.* 1999*b*). The absence of a spectrum of viral peptides means that neither the tetramers nor the intracellular IFN-γ-staining strategies used to measure the magnitude of the $CD8^+$ T-cell response (see §3(a)) have been available for the $CD4^+$ subset. Quantitative analysis of the $CD4^+$ T-cell response has relied instead on short-term *in vitro* culture with whole virus, followed by frequency determination using the single-cell IFN-γ ELISpot assay (Christensen & Doherty

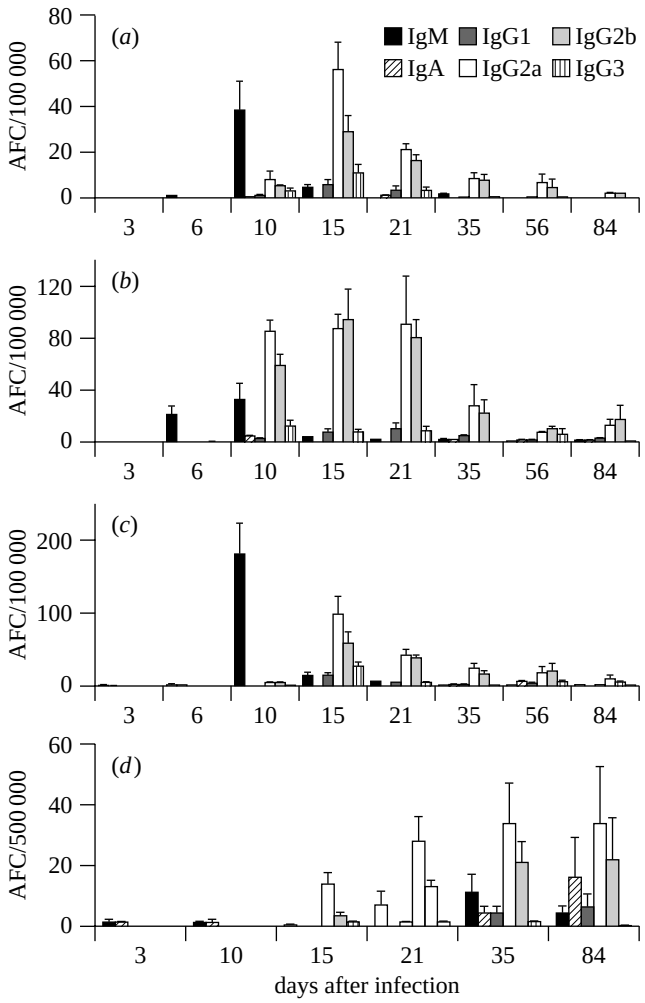

Figure 3. The AFC response. The kinetics of virus-specific AFC responses in the CLN (*a*), MLN (*b*), spleen (*c*) and BM (*d*) were determined for B6 mice infected i.n. with MHV-68. The ELISpot assay used purified MHV-68 as the immunoabsorbent to measure the number of cells producing virus-specific IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 at different times after infection. Results are expressed as the number of AFC per 1×10^5 or 5×10^5 nucleated cells. The mean + s.e. is shown for three to six individual mice. Cells sampled from days 6–35 post-infection were also assayed on plates coated with influenza virus antigens as control for non-specific binding. Small numbers of IgM AFC (0–12 AFC per 1×10^5 for CLN, MLN, and spleen; 0–48 AFC per 5×10^5 for the BM), and essentially no AFC producing switched isotypes, were detected on these control plates. Reproduced from Sangster *et al.* (2000).

1999). Earlier LDA experiments used interleukin-2 (IL-2) production as a read-out. The results from these studies are qualitatively similar to the finding from the ELISpot analysis, although, as with the LDA for $CD8^+$ T cells (§ 3(b)), the numbers tend to be lower.

The MHV-68-specific population peaks at about 1:20–1:50 of the $CD4^+$ set during the acute phase of the infectious process, with evidence of clonal expansion being found in both the MLN and the spleen (Christensen & Doherty 1999). Frequencies of about 1:200–1:500 are then maintained in the long term. Furthermore, cell sorting followed by ELISpot analysis indicates that the MHV-68-specific $CD4^+$ T cells retain an 'activated' $CD62L^{lo}$ $CD44^{hi}$ phenotype indefinitely. This is different from the situation for the readily eliminated Sendai virus (Ewing *et al.* 1995; Topham & Doherty 1998*b*), where much of the $CD4^+$ set reverts to being $CD62L^{hi}$, and is consistent with the profile that would be expected for a persistent infection.

5. THE B-CELL RESPONSE

Perhaps the most obvious feature of MHV-68 infection is the massive splenomegaly that peaks between 14 and 21 days after i.n. infection (Usherwood *et al.* 1996*a*). The greatest increase in cell numbers is observed for the $B220^+$ B-lymphocyte population, although the $CD4^+$ and $CD8^+$ (§ 3(c)) sets are also substantially expanded. The net consequence is that all three populations are maintained at higher prevalence in the PBL pool for at least five weeks after MHV-68 challenge (Doherty *et al.* 1997*a*). The *in vivo* expansion of the $B220^+$ population is, as with the proliferation of the $V\beta4^+CD8^+$ set (§ 3(c)), totally dependent on concurrent CD40L-mediated $CD4^+$ T-cell help (Cardin *et al.* 1996; Brooks *et al.* 1999; Flano *et al.* 1999). Also, although the prominence of the $CD8^+$ T cells in the circulation gives the appearance of an IM-like syndrome (§ 3(c)), the effect is relative; all categories of lymphocytes are proliferating and show higher blood counts for a prolonged interval (Hamilton-Easton *et al.* 1999). These findings have intriguing implications for models of lymphocyte homeostasis (Doherty *et al.* 1997*a*).

(a) *Proliferation* in vitro, *but without transformation*

A somewhat different, CD4-independent phenomenon is observed when B cells are infected *in vitro* with MHV-68. The lymphocytes proliferate, express higher levels of CD69 on the cell surface, produce substantial amounts of IL-6 and IgM, then die after about 72 h (Stevenson & Doherty 1999). The presence of IL-6 is not mandatory, and the same activation effect is seen for MHV-68 infection of IL-$6^{-/-}$ and IL-$6^{+/+}$ B cells, or B cells cultured with MHV-68 in the presence or absence of a mAb to this cytokine. Also, although these MHV-68-infected B lymphocytes all die, there is no evidence of productive (lytic) infection. The latency-associated MHV-68 tRNAs (but not the M2 gene) are transcribed, while the viral DNA remains linear and shows little of the tendency to circularize that is characteristic of γ-herpesvirus persistence (Dutia *et al.* 1999*b*). Numerous attempts have failed to promote the emergence *in vitro* of 'immortalized' B-cell clones, although it should be noted that only one MHV-68 isolate has yet been analysed in any detail.

(b) *Specific and non-specific Ig production* in vivo

The CD4-dependent, polyclonal B-cell activation that follows MHV-68 infection is accompanied by a massive, class-switched Ig response, much of which is clearly not specific for the virus (Stevenson & Doherty 1998; Sangster *et al.* 2000). This was measured as $\mu g\,ml^{-1}$ of serum Ig and by counting antibody-forming cells (AFCs) producing the κ light chain used by 90% of mouse Ig. At the peak of the acute response in the MLN, the numbers of κ-producing AFCs detected by ELISpot analysis were

more than 200-fold higher than those making antibody specific for purified MHV-68 (figure 3). Both spectrotyping and the constancy of κ/λ ratios indicated that this total serum Ig response, which was not significantly reduced in level by adsorption with purified virus, is indeed very polyclonal.

The numbers of MHV-68-specific AFCs peaked first in the MLN (figure 3*b*), then later in the CLN and spleen (figure 3*a*,*c*). The AFC response in the lymph nodes draining the respiratory mucosa was somewhat delayed, and of smaller magnitude when compared with that observed following i.n. infection with readily eliminated, negative-strand RNA viruses like influenza or murine parainfluenza type 1 (Sendai). Many of the AFCs producing antibody to MHV-68 were, as with other infections (Hyland *et al.* 1994; Slifka & Ahmed 1996), found in BM in the long term (figure 3*d*). There was no indication that the B-cell response to MHV-68 is being constantly stimulated as a consequence of virus reactivation to lytic phase.

An intriguing feature is that the distribution of the isotype patterns was characteristic of the Ig response overall, independent of specificity for the virus. The same IgG2a and IgG2b dominance was found for both virus-specific (figure 3) and total Ig profiles, determined by serum and AFC analysis. This suggests that the cytokines, and other molecular interactions that determine class switching, are operating in comparable ways for the MHV-68-specific and non-specific responses. Another unusual feature of MHV-68 infection in B6 mice is that virus-specific IgA-producing AFCs and serum IgA were, when compared with the situation for other respiratory viruses (Sangster *et al.* 1997), remarkably absent during the acute phase of the disease process.

(c) *Can MHV-68 trigger autoimmunity?*

Levels of Ig specific for double-stranded (ds) DNA and for type II collagen were increased in some, although not in all, MHV-68-infected B6 mice (Sangster *et al.* 2000). On the other hand, mice that had been immunized previously with an influenza-A virus showed a relative fall in serum antibody levels specific for this virus, suggesting that the massive total Ig response to MHV-68 accelerated the rate of clearance of the influenza-specific Ig. There is thus no indication that MHV-68 causes cross-reactive stimulation of memory B cells, although this needs to be looked at again using single-cell assays. Clearly, the nature of the Ig response induced by this virus merits further analysis, particularly from the aspect of determining the nature of the molecular events underlying the polyclonal B-cell activation and class-switched Ig production.

(d) *Is antibody involved in the control of viral persistence?*

The MHC class $II^{-/-}$ mice that lack $CD4^+$ T cells seem first to control MHV-68 infection, then show evidence of reversion to lytic phase and death within three to four months of the initial virus challenge (Cardin *et al.* 1996). This could be attributed to the lack of IFN-γ-producing $CD4^+$ T-cell effectors, or to the absence of a class-switched, virus-specific Ig response. The fact that $Ig^{-/-}$ μMT mice do not develop the wasting disease seen in the MHC $II^{-/-}$ group does not exclude a role for antibody as, due to the lack of B cells, the extent of MHV-68 persistence is lower in these animals. Set against this argument is other evidence suggesting that the lytic and latent phases of MHV-68 infection seem, once established, to be relatively independent.

Mice were thymectomized as adults (to prevent renewal of the T-cell pool), infected with MHV-68 and allowed to recover (Stevenson *et al.* 1999*c*). They were then depleted of both the major T-cell subsets by treatment, for a time, with mAbs to CD4 and CD8, a procedure that is lethal if performed prior to virus challenge. These mice remained clinically normal, and showed no evidence for the reactivation of lytic MHV-68 infection or an increase in the numbers of latently infected cells. Unfortunately, the mAb-depletion protocol did not remove all representatives of the $CD4^+$ and $CD8^+$ subsets in the very long term, and the experiment probably merits repeating now that we have sensitive assays for MHV-68-specific $CD4^+$ and $CD8^+$ T cells (Stevenson *et al.* 1999*a*; Christensen & Doherty 1999). Even so, the possibility that antibody plays a part in the control of the persistent phase of this infectious process cannot, at this stage, be discounted.

6. CYTOKINES AND CHEMOKINES

Cytokines and chemokines are likely to be enormously important for the control of this large DNA virus. It is very clear that type 1 IFNs (IFN-αβ) are essential, early mediators of MHV-68 resistance (Dutia *et al.* 1999*a*). Lung titres were 100- to 1000-fold higher in IFN-αβ receptor $(R)^{-/-}$ mice, which succumbed to a relatively low-dose challenge. The numbers of latently infected spleen cells were also increased tenfold in IFN-$\alpha\beta R^{-/-}$ mice, at least during the acute phase of the infection.

Lymphocytes recovered directly from MHV-68-infected B6 mice were found to produce substantial amounts of IFN-γ and IL-6, with lower levels of IL-10 and IL-2 being detected following *in vitro* culture (Sarawar *et al.* 1996). The 'Th2' lymphokines, IL-4 and IL-5, seem to be minimally expressed in this infection. Virus growth and control profiles were essentially normal in IL-$6^{-/-}$ mice (Sarawar *et al.* 1998), although there was evidence of enhanced natural killer cell activity early in the course of the infection. Otherwise intact IFN-$\gamma^{-/-}$ mice are not obviously compromised during the acute phase of the disease process (Sarawar *et al.* 1997). The same is true for LT-$\alpha^{-/-}$ mice, despite the extreme disruption of normal lymph node and splenic architecture that results from the absence of this cytokine throughout ontogeny (Lee *et al.* 2000). Both IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice develop abnormal pathology in the longer term, particularly associated with persistent MHV-68 replication in the smooth muscle cells of the large arteries (Dal Canto & Virgin 1999; Weck *et al.* 1997). This leads in turn to thickening of the blood vessel wall and narrowing of the arterial lumen, with eventual vascular occlusion and death. The MHV-68-infected IFN-$\gamma R^{-/-}$ animals can also show evidence of acute splenic atrophy, although the mechanism is yet to be clarified (Dutia *et al.* 1997).

The virus makes a chemokine-binding protein (M3), the role of which is currently being established by

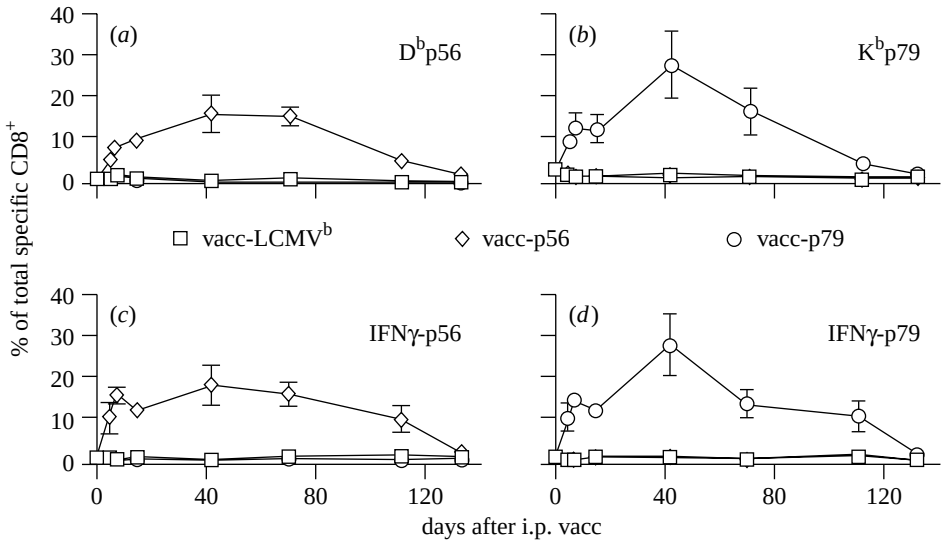

Figure 4. Effect of post-exposure vaccination on $CD8^+$ T-cell numbers. The $IA^{b+/+}$ B6 mice were infected i.n. with MHV-68 one month prior to i.p. challenge with the recombinant vacc viruses, vacc-p56, vacc-p79 or a vacc-$LCMV^b$ control. The numbers of virus-specific $CD8^+$ T cells in spleen were determined by staining with the D^bp56 or K^bp79 tetramers (*a*,*b*) or by the Pepγ assay (*c*,*d*). Reproduced from Belz *et al.* (2000).

experiments with an M3-deletion mutant (Parry *et al.* 2000; Van Berkel *et al.* 1999). This protein binds all subfamilies of chemokines in solution, thus inhibiting interaction with the appropriate receptors. The blocking effect is seen for the human CC and CXC chemokines, suggesting that the molecule may be of therapeutic value. The development of such a product would surely be a 'first' for experiments that have focused exclusively on a mouse model of viral pathogenesis!

7. EFFICACY OF IMMUNIZATION

Most of the vaccines that are currently used to protect human populations against virus infections work by promoting an antibody response. The virus-specific Ig functions to minimize the extent of virus replication at the point of entry and/or prevents dissemination via the blood. Mice were injected subcutaneously with a vaccinia (vacc) virus construct incorporating the MHV-68 gp150 gene (equivalent to EBV gp350), then challenged i.n. with MHV-68 (Stewart *et al.* 1999). Prior exposure to vacc-gp150 greatly diminished the extent of lytic infection, and virtually abrogated the development of the IM-like disease. However, the virus still established latency.

(a) *Pre-exposure priming of $CD8^+$ T cells*

The question of whether or not it is possible to control virus infections by priming only the $CD8^+$ T-cell response is currently of considerable interest, particularly from the aspect that such a vaccine might circumvent virus variation as a consequence of antibody-mediated selection processes. This is clearly important with the lentiviruses and the influenza-A viruses, although there is also evidence from both systems that a continuing (Price *et al.* 2000*b*) $CD8^+$ T-cell response can drive the emergence of viral escape mechanisms (Chen *et al.* 2000). Experiments with the influenza-A viruses have shown that the recall of a very large memory $CD8^+$ T-cell population can indeed limit virus replication, but the infectious process still becomes established in the lung and the protective effect is not obvious until about two to three days after virus challenge (Christensen *et al.* 2000).

Prime and boost experiments with vacc-p56 and influenza virus (WSN-p56) recombinants led to the development of a massive response to the D^b-restricted p56 epitope of MHV-68 (Stevenson *et al.* 1999*b*). This caused almost complete control of the lytic phase in the lung, and initially reduced the magnitude of latent infection in the lymphoid tissue. Within three weeks, however, there were as many persistently infected cells in the spleens of the primed and naive (prior to MHV-68 challenge) mice, and the IM-like disease (although delayed) also developed. Similar results were found in mice that had been immunized by the injection of dendritic cells pulsed with the peptides that stimulate MHV-68-specific $CD8^+$ T cells (Liu *et al.* 1999*b*). This analysis also included the one MHV-68 peptide known to be recognized by $CD4^+$ T cells, but the fact that there was no additional effect cannot be regarded as an adequate test of the consequences of priming the CD4 compartment. At this stage, what can be said with a reasonable level of certainty is that the establishment of a γ-herpesvirus infection cannot be prevented by priming only $CD8^+$ T cells specific for lytic-phase epitopes.

(b) *Post-exposure vaccination*

The fact that priming the $CD8^+$ T-cell response prior to i.n. MHV-68 challenge greatly diminished the extent of virus replication in the lung caused us to question whether it might be possible to modulate the reactivation of lytic infection in MHC class $II^{-/-}$ mice by vaccinating after the virus had become established (Belz *et al.* 2000). Post-exposure challenge of MHV-68-infected MHC $II^{+/+}$ (figure 4) and MHC $II^{-/-}$ mice with vacc-p56 or vacc-p79 caused a dramatic increase in the numbers of $CD8^+$ T-cells specific for D^bp56 or K^bp79, respectively. This was an intriguing result as we were, at least in the MHC $II^{-/-}$ mice, introducing the recombinant vacc viruses into a

situation where the reactivation of MHV-68 to lytic phase should be constantly boosting the $CD8^+$ T-cell response. Clearly, the vacc-p56 challenge in some way broke through the constraints regulating the magnitude of any such restimulation by persistent MHV-68.

The net consequence of this boost was, however, that the MHV-68-infected MHC $II^{-/-}$ mice survived (on average) for only a few weeks longer. All the secondarily stimulated mice eventually succumbed when the size of the MHV-68-specific $CD8^+$ T-cell set fell to the level (still more than 5% of the splenic $CD8^+$ population) found in the unmanipulated, MHV-68-infected MHC $II^{-/-}$ controls. Perhaps this reflects that the capacity of effector $CD8^+$ T cells to eliminate lytically infected targets by direct cell–cell interaction is limited by the capacity of the viral K3 gene to modulate epitope presentation. The $CD8^+$ T cells also produce IFN-γ, which, when virus-specific $CD8^+$ T-cell numbers are high, might be available at sufficient concentration to mimic the IFN-γ-dependent protective mechanism used by $CD4^+$ T cells, whatever that may be.

8. CONCLUSIONS

This experimental model has already provided us with a spectrum of novel information about the nature of γ-herpesvirus infections, and is likely to continue to do so. One of the more surprising insights to date is that there is no obvious correlation between the extent of the initial replicative infection and the level of MHV-68 latency in the long term. This message comes through from the vaccination experiments (Stevenson *et al*. 1999*b*), and from the consequences of infecting mice with viral mutants that lack functional genes important for either lytic infection or viral persistence (Clambey *et al*. 2000).

The quantitative protocols that have been developed for the analysis of the $CD8^+$-, $CD4^+$- and B-cell responses to MHV-68 offer a unique experimental system for analysing the consequences of various manipulations that may promote (or compromise) immunity to large, persistent DNA viruses. The finding that $CD4^+$ T cells producing IFN-γ are very important for the control of MHV-68 infection (Christensen *et al*. 1999) should cause us to think more seriously about this aspect of the host response to the human γ-herpesviruses. Is the reactivation of EBV to lytic phase and the oncogenesis associated with both EBV and HHV-8 in AIDS patients a function of the loss of $CD4^+$ T-cell help for the CD8 response, or a direct consequence of the lack of $CD4^+$ T-cell effectors?

Even so, it is clear that MHV-68 is neither EBV nor HHV-8. The latency-associated transcripts that are so central to EBV immunity and pathogenesis seem to be absent from, or as yet undetected in, cells that are persistently infected with MHV-68. Although there is one lymphoma line carrying MHV-68, the virus does not readily cause cell transformation. However, we do not know what happens in the bank vole, the species from which this virus was originally isolated, and it is possible that there are natural variants that may be oncogenic in mice.

Perhaps the most useful, general message that emerges from the experiments to date with MHV-68 is that all the key elements of immunity are required to control γ-herpesvirus infections. As with other, large DNA viruses, these pathogens have developed a spectrum of immune subversion strategies. The K3 molecules of HHV-8 and MHV-68 diminish the effectiveness of antigen presentation by MHC class I glycoproteins (Stevenson *et al*. 2000; Ishido *et al*. 2000; Coscoy & Ganem 2000). The MHV-68 genome encodes an inhibitor of complement activation (Kapadia *et al*. 1999). The MHV-68 chemokine-binding protein (M3) and the M11 protein (Wang *et al*. 1999) that inhibits apoptosis are likely to have a broad effect on both antigen-specific and non-specific elements of the host response. An optimal γ-herpesvirus vaccine should generate memory in the $CD8^+$ T-cell, $CD4^+$ T-cell and B-cell compartments.

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