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SELECTIVE MODULATION OF MUCOSAL IMMUNE RESPONSES BY CYTOKINES ENCODED IN VACCINE VECTORS

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SUMMARY

We have developed vector systems encoding vaccine antigens and cytokines and have used these constructs to selectively modulate mucosal antibody and cell-mediated immune (CMI) responses. Information gained from studies in cytokine-deficient mice pointed to the importance of Th2-type factors, particularly IL-4 and IL-6, for the development of mucosal antibody responses, whilst type 1 factors, such as IL-12, would be expected to promote CMI. In this article, we describe the use of three distinct vectors, each constructed to encode cytokine genes along with the gene encoding a model vaccine antigen, the haemagglutinin (HA) of influenza virus, for the delivery of these proteins *in vivo* for mucosal immunomodulation. The vectors included DNA vaccines and poxviral vectors, including vaccinia virus (VV) and fowlpoxvirus (FPV). Each of these systems elicited measurable mucosal antibody responses when delivered locally and these were, in all cases, significantly enhanced by the co-expression of IL-6. In contrast, the mucosal delivery of IL-12 by DNA vaccine had little influence on mucosal antibody responses but stimulated good CMI responses. Indeed, mucosal CMI was not detected unless IL-12 was co-expressed with the vaccine antigen. The combined use of DNA and FPV vectors encoding the same vaccine antigen in a consecutive immunisation strategy gave rise to mucosal antibody responses of greatly enhanced magnitude and duration, even when the DNA priming dose was given by a systemic route. In addition, mucosal delivery of DNA vaccine primed not only for strong local responses but also for specific antibody production at distant mucosal tissues. Finally, the co-expression of IL-6 significantly further enhanced responses stimulated by consecutive immunisation. Thus, vector-driven cytokine delivery represents a powerful approach for the selective modulation of the mucosal immune system.

INTRODUCTION

Cytokines are hormone-like molecules produced by immune cells and play a critical role in intercellular communication, being important determinants of both the type and magnitude of immune responses. T cells which bear the CD4 marker from mice undergoing vigorous immune responses commonly display

one of two major patterns of cytokine synthesis (*Mosmann* and *Coffman*, 1989), and a similar pattern of T cell diversity may exist in humans. The so-called Th1-type cells, whose development is driven by interleukin-12 (IL-12) produced largely by macrophages, secrete interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). Functions mediated by these cells are largely concerned with cytotoxic activity, thus a Th1-based immune response would be most appropriate in defence against viruses and other intracellular pathogens. In contrast, though not exclusively, help for antibody production by B cells is readily provided by another major subset, the Th2-type cells. Cytokines produced by these cells include IL-4, IL-5, IL-6 and IL-10, all of which stimulate B cell growth and differentiation *in vitro*, preferentially inducing antibody responses of the IgG1, IgE and IgA isotypes. In this article, we describe the use of molecular approaches to study the role of cytokines, particularly the Th2-type factors, in the development of immune responses *in vivo*. We also describe our experiments showing that they may act as strong, natural B cell adjuvants capable of modulating antigen-specific antibody responses, particularly at mucosae. Similarly, IL-12 and Th1-type factors are potential adjuvants for cell-mediated immunity. Our approach has been to present these factors as components of delivery vectors, including vaccinia virus, fowlpox virus and DNA vaccines in order to selectively modulate mucosal immunity. We also describe the use of DNA and fowlpox virus vectors in consecutive immunisation strategies which elicit enhanced immune responses, both locally and at distant mucosal sites.

CYTOKINE REGULATION OF MUCOSAL IMMUNE RESPONSES

There is now a plethora of data from *in vitro* and *in vivo* studies supporting a major role for the Th2-type cytokines, IL-4, IL-5 and IL-6, in the development of mucosal IgA reactivity. Th2-type cells occur at high frequency in mucosal tissues (*Taguchi* et al., 1990; *Xu-Amano* et al., 1992), while cells expressing mRNA for Th2 cytokines predominate in the murine small bowel (*Bao* et al., 1993). Message for IL-4 and IL-5 is detected in Peyer's patches and all three factors are abundantly expressed in the lamina propria at sites of IgA production (*Bao* et al., 1993). IL-4 has been shown to promote the production of antibodies of the IgA isotype in murine B cell lines (*Lin* et al., 1991) and has been considered an essential factor for the switching of surface (s)IgM$^+$ mucosal B cells to sIgA expression in both mice (*Ehrhardt* et al., 1992) and humans (*Islam* et al., 1991). Murine IL-5 appears to have no activity on mucosal B cells which do not express sIgA, but promotes the development of activated mucosal sIgA$^+$ B cells *in vitro*. In this respect, IL-5 may act alone (*Beagley* et al., 1988) or in synergy with IL-4 (*Murray* et al., 1987), IL-6 (*Kunimoto* et al., 1989) or transforming growth factor (TGF)-β, (*Coffman* et al., 1989). Thus, IL-5 has been regarded as a terminal differentiation factor rather than a switch factor for sIgA$^+$ B cells, although there is no direct evidence that it normally plays such a role *in vivo*. Interleukin-6 also selectively and potently enhances IgA production *in vitro* by isotype-committed B cells but not sIgA$^-$ B cells (*Beagley* et al., 1989). The presence of T cells, macrophages and other cells known to produce IL-6 (*Fujihashi* et al., 1991; *Mega* et al., 1992). and the high incidence and widespread distribution of

cells containing IL-6 mRNA in intestinal mucosa (*Bao* et al., 1993), are also suggestive of an important role for this factor in regulating IgA responses. On the basis of these and many other published studies, it is thought likely that Th2-type cytokines, particularly IL-5 and IL-6, direct the development of IgA B cells arriving in the submucosa following their exposure to antigen in the organised mucosal lymphoid tissues.

The recent development of strains of mice with targeted disruption of genes encoding IL-4 (*Kopf* et al., 1993), IL-5 (*Kopf* et al., 1996) or IL-6 (*Kopf* et al., 1994) has provided a novel approach to *in vivo* studies of cytokine regulation of mucosal immunity, allowing analysis of the development of immune responses in the absence of these factors. These mice show varying degrees of immune dysfunction, although all appeared to develop normally. The IL-4-deficient mice (IL-4$^{-/-}$) had normal numbers of B cells and T cells and unaltered distribution of typical surface markers, however their ability to produce Th2-type factors with or without antigenic stimulation was severely impaired (*Kopf* et al., 1993). Serum antibody levels of the IgG1 isotype were markedly diminished and IgE antibody was not detected, presumably as a direct result of the IL-4-deficiency. It was concluded that immune responses dependent on Th2-derived cytokines were disrupted in these animals. Interleukin-6$^{-/-}$ mice produced normal levels of cytokines other than IL-6 and had unaltered numbers of B cells but a significant reduction in T cell numbers (*Kopf* et al., 1994). They were unable to control infection with intracellular parasites such as vaccinia virus and *Listeria monocytogenes*. While baseline serum immunoglobulin levels appeared normal in IL-6$^{-/-}$ mice, their ability to mount specific serum IgG antibody responses following virus infection was markedly impaired. Their acute phase responses after tissue damage or infection were also compromised. These mice provided an ideal opportunity to study the relevance of Th2-type factors for mucosal IgA development.

The absence of IL-4 and the resultant down regulation of the Th2 phenotype in IL-4$^{-/-}$ mice suggested that these mutants may be impaired in their ability to produce mucosal IgA antibodies. However, we found that neither IgA antibody levels in lung lavage fluid nor numbers of IgA-secreting cells in the lungs differed in IL-4$^{-/-}$ mice and wild-type mice that had not been deliberately immunised (*Ramsay*, unpublished data). These results demonstrated that the ability of mucosal B cells to undergo isotype switching to IgA production was not dependent on the presence of IL-4. Nevertheless, we also found that IL-4$^{-/-}$ mice had smaller and fewer small intestinal Peyer's patches than wild-type mice and poor germinal centre development, a finding also reported by others (*Vajdy* et al., 1995). IL-4-deficiency also resulted in a marked inability to mount intestinal IgA responses following oral immunisation with soluble proteins in the face of a strong response to cholera toxin given as a component of the inoculum (*Vajdy* et al., 1995). This deficiency may have been due to a failure of IL-4$^{-/-}$ mice to develop the antigen-specific Th2 cells and B cells required to induce germinal centre activity in organised mucosal lymphoid tissues of the gut. We have found that antiviral IgA responses in the lung are also diminished but not ablated in IL-4$^{-/-}$ mice (*Ramsay*, unpublished data). Thus, the importance of IL-4 for the optimal development of Th2-driven immune responses is here illustrated in the context of mucosal immunity. Clearly however, isotype switching and mucosal IgA production is able to occur in the absence of this factor.

In contrast, IL-5$^{-/-}$ mice displayed little defect in systemic antibody or mucosal IgA responses despite the above

Table 1: Interleukin-6 regulates mucosal antibody responses *in vivo*

		Anti-HA antibody-secreting cells per 10^6 cells			
		IgA		IgG	
Mouse strain	Virus	day 8	day 15	day 8	day 15
IL-$6^{-/-}$	VV-HA	5 ± 1	2 ± 1	3 ± 1	6 ± 1
	VV-HA-IL-6	144 ± 15	96 ± 7	159 ± 10	98 ± 3
wild-type	VV-HA	32 ± 6	36 ± 6	15 ± 1	48 ± 3
	VV-HA-IL-6	76 ± 13	29 ± 7	40 ± 2	47 ± 2

Groups of five mice were given 10^7 plaque-forming units (PFU) rVV intranasally and lungs were removed on the days indicated for determination of numbers of HA-specific antibody-secreting cells by ELISPOT assay (*Ramsay* and *Kohonen-Corish*, 1993).

mentioned *in vitro* evidence to the contrary (*Kopf* et al., 1996). IgA B cell numbers in the intestinal lamina propria were similar in unimmunised IL-$5^{-/-}$ and wild-type mice and no significant differences were found in lung and small intestinal IgA responses in IL-$5^{-/-}$ and wild-type mice following local immunisation with recombinant vaccinia virus (rVV) constructs. In addition, specific lung antibody responses following sublethal intranasal infection with influenza virus were not affected by IL-5-deficiency (*Kopf* et al., 1996). Thus, whilst IL-5 is apparently abundantly expressed in mucosal tissues and may be used to stimulate mucosal IgA responses, it does not appear to play a crucial role in IgA production *in vivo*.

Interleukin-6, however appears to play a major role in the optimal development of IgA responses (*Ramsay* et al., 1994). In the absence of deliberate immunisation, IL-$6^{-/-}$ mice had fewer IgA plasma cells in mucosal tissues compared to wild type mice and these cells stained less intensely in the mutants. It is thought that over 40% of murine intestinal IgA cells are not conventional B2 cells, but B1 cells, many of which bear the CD5 marker and populate the gut from the peritoneal cavity rather than mucosal lymphoid tissues (*Pecquet* et al., 1992). Such cells do not require IL-6 for IgA secretion and may, therefore, account for some or all of the residual numbers of $sIgA^+$ cells in IL-$6^{-/-}$ mice (*Beagley* et al., 1995).

IL-6 deficiency also had major effects on the development of mucosal antibody responses to conventional B cell antigens (*Ramsay* et al., 1994). Mutants immunised locally with soluble protein mounted poor intestinal IgA responses. In addition, their production of anti-haemagglutinin (HA) IgA and IgG antibodies and numbers of HA-specific IgA and IgG ASC in lungs following intranasal immunisation with VV encoding HA (VV-HA) were dramatically lower than in wild-type mice. The ability of IL-$6^{-/-}$ mice to mount sustained mucosal antibody responses was dramatically restored when VV-HA-IL-6 was used to reconstitute the expression of IL-6 in the lungs (Table 1). These findings provide compelling evidence that IL-6 plays a major role in the development of mucosal antibody responses to virus infection and, perhaps, conventional B cell antigens in general. Indeed, deficient mucosal IgA responses and, in some cases IgG responses, were found in IL-$6^{-/-}$ mice challenged mucosally with *Candida albicans* (*Romani* et al., 1996), although not when given *Helicobacter*

felis or soluble protein together with the mucosal adjuvant cholera toxin (*Bromander* et al., 1996). IL-6 may be less important for the development of B1 cells, which supply half of the IgA-producing cells in the gut and other tissues and which apparently respond to a different set of antigens than conventional B cells (*Beagley* et al., 1995).

In summary, these studies have confirmed that Th2 factors are important for some but not all of the activities attributed to them in earlier published work. It should be remembered, however, that mutant mice develop in the absence of the factor encoded by the disrupted gene and also that alternative mechanisms may have compensated where no observable effect of a particular cytokine deficiency was observed. It appears that IL-4 is important for the optimal development of at least some mucosal IgA responses and of functional mucosal lymphoid tissues, but is not crucial for IgA isotype switching. IL-6 also seems to play an important role at mucosae, probably in the terminal differentiation of antibody-secreting plasma cells. Conventional mucosal IgA and IgG B cell responses, but not B1 cell development, are impaired in IL-$6^{-/-}$ mice.

Thus, both *in vitro* and *in vivo* studies in several species indicate that cytokines secreted by Th2-type cells play important roles in mucosal B cell development. Such findings suggest that these factors, particularly IL-4 and IL-6, are worthwhile candidates for testing as adjuvants for antibody responses at mucosae. The corollary of this assumption is that type 1 cytokines, including IL-12, may be useful adjuvants for cell-mediated immune responses. Thus, vector-directed expression of different cytokines may represent an effective approach to the selective immunomodulation of the mucosal immune response.

VECTOR DELIVERY OF CYTOKINES FOR MUCOSAL IMMUNOMODULATION

The above mentioned findings that type 2 cytokines are important for the development of B cell responses led us to examine their potential for selective manipulation of vector-driven mucosal immunity. Enhancement of the magnitude and longevity of specific IgG and mucosal IgA responses would have major implications for successful immunoprophylaxis, and to this end, we have established a murine model to test the ability of co-expressed cytokines to modulate immune responses to vaccine antigen. This has involved the construction and testing of a range of replicating and non-replicating vectors which co-express different cytokine genes along with the HA gene of influenza virus A/PR/8/34 as a model vaccine antigen (*Ramshaw* et al., 1992, 1997).

Attempts have been made to modify immune responses by the administration of recombinant cytokines, however this work has been hampered by difficulties in targeting these factors to sites of immune reactivity and by their short half-life *in vivo*. However, virus constructs produce the encoded factor and secrete it from infected cells such that the pattern of virus replication determines the level and sites of production of the cytokine. Using this system, we have studied the immunoregulatory and antiviral properties of a number of factors. Vaccinia and other poxvirus vectors now being developed have the capacity to carry enough heterologous DNA to encode multiple genes, to allow faithful tran-

Table 2: Sustained mucosal antibody responses elicited against vaccine antigen encoded in fowlpoxvirus vectors are enhanced by co-expression of IL-6

		Anti-HA antibody-producing cells / 10^6 lung lymphoid cells	
	Virus	IgG	IgA
Day 14	FPV-HA	5.5	3.0
	FPV-HA-IL-2	5.0	3.2
	FPV-HA-IL-6	38.9	30.6
Day 28	FPV-HA	11.4	9.5
	FPV-HA-IL-2	15.9	10.2
	FPV-HA-IL-6	93.3	15.6

Groups of five mice were given 10^7 PFU rFPV intranasally and lungs were removed on the days indicated for determination of HA-specific ASC numbers by ELISPOT.

scription and translation from these inserted genes and appropriate post-translational processing and transport (*Moss* and *Flexner*, 1987) and, therefore, to facilitate prolonged and enhanced production of desired antigens in host cells. We have expressed HA along with genes encoding cytokines in recombinant vaccinia virus (rVV), fowlpoxvirus (FPV) and DNA vaccine vectors in attempts to enhance mucosal and systemic antibody responses.

Initially we found that type 2 cytokines encoded in rVV vectors may increase mucosal IgA and IgG responses against HA five to ten-fold. Lung immunocytes secreting antibodies specific for the co-expressed HA glycoprotein were significantly enhanced in mice given VV-HA-IL-5 than in those given the control virus (*Ramsay* and *Kohonen-Corish*, 1993). The elevated response peaked on day 14 after infection at 4-fold greater than control levels but had declined by day 28, much later than in controls. Interleukin-5 given in this manner did not appear to affect local IgG responses or systemic reactivity. We also found four-fold increases in secretory IgA ELISA titres in lung fluids of mice given rVV expressing IL-5 by 28 days post-infection, and in those given VV-HA-IL-6 at both 21 and 28 days (*Ramsay*, 1995). Thus, both IL-5 (notwithstanding our findings in IL-5-deficient mice) and IL-6, when expressed in replicating rVV, were effective stimulators of antigen-specific mucosal IgA responses (Table 1).

Fowlpoxvirus is another poxvirus currently being tested as a vaccine vector. This agent has a highly restricted host range, conferring the potential advantage that its replication is blocked in mammalian cells, although heterologous genes under the control of early promoters are expressed, resulting in presentation of the encoded vaccine antigen to the immune system (*Somogyi* et al., 1993). This makes the virus extremely safe but nonetheless highly immunogenic. We have studied the capacity of rFPV to deliver IL-6 as an adjuvant for systemic IgG and mucosal IgA responses and found marked increases in these responses (*Leong* et al., 1994). Strong specific IgA and IgG responses were found in the lungs of mice given intranasal inocula of FPV-HA-IL-6 by 2 weeks after immunisation which were up to ten-fold higher than those detected in mice given control virus or FPV expressing IL-2 (Table 2). Responses at 4 weeks were still elevated in mice given

Table 3: Selective modulation of mucosal antibody and CTL responses by cytokines encoded in DNA vaccines

DNA vaccine	Antibody response in lungs (anti-HA ASC /10^6 cells)		Anti-HA CTL in lungs
	IgA	IgG	(% specific lysis)
pHA	94 ± 15	152 ± 35	<5
pHA + pIL-6	320 ± 47	510 ± 52	<5
pHA + pIL-12	72 ± 19	318 ± 30	32

Mice were immunised twice (day 0, day 21) intra-tracheally with DNA in lipofectin and sacrificed on day 35 following exsanguination of lungs. Lung cell isolates were assayed for specific antibody production (ELISPOT) and CTL (^{51}Cr release assay).

FPV-HA-IL-6, particularly numbers of specific IgG-secreting cells. Reactivity was further elevated when mice were boosted with FPV-HA-IL-6 or challenged with a sublethal dose of homologous wild-type influenza virus (*Leong* et al., 1994), suggesting that vector-driven IL-6 may both prime for enhanced responses as well as stimulating the development of mature IgA- and IgG-producing cells.

DNA plasmids (naked DNA or nucleic acid vaccines) represent novel vectors offering great promise as vaccines. The features of these constructs most relevant for improved vaccination are now well known (*Fynan* et al., 1993; *Pardoll* and *Beckerleg*, 1995; *Ramsay* et al., 1997). Briefly, DNA plasmids are non-replicating, non-infectious and non-integrating and are stable and easier to prepare at lower cost than other vectors or protein immunogens. Multiple genes may be expressed in these constructs and several different routes of inoculation have been shown to be effective (including direct mucosal administration). At least systemically, long-lived immune responses have been generated in many species following DNA immunisation, with the early development of high affinity antibodies. The major characteristic of DNA plasmids of relevance for vaccination and immunomodulation is an ability to persist in the host with sustained presentation of the encoded gene and the resultant potential for sustained immune responses. DNA vaccines have most often been administered via intramuscular injection or particle-mediated ballistic transfer into the dermal layer of the skin (the "gene gun" approach), but have also induced protection in mice when given intranasally prior to an otherwise lethal challenge with influenza virus (*Fynan* et al., 1993). We have confirmed that HA encoded in DNA plasmids elicits sustained serum antibody responses over several months in mice when given via the gene gun or intramuscularly (*Ramsay* et al., 1997). We have also encoded type 2 cytokines in DNA plasmids and observed a clear enhancement of these responses when given in a cocktail along with the HA-encoding DNA vaccine (unpublished data). More recently, we have administered DNA vaccines encoding either IL-6 or IL-12 and have administered these intra-tracheally in combination with DNA encoding HA in attempts to boost mucosal antibody and cell-mediated immune responses, respectively. In order to "target" the DNA constructs for uptake by mucosal cells, they were delivered in a lipid solution, as our previous studies had shown little evidence

Table 4: Mucosal anti-HA antibody responses after consecutive immunisation with DNA and FPV

Immunisation (i.m.)	Boosting (i.n.)	Anti-HA ASC per 10^6 cells (mean ± SD)	
		IgG2a	IgA
Week 1 post-boost			
pCMV/HA	FPV-HA	382 ± 46	195 ± 54
pCMV/HA	FPV-HA-IL6	1,324 ± 93	769 ± 48
pCMV/control	FPV-HA	145 ± 10	60 ± 21
pCMV/control	FPV-HA-IL6	359 ± 111	89 ± 40
Week 3 post-boost			
pCMV/HA	FPV-HA	135 ± 15	282 ± 23
pCMV/HA	FPV-HA-IL6	540 ± 63	793 ± 63
pCMV/control	FPV-HA	<2	<2
pCMV/control	FPV-HA-IL6	63 ± 20	87 ± 18

Mice were given FPV 28 days after priming with DNA and lungs were taken at 1 or 3 weeks thereafter for assessment of anti-HA antibody responses by ELISPOT.

for responsiveness following delivery in saline. Marked increases in mucosal anti-HA IgA and IgG antibody responses were observed in mice given DNA-IL-6 in lipid solution along with DNA-HA, whilst significant levels of specific cytotoxic T cell activity were found in the lungs of those given DNA-IL-12 together with DNA-HA (Table 3).

ENHANCEMENT AND MODULATION OF MUCOSAL IMMUNITY BY CONSECUTIVE VACCINATION WITH DNA AND FPV VECTORS

We have also devised a consecutive immunisation strategy involving intramuscular priming by DNA vaccination and boosting with poxvirus vectors encoding the same vaccine antigens in attempts to generate improved specific immune responses. The viruses used in these studies were rVV and FPV, which we have previously developed as vectors for the induction of long-lasting immune responses to heterologous vaccine antigens (*Ramshaw* et al., 1992; *Leong* et al., 1994). These responses have been enhanced, as described above, by the co-expression of genes encoding cytokines (*Ramshaw* et al., 1992; *Leong* et al., 1994; *Ramsay* et al., 1994). The rationale behind this consecutive vaccination strategy was that DNA immunisation, which elicits low-level but persistent immunity, may prime for greatly enhanced responsiveness following boosting with another persistent vector such as FPV, which expresses somewhat greater levels of vaccine antigen. In addition, immune responsiveness is likely to be directed almost entirely against the encoded vaccine antigens as the vectors themselves, which do not replicate, elicit poor responses.

Initially, mice given an intravenous booster inoculum of rFPV encoding the HA gene of influenza virus (FPV-HA)

Table 5: Priming for both local (lung) and distal (intestinal) mucosal antibody responses following consecutive immunisation with pCMV/HA and FPV-HA

DNA vaccine	Boosting	ASC per 10^6 cells in lung	Ab in faecal pellet (ELISA OD units)
pCMV/HA i.t.	nil	106 ± 16	<0.1
pCMV/HA i.n.	nil	NT	<0.1
pCMV/HA i.t.	FPV-HA i.n.	512 ± 48	0.94 ± 0.25
pCMV/HA i.n.	FPV-HA i.n.	NT	1.22 ± 0.36

Mice were given FPV 21 d after priming with DNA in lipofectin and lungs and faecal pellets were taken for assay 10 d later. ASC were <140 and OD readings were <0.15 units in mice given FPV-HA only. NT = not tested.

four weeks after intramuscular immunisation with DNA vaccine (pCMV/HA) exhibited high levels of anti-HA antibody within one week of boosting. Antibody titres peaked at extremely high levels (over 1 mg/ml, resembling those found in convalescent sera) by 3 weeks post-boosting and were maintained at significant titres for at least 15 weeks. We considered that this approach might offer prospects for improved mucosal vaccination, given that effective mucosal immunity has been notoriously difficult to achieve, particularly via systemic immunisation. Previous reports, however, have indicated that systemic DNA vaccination may, in fact, prime for immune responses at mucosae (*Fynan* et al., 1993; *Ulmer* et al., 1993). We have primed mice with pCMV/HA via the intramuscular route 4 weeks prior to intranasal boosting with FPV-HA in attempts to elicit strong, sustained mucosal responses. Although specific antibody-secreting cells (ASC) were not detected in the lungs of mice given pCMV/HA only, both mucosal anti-HA IgG (particularly IgG2a) and IgA antibody responses were markedly enhanced in DNA-primed animals given FPV-HA and were sustained for at least 3 weeks (Table 4). These augmented responses were further elevated in mice boosted with FPV-HA which co-expressed IL-6 (Table 4). Priming with pCMV/HA via gene-gun immunisation prior to intranasal delivery of FPV-HA was similarly effective for the development of strong mucosal immune responses, with IL-6 co-expression also significantly enhancing the levels of antibody which were secreted (data not shown).

Finally, we have studied the capacity of mucosal priming and boosting in our consecutive immunisation strategy to elicit enhanced mucosal responses both locally and at distant sites, particularly in the light of recent evidence that the intranasal route may more effectively prime for both systemic and distal mucosal antibody and cell-mediated immunity (*Porgador* et al., 1997; *Staats* et al., 1997). Whilst immunisation with DNA vaccine via the intra-tracheal route was superior to intranasal delivery for induction of mucosal antibody responses in the lung (data not shown), neither route elicited significant levels of specific intestinal antibodies (Table 5). However, both routes of immunisation primed for enhanced responses both in the lungs and intestines following intranasal boosting with FPV encoding the same vaccine antigen (Table 5). We are currently investigating the characteristics of this prime-boost strategy which underlie its capacity to elicit immune re-

sponses at distant mucosal sites and the ability of co-expressed cytokines to further enhance local and distal antibody and, perhaps cell-mediated immunity.

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