JOURNAL OF VIROLOGY, Nov. 2001, p. 10467–10471 Vol. 75, No. 21
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.21.10467–10471.2001

Role of Tachykinins in the Host Response to Murine Gammaherpesvirus Infection

CATHERINE M. PAYNE,† CAROLINE J. HEGGIE, DAVID G. BROWNSTEIN, JAMES P. STEWART,* AND JOHN P. QUINN‡

Laboratory for Clinical and Molecular Virology, The University of Edinburgh, Edinburgh EH9 1QH, United Kingdom

Received 29 May 2001/Accepted 26 July 2001

Tachykinins function not only as neurotransmitters but also as immunological mediators. We used infection of tachykinin-deficient (*PPT-A*$^{-/-}$) mice and wild-type controls with murine gammaherpesvirus to assess the role of tachykinins in the host response to a virus infection. Although infection was ultimately controlled in *PPT-A*$^{-/-}$ mice, there were higher titers of infectious virus in the lungs, accompanied by a more rapid influx of inflammatory cells. Clearance of latently infected cells from the spleen was also delayed. This is the first report of the direct influence of tachykinins in the host response to a virus infection.

The tachykinin family of neurotransmitters not only is involved in both central and peripheral nervous system function but also has a role in inflammation (termed neurogenic inflammation) and adaptive immunity (9, 16). The most characterized member of the family is substance P (SP). This is encoded by the preprotachykinin A (*PPT-A*) locus. Alternative splicing of *PPT-A* RNA and processing of the propeptide yields, in addition to SP, neurokinin A and neuropeptides K and γ. A major physiological source of SP is primary sensory neurons, whose cell bodies in the dorsal root ganglia produce SP and transport it to peripheral and central sites, where it is stored and later released from nerve endings. However, cells of the immune system such as T cells, monocytes-macrophages, and dendritic cells can also produce SP (12). The effects of SP are mediated by signaling through the G protein-coupled receptor NK-1 or NK-2 (13). A number of cell types involved in inflammation and immunity express the NK-1 receptor, including T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, and mast cells (10). Consequently, SP can enhance immunoglobulin production by B cells, stimulate mitogen-induced proliferation of lymphocytes, and induce the production of several important inflammatory cytokines, including interleukin 1 (IL-1), IL-2, IL-6, gamma interferon (IFN-γ), and tumor necrosis factor alpha (for a review, see reference 18).

The demonstration that most immunocytes producing SP also express its receptor led to the hypothesis that SP not only acts as a mediator of the cross talk between the nervous and immune systems but also is biologically involved in the direct interaction between immune cells in a paracrine and/or autocrine fashion, independently of sensory nerves or neurogenic inflammation (10, 11).

* Corresponding author. Mailing address: Laboratory for Clinical and Molecular Virology, The University of Edinburgh, Summerhall, Edinburgh EH9 1QH, United Kingdom. Phone: 44 131 650 7939. Fax: 44 131 650 6511. E-mail: james.stewart@ed.ac.uk.

† Present address: Department of Pathology, The University of Edinburgh, Edinburgh EH8 9AG, United Kingdom.

‡ Present address: Physiological Laboratory and Department of Human Anatomy and Cell Biology, University of Liverpool, Liverpool L69 3BX, United Kingdom.

A number of studies have shown that viruses (e.g., respiratory syncytial virus) can induce SP and neurogenic inflammation, particularly in the lungs, in the context of a respiratory challenge (19, 26). However, there are no published data on the influence of SP in combating virus infection. The aim of this study was to assess the role of tachykinins in the host response to virus infection using murine gammaherpesvirus infection of mice as a model system.

Infection of laboratory mice by murine gammaherpesvirus 68 (MHV-68; International Committee on Taxonomy of Viruses name, murid herpesvirus 4) (30) is a well-characterized system for the study of gammaherpesvirus pathogenesis and, via the use of transgenic mice, for the study of host components important in combating infection (for reviews, see references 17 and 20). Intranasal inoculation of mice with MHV-68 results in a productive infection in the lungs (23) that is resolved at about day 10 postinfection (p.i.) by the action of $CD8^+$ T cells (7). The virus then persists in a latent form in epithelial cells at this site (21). MHV-68 spreads to the spleen during the subsequent viremia, where it becomes latent in B lymphocytes, macrophages, and dendritic cells (8, 24, 29). Establishment of latency in the spleen is associated with marked splenomegaly and mononucleosis that resembles that caused by primary infection of humans by Epstein-Barr virus (25). Splenomegaly is driven by $CD4^+$ T cells (7, 27) and is dependent on the presence of MHV-68-infected B cells in the spleen (29, 31). The resolution of splenomegaly is achieved by $CD8^+$ T cells (7), which are also important in the long-term control of persistent infection (4, 21).

To elucidate the part played by tachykinins after MHV-68 infection, we used transgenic mice with lesions in the *PPT-A* locus (*PPT-A*$^{-/-}$) such that coding sequences for SP and neurokinin A were both deleted (3). It has been shown that these mice express none of the tachykinins encoded by *PPT-A*. Aside from modulation of several nociceptive parameters, these mice have no obvious deleterious phenotypic abnormalities and have been used to address the role of tachykinins in other neuronal challenge models (1, 14). The genetic background for these mice is CD1. There are no data on the course of MHV-68 infection in this strain. However, with minor varia-

tions, the typical course of infection described above has been observed in all strains tested to date (17). Wild-type (wt) CD1 mice were therefore used as controls and were purchased from Harlan United Kingdom (Bicester, England). MHV-68 (strain g2.4) was grown and titers were determined on BHK-21 cells, and the virus was used to infect mice exactly as described previously (23). Groups of *PPT-A*$^{-/-}$ and wt mice were infected intranasally with 4×10^5 PFU of MHV-68 at 4 to 5 weeks of age. The mice were then monitored over a period of 79 days p.i. for clinical signs, histopathological changes, and the levels of infectious and latent viruses in the lungs and spleens. The whole experiment was performed twice, with comparable results.

Infectious virus is recoverable from *PPT-A*$^{-/-}$ but not wt mouse lungs. To assess the extent of virus replication in lungs, groups of four mice were euthanized by CO_2 asphyxiation at days 3, 5, 7, 10, and 14 p.i. The amount of infectious virus was then measured by plaque titration (23). No infectious virus was detected in the lungs of wt mice at any time p.i. In two separate experiments, means of 2.3 and 2.5 $\log_{10}$ PFU per mouse were observed in the lungs of *PPT-A*$^{-/-}$ mice, but only at day 7 p.i.; actual values were 1.6, 3, 2.7, and 2 $\log_{10}$ PFU in experiment 1 and 2.4, 2, 2.9, and 2.6 $\log_{10}$ PFU in experiment 2.

This result is highly unusual. The level of infectious MHV-68 present in the lungs of mice after intranasal infection is known to vary between strains and is independent of clinical signs of infection (22). However, peak levels varying from 10^2 to 10^6 PFU per mouse are always seen. The experiment was performed on two separate occasions, with comparable results. Also, the virus stock used in these experiments generates expected lung titers after infection of BALB/c mice (15). CD1 (wt) mice therefore appear to be unusually highly resistant to the replication of MHV-68 in the lungs to the point at which the amount of infectious virus recovered is below the level of detection of the plaque assay. In spite of the low level, the detection of infectious virus in *PPT-A*$^{-/-}$ mice at day 7 p.i. indicates that tachykinins play a role in the host control of virus infection in the lungs.

Splenomegaly is unaffected, but latently infected cells are cleared less efficiently in *PPT-A*$^{-/-}$ mice. To investigate changes in the spleen following infection, samples were obtained from groups of four mice at 10, 14, 21, 28, 38, and 79 days p.i. The number of cells present in the spleen was ascertained, and the amount of latent virus present in the spleen was measured by using an infective-center (i.c.) assay as described previously (15). As shown in Fig 1A, there was a characteristic transient rise in the numbers of splenocytes (splenomegaly) in both wt and *PPT-A*$^{-/-}$ mice that peaked at day 14 p.i. However, there was no significant difference between the spleen cell numbers in the two groups of mice, as determined by two-way analysis of variance (ANOVA) ($P = 0.18$). Figure 1B shows that concomitant with splenomegaly, there was a rise in the i.c. numbers in the spleens of both wt and *PPT-A*$^{-/-}$ animals. In the wt mice, the increase peaked at day 14 p.i. and rapidly resolved to a near-baseline level by day 21 p.i. In the *PPT-A*$^{-/-}$ mice, the i.c. numbers increased to a level similar to that in wt mice up to day 14 p.i. However, after this time, the i.c. numbers in *PPT-A*$^{-/-}$ mice remained at the same level through 21 days p.i. and then decreased less rapidly to a level that was still higher than that in wt mice at day 79 p.i. The higher i.c. level

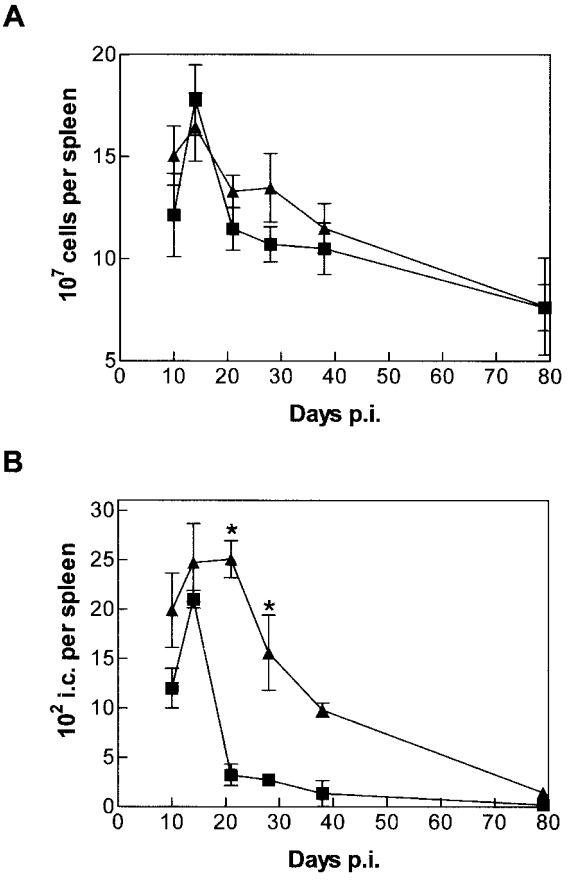

FIG. 1. Cellular and virological changes in the spleens of mice following infection. CD1 wt (■) or *PPT-A*$^{-/-}$ (▲) mice were infected intranasally with 4×10^5 PFU of MHV-68 and euthanized at the indicated days p.i. (A). Mean total numbers of splenocytes and standard error for four mice per group are shown for each time point. (B) Latent virus in the spleens, as determined by an i.c. assay. Infectious virus was not seen in the spleens in this experiment. Mean i.c. numbers per spleen and standard error for four mice per group are shown for each time point. Statistically significant differences between points on the curves were determined by two-way ANOVA with Bonferroni posttests and are indicated by asterisks above the relevant points.

in the *PPT-A*$^{-/-}$ mice than in the wt mice was statistically significant at days 21 and 28 p.i. ($P = <0.001$ and $P = <0.01$, respectively), as determined by two-way ANOVA with Bonferroni posttests. Similar results were obtained in a repeat of this experiment, with the higher i.c. levels in PPT-A$^{-/-}$ mice being statistically significant at days 21 and 28 p.i. (data not shown).

In spite of the unusually low titers of infectious virus present in the lungs of both CD1 and *PPT-A*$^{-/-}$ mice, MHV-68 trafficked to the spleen and was associated with a rise in latently infected splenocytes that was entirely typical of that seen in other strains of mice. However, the observed delay in the i.c. decline in *PPT-A*$^{-/-}$ mice indicates that tachykinins play a role in the host response to control cells that are latently infected with MHV-68 in the spleen.

Pathological changes in lungs following MHV-68 infection. Although small, there were consistently repeatable differences between the levels of virus in the lungs of wt and *PPT-A*$^{-/-}$ mice. Also, the lung is an area where SP is known to influence

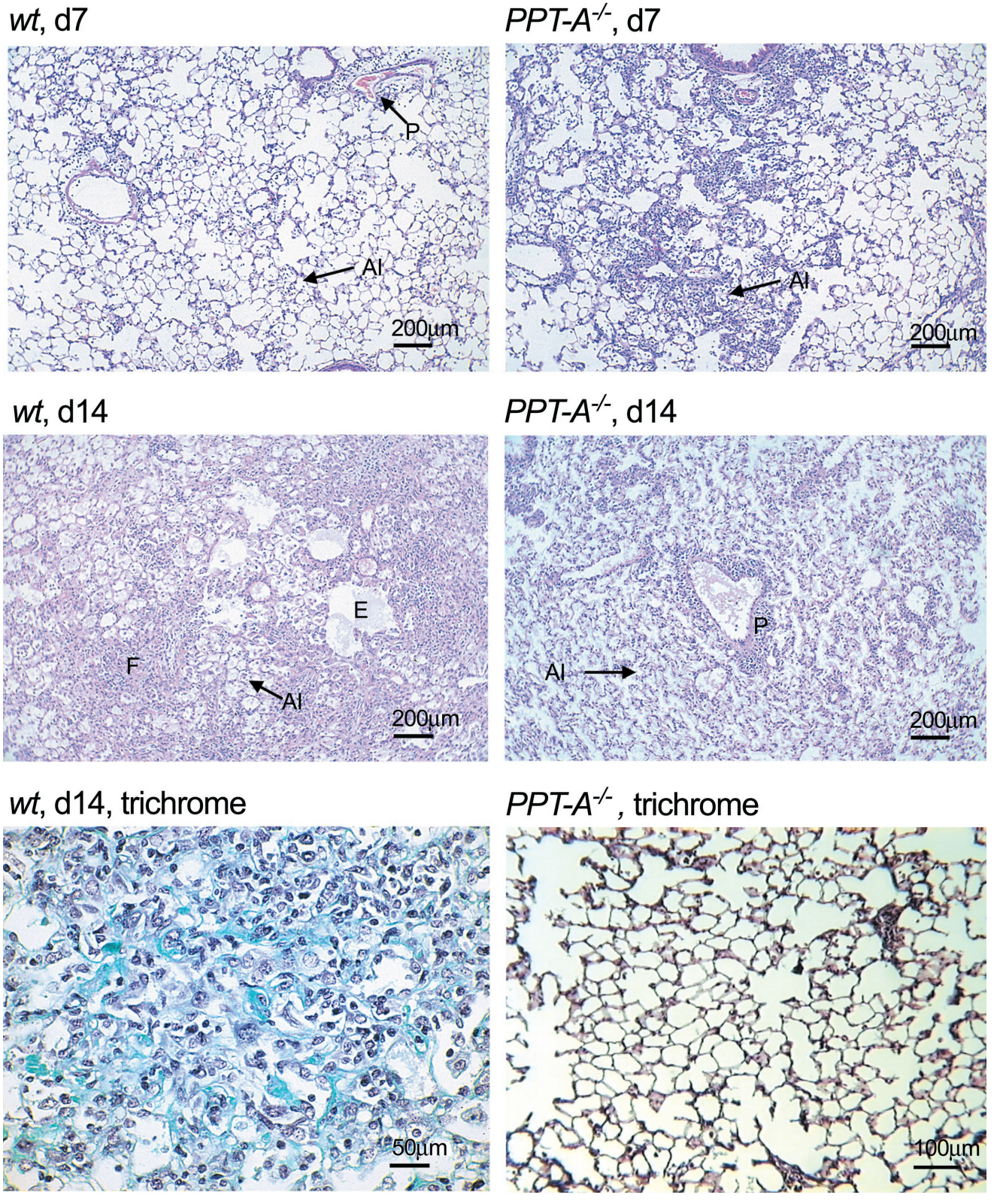

FIG. 2. Histopathological changes in the lungs of mice infected with MHV-68. Representative lung sections stained with hematoxylin and eosin or Masson trichrome are shown for mice infected for the times indicated (d, days). Salient pathological features are highlighted as follows: P, perivascular infiltration; AI, alveolar inflammation; F, fibrosis; E, emphysema.

host responses to virus infection (19, 26). As a preliminary step in assessing the influence of tachykinins in the response to MHV-68 in the lungs, we performed a sequential histopathological study. Groups of four mice were euthanatized at 7 and 14 days p.i. and perfused through the left ventricle with phosphate-buffered saline followed by Formol saline. Sections cut from paraffin-embedded blocks were then stained with hematoxylin and eosin and examined by microscopy. The results are shown in Fig. 2. In wt mice at day 7 p.i. there were mild lymphoid cell infiltrates around blood vessels, in alveolar spaces, and in alveolar septa. These findings are typical of the response seen at this time after MHV-68 infection (15, 22). In *PPT-A*$^{-/-}$ mice, however, there was unusually severe inflammation of lung parenchyma consisting of more florid alveolar exudate and greater thickening of alveolar septa by lymphocytes than were seen in wt mice. At day 14 p.i., this parenchymal inflammation had largely resolved in *PPT-A*$^{-/-}$ mice, with the persistence of perivascular lymphoid infiltrates and small numbers of lymphocytes in thickened alveolar septa. In contrast, in the wt mice at the same time p.i., there were extensive intra-alveolar exudate of both macrophages and lymphocytes and extensive interstitial fibrosis. In addition, there was also evidence of emphysema, indicating some breakdown of architecture. Fibrosis was confirmed by the presence of collagen fibrils when serial sections were stained with Masson trichrome. No such staining was observed in sections derived from *PPT-A*$^{-/-}$ mice.

Like the lung titers, the pathological changes seen in the lungs of wt (CD1) mice were unusual. However, this experiment was performed twice, with consistent results. While the kinetics of the appearance and distribution of infiltration are extremely similar to those seen after the intranasal infection of other strains of mice (e.g., BALB/c and C57BL/6) (15), the appearance of fibrosis and emphysema is atypical, although multiorgan fibrosis has been observed in the context of MHV-68 infection of IFN-γ receptor-deficient mice (6). Taken together with the unusually low level of infectious virus recovered from the lungs, the pathological findings are suggestive of a vigorous host response that is reactive to MHV-68 infection in this strain, resulting in highly effective control of virus replication but also in tissue damage and inappropriate repair (fibrosis). This first report of infection of CD1 mice with MHV-68 indicates that it may be a good mouse model for future studies of the treatment of virus-associated pulmonary fibrosis.

The kinetics of the appearance of reactive inflammatory cells in *PPT-A*$^{-/-}$ mice were far more rapid than those in wt mice, but the control of MHV-68 productive replication was less effective. There could be a number of possible reasons for these findings. First, tachykinins could be involved in a host response that does not involve inflammatory cells (e.g., interferon or apoptosis). In this context, it is interesting to note the role of SP as a regulatory molecule upstream of the well-characterized apoptosis molecules bax and caspase 3 in status epilepticus (14). Second, tachykinins are known to be involved in the recruitment of inflammatory cells (2); this could be a highly effective strategy for the control of MHV-68 infection. Finally, tachykinins could be involved in the effective function of recruited inflammatory cells (e.g., secretion of IFN-γ or tumor necrosis factor alpha). In each instance, the greater and more rapid recruitment of inflammatory cells to the site of infection seen in the *PPT-A*$^{-/-}$ mice could occur to compensate for the deficit.

In spite of the unusually low level of infectious virus in CD1 mice, MHV-68 caused splenomegaly and established latency with an efficiency comparable to that seen in other strains, such as BALB/c and C57BL/6. The factors controlling the number of latently infected cells in the spleens of mice have not been fully delineated. However, while the host response early during infection in the lungs is largely innate (5), a major contributory factor to the response in the spleen is known to be adaptive, i.e., virus antigen-specific CD8$^+$ T cells (7, 28). The delay in the clearance of latently infected cells in *PPT-A*$^{-/-}$ mice relative to wt mice suggests that tachykinins could be involved in this response.

Although other studies have shown that tachykinins are produced in response to virus infection (19, 26), this study is the first to show directly that tachykinins play a role in the host response to control a virus infection. However, these peptides could function in a number of ways and at a number of sites during infection. Since the completion of this study, tachykinin-deficient mice in a 129Sv/Ev background have become available. Since productive infection in 129 mice is more akin to the pattern seen in other strains, these mice may be more amenable for use in future studies of tachykinin function. The delineation of the precise function of these molecules in this context therefore awaits the results of detailed immunological studies using these mice along with NK-1 receptor-deficient transgenic strains.

James P. Stewart and John P. Quinn made equal contributions to the manuscript.

This work was supported by The Royal Society (London), the Biotechnology and Biological Sciences Research Council, and the Medical Research Council.

We thank Alan Basbaum, University of California, San Francisco, for the generous gift of the *PPT-A*$^{-/-}$ mice. J.P.S. is a Royal Society university research fellow.

REFERENCES

1. **Basbaum, A. I.** 1999. Distinct neurochemical features of acute and persistent pain. Proc. Natl. Acad. Sci. USA **96:**7739–7743.
2. **Campos, M. M., and J. B. Calixto.** 2000. Neurokinin mediation of edema and inflammation. Neuropeptides **34:**314–322.
3. **Cao, Y. Q., P. W. Mantyh, E. J. Carlson, A. M. Gillespie, C. J. Epstein, and A. I. Basbaum.** 1998. Primary afferent tachykinins are required to experience moderate to intense pain. Nature **392:**390–394.
4. **Cardin, R. D., J. W. Brooks, S. R. Sarawar, and P. C. Doherty.** 1996. Progressive loss of CD8$^+$ T cell-mediated control of a gamma-herpesvirus in the absence of CD4$^+$ T cells. J. Exp. Med. **184:**863–871.
5. **Dutia, B. M., D. J. Allen, H. Dyson, and A. A. Nash.** 1999. Type I interferons and IRF-1 play a critical role in the control of a gammaherpesvirus infection. Virology **261:**173–179.
6. **Ebrahimi, B., B. M. Dutia, D. G. Brownstein, and A. A. Nash.** 2001. Murine gammaherpesvirus-68 infection causes multi-organ fibrosis and alters leukocyte trafficking in interferon-gamma receptor knockout mice. Am. J. Pathol. **158:**2117–2125.
7. **Ehtisham, S., N. P. Sunil-Chandra, and A. A. Nash.** 1993. Pathogenesis of murine gammaherpesvirus infection in mice deficient in CD4 and CD8 T cells. J. Virol. **67:**5247–5252.
8. **Flano, E., S. M. Husain, J. T. Sample, D. L. Woodland, and M. A. Blackman.** 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J. Immunol. **165:**1074–1081.
9. **Goetzl, E. J., and S. P. Sreedharan.** 1992. Mediators of communication and adaptation in the neuroendocrine and immune systems. FASEB J. **6:**2646–2652.
10. **Ho, W. Z., J. P. Lai, X. H. Zhu, M. Uvaydova, and S. D. Douglas.** 1997. Human monocytes and macrophages express substance P and neurokinin-1 receptor. J. Immunol. **159:**5654–5660.
11. **Lai, J. P., S. D. Douglas, and W. Z. Ho.** 1998. Human lymphocytes express

substance P and its receptor. J. Neuroimmunol. **86:**80–86.
12. **Lambrecht, B. N., P. R. Germonpre, E. G. Everaert, I. Carro-Muino, M. De Veerman, C. de Felipe, S. P. Hunt, K. Thielemans, G. F. Joos, and R. A. Pauwels.** 1999. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. Eur. J. Immunol. **29:**3815–3825.
13. **Lecci, A., S. Giuliani, M. Tramontana, F. Carini, and C. A. Maggi.** 2000. Peripheral actions of tachykinins. Neuropeptides **34:**303–313.
14. **Liu, H., Y. Cao, A. I. Basbaum, A. M. Mazarati, R. Sankar, and C. G. Wasterlain.** 1999. Resistance to excitotoxin-induced seizures and neuronal death in mice lacking the preprotachykinin A gene. Proc. Natl. Acad. Sci. USA **96:**12096–12101.
15. **Macrae, A. I., B. M. Dutia, S. Milligan, D. G. Brownstein, D. J. Allen, J. Mistrikova, A. J. Davison, A. A. Nash, and J. P. Stewart.** 2001. Analysis of a novel strain of murine gammaherpesvirus reveals a genomic locus important for acute pathogenesis. J. Virol. **75:**5315–5327.
16. **Maggi, C. A.** 1997. The effects of tachykinins on inflammatory and immune cells. Regul. Pept. **70:**75–90.
17. **Nash, A. A., B. M. Dutia, J. P. Stewart, and A. J. Davison.** 2001. Natural history of murine gamma-herpesvirus infection. Philos. Trans. R. Soc. London B Biol. Sci. **356:**569–579.
18. **Pascual, D. W., A. M. Stanisz, J. Bienenstock, and K. L. Bost.** 1999. Neuronal intervention in mucosal immunity, p. 631–642. *In* P. L. Ogra, J. Mestscky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee (ed.), Mucosal immunology. Academic Press, San Diego, Calif.
19. **Piedimonte, G., M. M. Rodriguez, K. A. King, S. McLean, and X. Jiang.** 1999. Respiratory syncytial virus upregulates expression of the substance P receptor in rat lungs. Am. J. Physiol. **277:**L831–L840.
20. **Stewart, J. P.** 1999. Of mice and men: murine gammaherpesvirus 68 as a model. EBV Rep. **6:**31–35.
21. **Stewart, J. P., E. J. Usherwood, A. Ross, H. Dyson, and T. Nash.** 1998. Lung epithelial cells are a major site of murine gammaherpesvirus persistence. J. Exp. Med. **187:**1941–1951.
22. **Sunil-Chandra, N. P.** 1991. Studies on the pathogenesis of a murine gammaherpesvirus (MHV-68). Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
23. **Sunil-Chandra, N. P., S. Efstathiou, J. Arno, and A. A. Nash.** 1992. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J. Gen. Virol. **73:**2347–2356.
24. **Sunil-Chandra, N. P., S. Efstathiou, and A. A. Nash.** 1992. Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. J. Gen. Virol. **73:**3275–3279.
25. **Tripp, R. A., A. M. Hamilton-Easton, R. D. Cardin, P. Nguyen, F. G. Behm, D. L. Woodland, P. C. Doherty, and M. A. Blackman.** 1997. Pathogenesis of an infectious mononucleosis-like disease induced by a murine gamma-herpesvirus: role for a viral superantigen? J. Exp. Med. **185:**1641–1650.
26. **Tripp, R. A., D. Moore, J. Winter, and L. J. Anderson.** 2000. Respiratory syncytial virus infection and G and/or SH protein expression contribute to substance P, which mediates inflammation and enhanced pulmonary disease in BALB/c mice. J. Virol. **74:**1614–1622.
27. **Usherwood, E. J., A. J. Ross, D. J. Allen, and A. A. Nash.** 1996. Murine gammaherpesvirus-induced splenomegaly: a critical role for CD4 T cells. J. Gen. Virol. **77:**627–630.
28. **Usherwood, E. J., D. J. Roy, K. Ward, S. L. Surman, B. M. Dutia, M. A. Blackman, J. P. Stewart, and D. L. Woodland.** 2000. Control of gammaherpesvirus latency by latent antigen-specific CD8(+) T cells. J. Exp. Med. **192:**943–952.
29. **Usherwood, E. J., J. P. Stewart, K. Robertson, D. J. Allen, and A. A. Nash.** 1996. Absence of splenic latency in murine gammaherpesvirus 68-infected B cell-deficient mice. J. Gen. Virol. **77:**2819–2825.
30. **van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner.** 2000. Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
31. **Weck, K. E., M. L. Barkon, L. I. Yoo, S. H. Speck, and H. W. Virgin.** 1996. Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68. J. Virol. **70:**6775–6780.