Modulation of antiviral immune responses by exogenous cytokines: effects of tumour necrosis factor-α, interleukin-1α, interleukin-2 and interferon-γ on the immunogenicity of an inactivated rabies vaccine

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In vivo administration of exogenous cytokines may influence elicited immune responses, and hence may change the efficacy of a vaccine. We investigated the effects of tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α), interleukin-2 (IL-2) and interferon-γ (IFN-γ) on the immune response elicited by inactivated rabies virus vaccine in a mouse model. Each of the cytokines increased virus-specific IgG responses after primary and after secondary immunization. A single dose of 1·3 ng TNF-α or IL-1α, when injected shortly before vaccination, only marginally stimulated resistance to challenge infection (four- and seven-fold, respectively) without enhancing virus neutralizing antibody (VNAb) responses. In contrast, a single injection of 10^3 units of IFN-γ or five daily injections of 1·6 μg IL-2 increased vaccine dilutions protecting 50% of mice (PD_{50} values) 77- to 50-fold, respectively, with a concomitant enhancement of VNAb. At a 1:10000 dilution of a standard inactivated rabies vaccine preparation both IFN-γ and IL-2 increased protective immunity without enhancing VNAb responses; in non-vaccinated animals this treatment had no effect on resistance to challenge. Combined administration of IFN-γ and IL-2 synergistically enhanced VNAb responses. In contrast to the other cytokines tested, IFN-γ preferentially stimulated virus-specific IgG2a production. It also augmented the vaccine-induced priming of rabies virus-specific splenocyte proliferation. These results document that certain cytokines alone or in combination are potent immunological adjuvants which may direct and modulate immunization-induced antiviral immune responses.

Introduction

Antiviral protection by immunization is established after a cascade of cellular interactions in which soluble mediators collectively termed cytokines are involved. Certain macrophage-derived cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor (TNF) and interferon (IFN) play a critical role in the early activation of accessory cells and in the subsequent co-stimulation of helper and effector lymphoid cells (Unanue & Allen, 1987; Weaver & Unanue, 1990). The secreted cytokine profile of antigen-stimulated T helper cells largely determines the nature of the immune response (Janeway *et al.*, 1988; Mosmann & Coffman, 1989). The factors influencing the distinct lymphokine gene expression in antigen-specific T helper cells are less well understood but appear to depend upon the type and dose of antigen (Parish, 1972; Carding *et al.*, 1989), the lymphoid microenvironment where helper cell activation occurs (Daynes *et al.*, 1990) and the local cytokine profile at the onset of antigen recognition (Seder *et al.*, 1992; Locksley *et al.*, 1991). The latter may follow initial non-specific immune responses including IFN production, activation of natural killer cells or triggering of mast cells and basophils (Romagnani, 1992). Administration of exogenous cytokines may therefore represent a means of improving the magnitude and nature of immune responses elicited by vaccines.

Improvement of the immunogenicity of a vaccine or model antigen has been demonstrated in several systems when exogenous cytokines were administered during the immunization period, for example IL-1 (Staruch & Wood, 1983), TNF (Ghiara *et al.*, 1987), IL-2 (Nunberg *et al.*, 1989), IFN-γ (Playfair & De Souza, 1987; Finkelman *et al.*, 1988; Heath *et al.*, 1991) and IL-6 (Takatsuki *et al.*, 1988). Moreover, the action of

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traditional adjuvants like bacterial lipopolysaccharide, Freund's complete adjuvant, $Al(OH)_3$ (Mannhalter *et al.*, 1985) and muramyl dipeptides (Bahr *et al.*, 1987; Oppenheim *et al.*, 1980) is at least partly based on the induction of cytokine secretion (Tomai & Johnson, 1982; Odean *et al.*, 1990). Thus, particular cytokines either administered or induced, may selectively stimulate or inhibit immune reactions. These phenomena may have direct implications for immunization strategies, because pathogens may be eliminated by one effector mechanism but may escape or cause exacerbated reactions during others (Mosmann & Coffman, 1989). Furthermore, studies in this field may contribute to the understanding of the *in vivo* immunoregulatory activities of cytokines.

In this study we investigated the effects of putative stimulatory cytokines, including TNF-α, IL-1α, IL-2 and IFN-γ and of a combination of IL-2 and IFN-γ on the immunogenicity of an inactivated rabies vaccine. For potency assessment we used an *in vivo* protection test, recommended by the WHO for the assessment of human rabies vaccines. Induction of protective immunity by rabies vaccines has largely been attributed to virus neutralizing antibody (VNAb) (Kaplan *et al.*, 1975; Turner, 1976; Wunderli *et al.*, 1991). However, Dietzschold and coworkers (1992) have suggested that it is not solely correlated with neutralizing activity of antibodies but also with their ability to prevent virus spread between cells and to inhibit transcription of viral RNA. Other investigators have demonstrated the induction of rabies virus-specific cytotoxic T lymphocytes (CTLs) by this type of vaccine (Wiktor *et al.*, 1977; Morgeaux *et al.*, 1989; Sugamata *et al.*, 1990), which are not usually primed by non-replicating vaccines. Here we demonstrate that each of the cytokines tested enhance the antiviral immunity induced by an inactivated rabies vaccine to various degrees. IFN-γ, which was shown to enhance the production of antiviral IgG2a, proved to be the most potent and suitable for practical use.

Methods

Mice. Outbred NIH (RIVM:NIH) mice and inbred C57BL/6 (H-2^b) mice were obtained from the National Institute of Health and Environmental Protection and used at 4 to 5 weeks of age. The mice were kept in barrier-contained animal facilities and both sexes were used. The experimental protocols were approved by the Animals Welfare Officer of the National Institute of Public Health and Environmental Protection.

Viruses. The Pitman-Moore (PM) strain of rabies virus (PM/w1-38-1503-3m) was propagated in dog kidney cells (PM-DKCV). The challenge virus standard (CVS) strain of rabies virus was propagated in BHK-21 cells and purified by gradient centrifugation.

Vaccine. The inactivated rabies vaccine (batch R41-A) had been prepared from a concentrated, purified PM virus suspension by inactivation with β-propiolactone according to standard methods (van Wezel *et al.*, 1987). It contained about 200 µg protein per 1 ml dose, of which about 50 µg proved to be glycoprotein as determined by ELISA (Osterhaus *et al.*, 1990).

Cytokines. Recombinant (r) human IL-1α [3×10^8 units (U)/mg protein] and murine rTNF-α (7×10^7 U/mg protein) were diluted in PBS directly before use and injected intraperitoneally (i.p.) 15 min before vaccination on days 0 and 7, at a concentration of 1·3 ng/mouse (i.e. 4×10^2 U IL-1/mouse and 10^2 lytic U TNF/mouse). Human rIL-2 (Proleukin, EuroCetus, 3×10^6 Cetus U/mg, equivalent to $1{\cdot}8 \times 10^7$ International U/mg protein) was reconstituted in sterile water, diluted in PBS and injected i.p. daily on days 0 to 4 and 7 to 11 at a concentration of 1·6 µg/mouse (i.e. 3×10^4 U/mouse). The rat rIFN-γ (4×10^6 U/mg protein), which displays biological activity in both rats and mice, had been produced in Chinese hamster ovary cells and was purified by monoclonal antibody affinity chromatography as previously described (van der Meide *et al.*, 1986). Preparations were assayed in a vesicular stomatitis virus cytopathic effect inhibition assay on rat embryo fibroblasts, and their activity is expressed in U standardized against a laboratory reference. In most experiments 10^3 U IFN-γ/mouse (i.e. 0·25 µg/mouse) was injected i.p. at the indicated times.

Immunizations. Groups of eight to 20 mice were immunized with two injections of an R41-A vaccine dilution (0·25 ml) via the i.p. route on days 0 and 7. On day 14 the animals were challenged by intracerebral (i.c.) injection with 30 µl of CVS-26 rabies virus containing approximately 30 LD_{50}. In the subsequent period the animals were observed for the development of rabies-related symptoms including paralysis. Mortality in the non-vaccinated control animals generally occurred between 7 and 14 days after infection. Other groups of mice received the vaccine in conjunction with cytokine treatment. Titration of the CVS challenge virus was included in each experiment; this is mandatory since reproducibility of LD_{50} values is poor, a problem generally recognized in vaccine potency testing. Therefore only data from the same experiment were compared.

Antibody responses. On days 6 and 13, mice were anaesthetized and bled from the retro-orbital plexus. In individual serum samples rabies virus-specific Ig titres were determined by ELISA and VNAb titres were measured by a rapid fluorescence focus inhibition test (Zalan *et al.*, 1979). The ELISA titre is defined as the reciprocal of the highest serum dilution at which the absorbance was equal to three times the background value.

Proliferative splenocyte responses. C57BL/6 mouse splenocytes isolated 2 weeks after the last immunization were cultured in 96-well round-bottom plates at a density of 3×10^5 cells per well in Iscove's modified Dulbecco's medium containing 1% C57BL/6 mouse serum. Inactivated rabies virus was added at the concentrations indicated in Fig. 3. After 4 days of incubation at 37 °C in a humidified atmosphere containing 5% CO_2, cultures were pulse-labelled with 0·5 µCi [^{3}H]thymidine (Amersham) for 16 h. Cells were harvested on glass filters and the incorporated radioactivity was measured in a Betaplate flat bed scintillation counter (LKB-Wallac).

Statistical analysis. To compare group means of VNAb titres, data were evaluated using the Wilcoxon-Mann-Whitney test. In order to take into account the greater probability of a type I error due to multiple comparisons, the level of significance was pre-set according to Bonferroni's adaptation at $P <$ [0·05/number of comparisons] instead of a fixed $P < 0{\cdot}05$. Group means of splenocyte proliferative responses and Ig isotype ratios of PBS-treated vaccine controls and IFN-γ-treated mice were compared by using the two-tailed Student's t-test. The 50% protective vaccine doses (PD_{50}) were calculated according to Reed & Muench (1938).

Results

Exogenous cytokines augment immunization-induced protective antiviral immunity

Two immunizations of mice with PM-DKCV (dilutions 1:100, 1:1000 or 1:10000) resulted in a dose-dependent resistance to challenge. At the highest vaccine concentration (dilution 1:100) TNF-α and IL-1α improved survival upon challenge, resulting in high (90%) or complete survival, respectively, compared to 40% survival of mock-vaccinated mice (Fig. 1*a*). At a vaccine dilution of 1:1000, TNF-α treatment had no effect on survival, whereas IL-1α treatment caused a prolongation of survival times, with a mean time to death of 9·6 days as compared to 8·3 days in the control group (data not shown). No increase in numbers of long-term survivors was observed (Fig. 1*a*). At the 1:10000 vaccine dilution TNF-α and IL-1α did not influence resistance to challenge.

In contrast, IFN-γ and IL-2 dramatically increased survival upon challenge at all the vaccine concentrations tested (Fig. 1*a*). This effect was still observed in groups of mice injected with a non-protective vaccine dilution of 1:10000. Co-administration of IFN-γ or IL-2 induced 45 and 60% survival, respectively. IFN-γ or IL-2 treatment in non-immunized animals had no effect (data not shown). When compared to the PBS-treated vaccine control group, PD_{50} values were increased 4·2-fold by TNF ($PD_{50} = 10^{2.6}$), 6·8-fold by IL-1 ($PD_{50} = 10^{2.8}$), 50-fold by IL-2 ($PD_{50} = 10^{3.7}$) and 30-fold by IFN-γ ($PD_{50} = 10^{3.5}$); PD_{50} of PBS = 10^2.

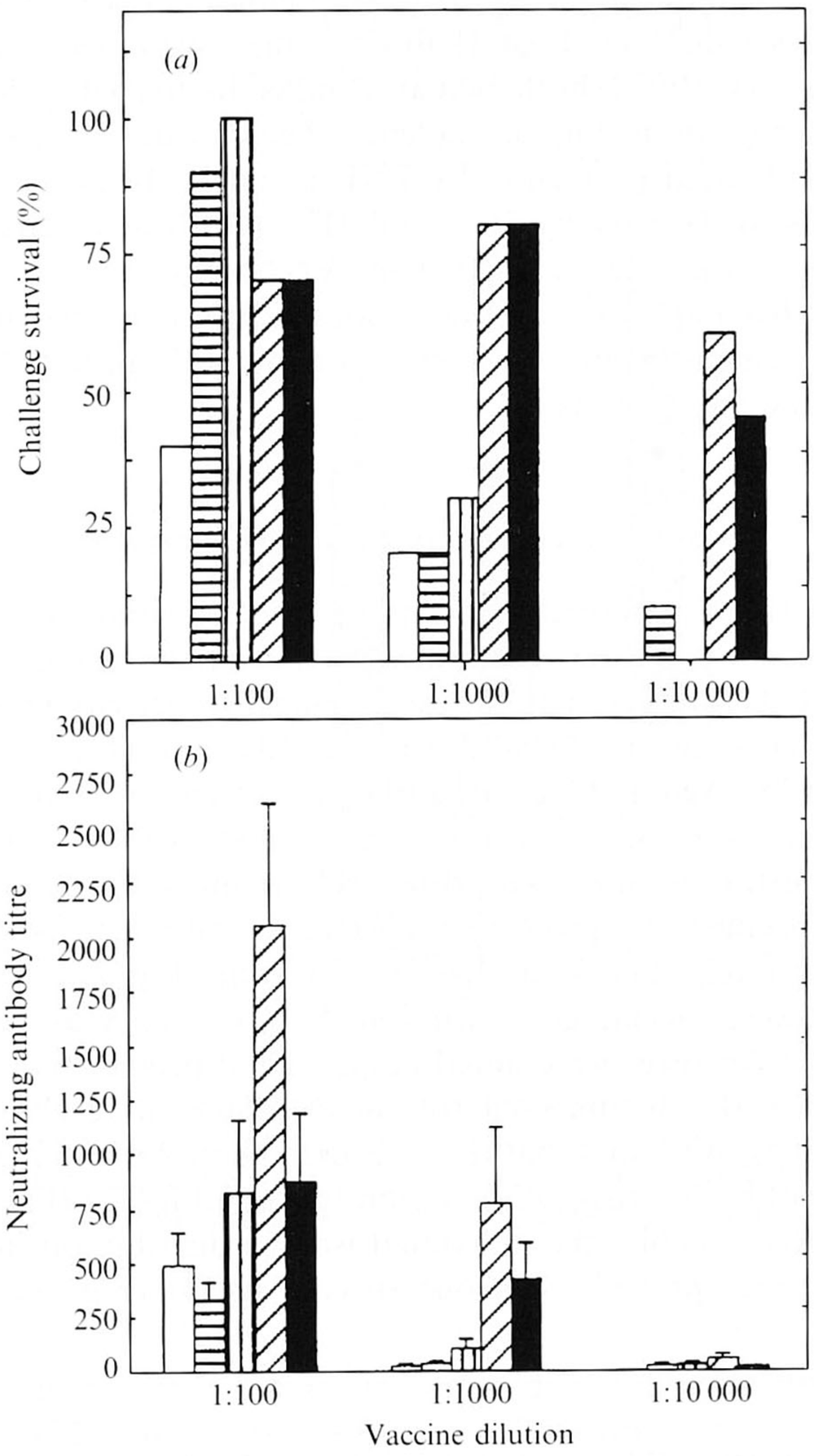


Fig. 1. Effect of TNF-α, IL-1α, IL-2 and IFN-γ on the protective activity (*a*) and VNAb responses (*b*) induced by inactivated rabies virus. NIH mice (10 per group) were immunized i.p. with the indicated dilutions of inactivated rabies virus vaccine (R41-A) at days 0 and 7; 15 min before each immunization the animals were injected i.p. with murine rTNF-α (▤) (1·3 ng/mouse), human rIL-1α (▥) (1·3 ng/mouse), rat rIFN-γ (■) (10^3 U = 0·25 μg/mouse) or PBS (□). Another group of mice received human rIL-2 (▨) (3×10^4 U = 1·6 μg/mouse) administered daily on days 0 to 4 and days 7 to 11. The animals were bled on day 13 and challenged i.c. with CVS-26 (34 LD_{50}) on day 14. VNAb titres (mean values ± S.E.M.) were determined for five individual mice per group.

Table 1. *Effect of cytokines on induction of rabies virus-specific serum IgG**

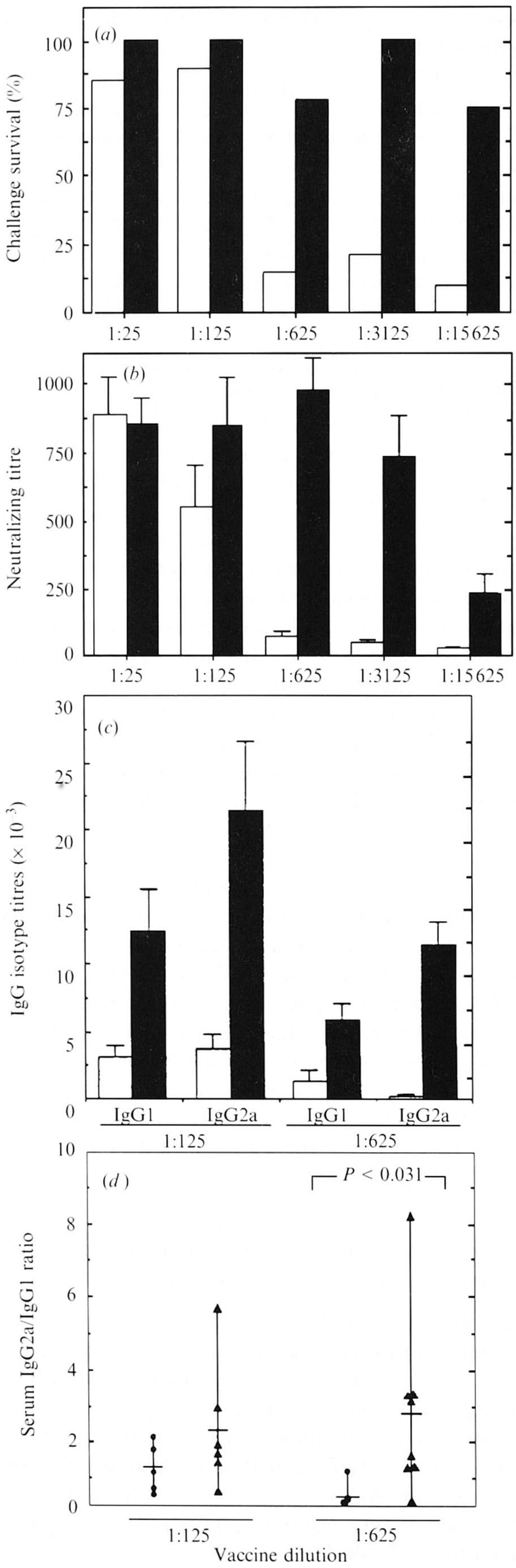
	Virus-specific serum IgG†			
	Vaccine dilution 1:100		Vaccine dilution 1:1000	
Treatment	Day 6	Day 13	Day 6	Day 13
PBS	45 ± 28	732 ± 150	< 10	167 ± 93
TNF-α	192 ± 62	3560 ± 1149	18 ± 7	270 ± 140
IL-1α	208 ± 68	8060 ± 3089	28 ± 11	1332 ± 518
IL-2	560 ± 93	8680 ± 1819	92 ± 44	3380 ± 655
IFN-γ	398 ± 166	3080 ± 773	86 ± 30	1364 ± 580

* NIH mice (five per group) were i.p. immunized with a 1:100 or a 1:1000 vaccine dilution at day 0 and boosted with the same vaccine dose at day 7. The animals were injected with PBS or different cytokines as indicated in the legend of Fig. 1.

† The animals were bled on days 6 and 13. Geometric means (± S.E.M.) of anti-rabies virus IgG titres were determined as previously described (Zalan *et al.*, 1979).

Exogenous cytokines cause an increase in antiviral IgG responses after primary and secondary immunization

The levels of the humoral immune response were determined by measuring antibody titres in sera collected from immunized mice at days 6 and 13, 6 days after primary and secondary immunization, respectively. All cytokines tested augmented rabies virus-specific IgG levels four- to 12-fold within 6 days after primary immunization. At day 13 a five- to 10-fold increase in specific serum IgG response was noted in all cytokine-treated mice (Table 1).



IL-2 and IFN-γ enhance virus-neutralizing antibody responses

VNAb titres were determined in day 13 sera of vaccinated mice. In vaccine control mice the VNAb titres decreased with a lowering of the vaccine dose, which correlated well with survival upon challenge. Neither TNF-α nor IL-1α influenced the VNAb response at any vaccine concentration tested, although both enhanced protective immunity at the 1:100 vaccine dilution (Fig. 1*a*).

At the 1:100 and 1:1000 vaccine dilutions IL-2 and IFN-γ significantly ($P < 0{\cdot}0125$) enhanced VNAb responses (Fig. 1*b*). Remarkably, at the lowest vaccine dilution (1:10000) both mediators failed to do so despite increasing survival upon challenge. These results suggest that enhanced protection by TNF-α and IL-1α at high vaccine doses and by IL-2 and IFN-γ at low vaccine doses is not solely mediated by VNAb but may also result from antibody that does not neutralize *in vitro* or from enhancement of other, probably cell-mediated, immune mechanisms.

INF-γ causes an increase in antiviral IgG2a production

Since IFN-γ emerged as the most practical immunity-enhancing cytokine, requiring only a single injection shortly before vaccination, we examined its activity in a broader range of fivefold vaccine dilutions (1:25 to 1:15625). Again, it increased PD_{50} values 25- to 77-fold. At all vaccine concentrations tested IFN-γ co-administration increased protective immunity (Fig. 2*a*). Enhancement of protection correlated with increased VNAb levels at each vaccine concentration (Fig. 2*b*). At the lowest vaccine concentration, however, once again low VNAb titres were noted despite good protection.

Given the documented role of cytokines in Ig class switching, we found that IFN-γ caused an increase in the levels of both virus-specific serum IgG1 and IgG2a (Fig. 2*c*). Remarkably, the individual isotype distribution in these mice proved to favour specific IgG2a (Fig. 2*d*)

Fig. 2. Effect of IFN-γ on protective immunity (*a*), development of rabies VNAb (*b*) and antiviral IgG1 and IgG2a (*c* and *d*). NIH mice (10 per group) were immunized i.p. with the indicated dilutions of inactivated rabies virus vaccine (R41-A) at days 0 and 7; two hours before each immunization the animals were injected i.p. with rat rIFN-γ (■) (10^3 U = 0·25 μg/mouse) or mock-injected (□). The animals were bled on day 13 and challenged i.c. on day 14 with CVS-26 (10 LD_{50}). (*a*) Percentage of long-term challenge survivors. (*b*) VNAb titres (mean values ± S.E.M.) determined for eight individual mice per group. (*c*) Antiviral IgG1 and IgG2a serum antibody (mean ± S.E.M) determined for four to eight mice per group. (*d*) Individual IgG2a/IgG1 ratios of antiviral antibody of four to eight mice per group. No IFN-γ (●); IFN-γ (▲).

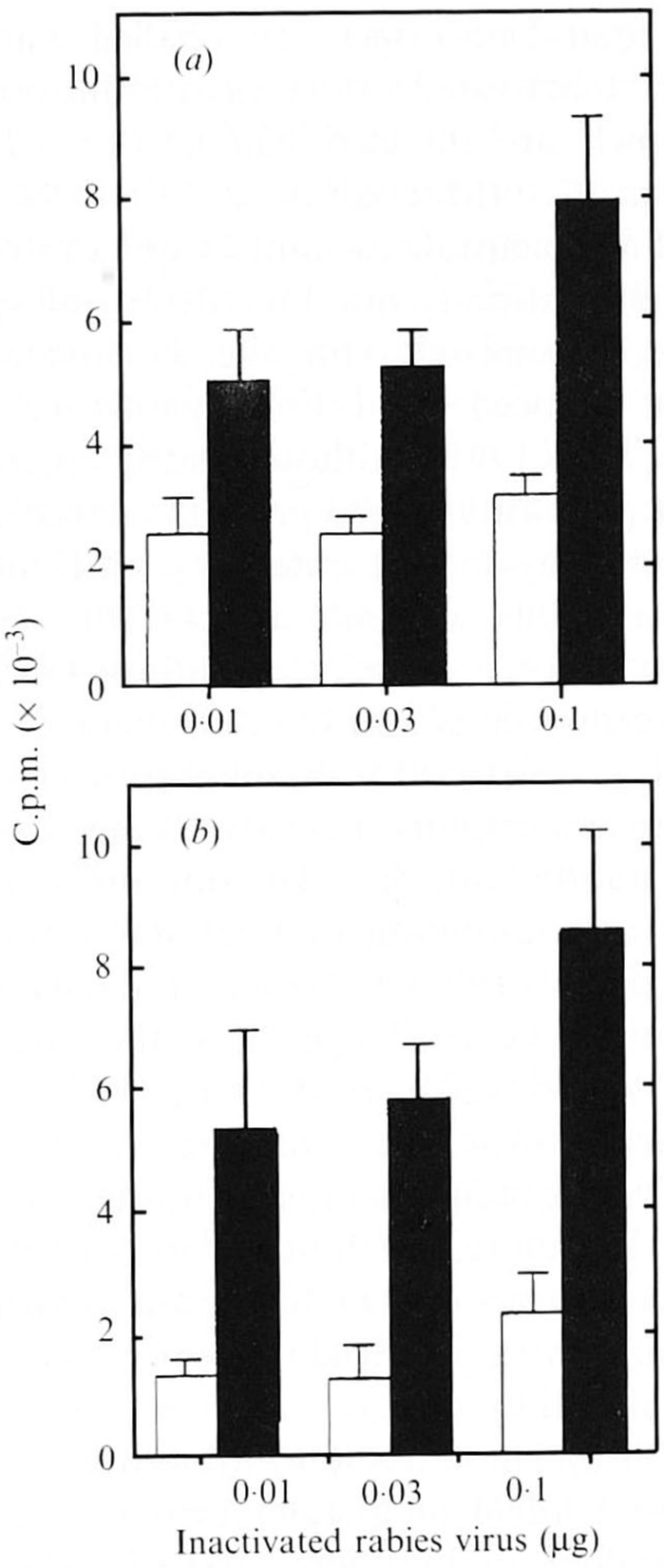


Fig. 3. Effect of *in vivo* IFN-γ administration on *in vitro* proliferative responses of rabies virus-specific splenocytes. C57BL/6 mice (two per group) were given 10^3 U rat rIFN-γ (■) and immunized 2 h later with a 1:100 (*a*) or 1:1000 (*b*) dilution of the inactivated rabies virus vaccine R41-A, on days 0 and 7; all inoculations were via the i.p. route; PBS was used as a control (□). Spleen cells isolated on day 21 were stimulated with different concentrations of inactivated rabies virus antigen. Proliferative response was measured by the incorporation of [^{3}H]thymidine. Results are shown as the net mean ± S.E.M. [response − background (medium)] of quadruplicate values obtained from two mice per group.

whereas the other cytokines did not alter specific IgG2a/IgG1 subclass ratios (not shown).

IFN-γ enhances the priming of rabies virus-specific splenocyte proliferative responses

To determine the effect of *in vivo* administration of IFN-γ on cell-mediated immune reactivity, *in vitro* lymphocyte stimulation tests were performed. To this end, spleen cells isolated 2 weeks after the last immunization were stimulated with PM-DKCV. Fig. 3 shows that *in vivo* IFN-γ administration induced a significant enhancement

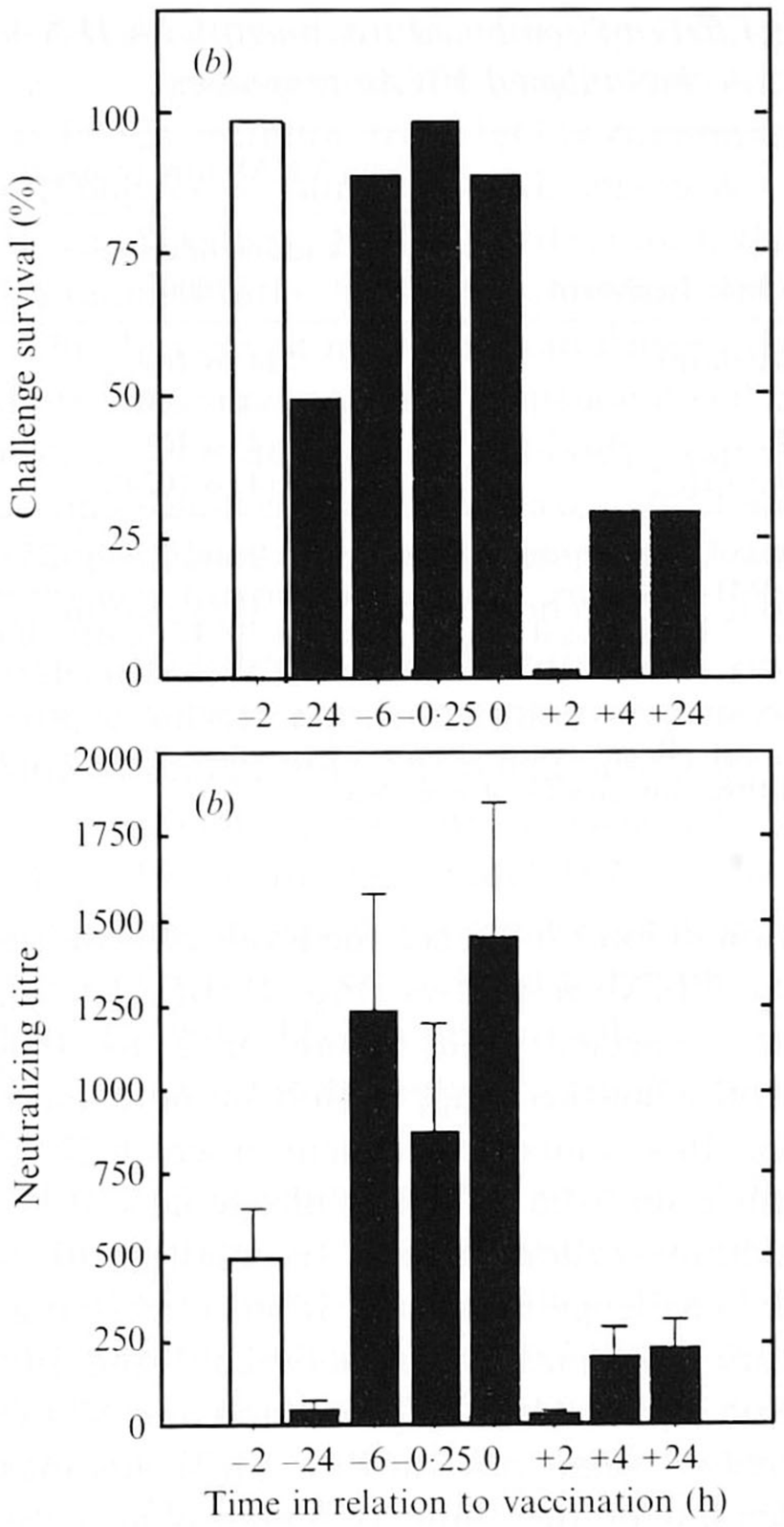


Fig. 4. Effect of the time of IFN-γ administration on inactivated rabies virus vaccine (R41-A)-induced challenge protection (*a*) and VNAb responses (*b*). NIH mice (10 per group) were immunized i.p. with a 1:1000 vaccine dilution on days 0 and 7 and i.p. injected with rat rIFN-γ (■) (10^3 U = 0·25 μg/mouse) at different times before or after immunization as indicated; PBS was used as a control (□). The animals were bled on day 13 and challenged i.c. with 128 LD_{50} CVS-26 on day 14. (*a*) Percentage of long-term challenge survivors. (*b*) VNAb titres (mean values ± S.E.M.) determined for five individual mice per group.

of the proliferative responses ranging between two- and 3·7-fold ($P < 0·04$), depending upon the concentration of PM-DKCV. These findings demonstrate an IFN-γ-mediated enhancement of the *in vivo* priming of splenocyte responses, which may have contributed to the observed augmentation of protective immunity.

IFN-γ administration post-immunization causes a decrease in protective immunity and VNAb responses

The time of IFN-γ injection proved to play a critical role. Under conditions where the vaccine alone induced complete resistance against a low challenge dose (128 LD_{50}), IFN-γ injection in the 6 h interval before

Table 2. *Effect of combined treatment with IFN-γ and IL-2 on vaccine-induced VNAb responses**

Cytokine treatment	Mean VNAb titre (range)† Vaccine dilution 1:10000
PBS control	7 (4 to 16)
IFN-γ	22 (4 to 64)
IL-2	9 (4 to 16)
IFN-γ + IL-2	417 (4 to 1024)

* Groups of mice, i.p. immunized on days 0 and 7 with 0·25 ml of the indicated R41-A vaccine dilution, were treated with either IFN-γ (10^3 U, at −2 h on days 0 and 7), IL-2 (3×10^4 U on days 0 to 4 and days 7 to 11), or both IFN-γ and IL-2, and bled on day 13.

† VNAb titres were determined in a rapid fluorescence focus inhibition test. Results are shown as geometric mean (range) of individual titres for groups of five mice.

vaccination did not influence the levels of protection but stimulated VNAb responses (Fig. 4). In contrast, when IFN-γ was injected 24 h before or 2 to 48 h after vaccination a marked suppression of both parameters was noted. In a similar experiment in which 20% of the control mice survived a high challenge dose (617 LD_{50}), IFN-γ administration before vaccination (0 to 2 h) stimulated challenge resistance. In mice receiving IFN-γ 24 h before immunization or between 2 and 24 h after vaccination, again both the protection and VNAb titres had decreased (data not shown). These findings show that depending on the timing of lymphokine administration, either a stimulation or an inhibition of immune responsiveness could be observed.

Combined treatment with IL-2 and IFN-γ further increases VNAb responses

Since both IL-2 and IFN-γ enhanced vaccine efficacy it was of interest to examine whether they would act additively or synergistically. Combined IFN-γ and IL-2 treatment did not further enhance resistance to challenge as compared with immunization in conjunction with IL-2 only. Only a 3-day increase in survival times was noted under the conditions tested (data not shown). At a low vaccine concentration (dilution 1:10000) neither IFN-γ nor IL-2 alone affected antibody responses. The combined cytokine treatment, however, synergistically enhanced the development of VNAb (Table 2).

Discussion

In this study, we demonstrate that co-injection of each of the cytokines TNF-α, IL-1α, IL-2 and IFN-γ with inactivated rabies vaccine increases the induced protective antiviral immunity. The mechanisms underlying protection against i.c. rabies virus challenge are not fully understood. Inactivated rabies virus induces circulating VNAbs which are directed against the viral surface glycoprotein (G) (MacFarlan *et al.*, 1986). Neither injection of non-neutralizing anti-G and anti-rabies virus nucleoprotein antibody nor inoculation of spleen cells from mice immunized with the inactivated vaccine preparation induced protection against i.c. challenge (Wunderli *et al.*, 1991). Although antigens from inactivated virus preparations are probably processed by the endocytic pathway and presented by class II molecules to $CD4^+$ helper T cells, we could detect $CD8^+$ CTL activity in vaccinated mice, as have other authors (Wiktor *et al.*, 1977; Morgeaux *et al.*, 1989; Sugamata *et al.*, 1990; MacFarlan *et al.*, 1986). Immunoprotection against rabies encephalomyelitis has also been suggested to result from antibody-mediated disruption of viral cell-to-cell spread and inhibition of viral RNA transcription (Dietzschold *et al.*, 1992). Destruction of the blood–brain barrier following i.c. challenge allows the inoculum to be distributed to the periphery (Cairns, 1950). Part of the i.c. inoculated virus may activate vaccine-sensitized peripheral T cells before being neutralized by circulating antibodies. Therefore, the contribution of cell-mediated immune mechanisms in the elimination of virus-infected cells in the central nervous system should also be considered in our system.

The establishment of immunization-induced immunity is under the control of distinct cytokine cascades. In mice, physiological cytokine production reflects the expansion of functionally distinct T helper cell subsets: Th-1 cells produce TNF, IL-2 and IFN-γ and give rise to cellular reactions, whereas Th-2 cells produce IL-4, IL-5, IL-6 and IL-10 and lead to antibody production. The subset-specific cytokines stimulate expansion of their own subset while inhibiting the other (Fiorentino *et al.*, 1989); activation of Th-1 or Th-2 subsets is often mutually exclusive. Thus, administration of particular cytokines during or shortly before the immunization period may be expected to influence the physiological cytokine balance and may inhibit or stimulate antigen-specific immune reactions.

In immunized control mice (no cytokine added) we found a correlation between protection and induction of VNAb, as reported before by Wunderli and co-workers (1991). In mice immunized in conjunction with a cytokine however, discrepancies between VNAb titres and protection were noted. Both TNF-α and IL-1α, when given shortly before the vaccination, stimulated protection at the highest vaccine concentration (1:100 dilution) without increasing VNAb titres. In contrast, both IL-2 and IFN-γ stimulated protective immunity which correlated well with increased VNAb responses. Notably, at the lowest vaccine concentrations tested

(1:10000 and 1:15625 dilution), which gave minimal or no protection, both cytokines prolonged survival in the absence of increased VNAb titres (Fig. 1 and 2). These data suggest that other protective immune mechanisms are stimulated. These are antigen-dependent and specific, since injection of the cytokines alone did not confer protection. Indeed, virus-specific IgG responses were augmented four- to 12-fold as early as 6 days after primary immunization and five- to 10-fold after secondary immunization in cytokine-treated mice. These non-neutralizing antibodies may be expected to contribute to protective immunity.

The mechanism of the *in vivo* cytokine actions is difficult to define because of their numerous and complex activities. The effects resulting from early cytokine administration demonstrate a modulatory activity at the beginning of the immune response. Indeed, the commitment to a Th-1 or Th-2 response appears to occur very soon after antigen exposure (Scott, 1991). Although inactivated rabies virus by itself can stimulate secretion of IFN-γ (Celis *et al.*, 1986) and IL-2 (Ertl *et al.*, 1989) by T helper cells *in vitro*, it is unknown whether *in vivo* Th-2-characteristic lymphokine secretion is stimulated as well and eventually to a larger extent. Since the PM-DKCV induces both VNAb responses and cell-mediated immunity, including CTL priming and delayed type hypersensitivity (not shown), it is difficult to speculate about induction of either T helper subset.

In contrast to the moderate effects of TNF-α and IL-1α, the Th-1-derived lymphokines IFN-γ and IL-2 vigorously stimulated vaccine potency and VNAb responses at high vaccine doses. IL-2, given daily over a 5-day period, probably acts by stimulating the proliferation of antigen-reactive T cells. IL-2 is the principal lymphokine produced by naive helper T cells (Swain *et al.*, 1991); it acts as a growth factor for both Th-1 and Th-2 cells (Williams *et al.*, 1991), and has a direct role in the induction of B cells (Zubler *et al.*, 1984). Our IL-2 data are consistent with the findings of Nunberg and coworkers (1989) who suggested that IL-2 acts by increasing the cellular immune response to the rabies vaccine. However, because of the requirement for repeated injections the use of IL-2 as an immunological adjuvant is not practical for routine pre-exposure vaccination protocols.

In contrast, IFN-γ is a practical and potent modulator. It is the key regulatory Th-1 lymphokine which favours Th-1-type responses and antagonizes Th-2 reactions. Recent studies in murine *Leishmania* infection models have demonstrated that a single injection of IFN-γ, when included in the parasite inoculum, enhanced Th-1 and decreased Th-2 responses in BALB/c mice (Scott, 1991). Early injection in our system provided enhanced challenge protection, increased VNAb responses and augmented the priming of rabies virus-reactive splenocyte proliferation. In the mouse, the lymphokine is known to promote IgG2a secretion from IgG2a-committed B cells *in vitro* (Snapper & Paul, 1987; Mosmann & Coffman, 1989; Bossie & Vitetta, 1991). Our data show that *in vivo* IFN-γ administration facilitates antiviral IgG2a synthesis. This Ig isotype mediates complement-dependent virus destruction and antibody-dependent cell-mediated cytotoxicity. The augmented antibody responses and elimination of infected cells may have contributed to the increased protective immunity observed. If we assume that physiological IFN-γ production requires a few hours to become established, our finding that post-vaccination IFN-γ injection decreases immune responsiveness is surprising. A similar time-dependent inhibitory effect has been noted with an experimental malaria vaccine (Heath & Playfair, 1991). In this model IFN-γ adjuvanticity correlated with increased expression of MHC class II molecules but was inversely related to lymphocyte influx (Heath *et al.*, 1991). The stimulatory effect of IFN-γ may therefore also result from improved antigen presentation or uptake.

Th-1-like responses are believed to play a major role in the control of most acute virus infections (Mosmann & Coffman, 1989). However, immunization-induced Th-2 reactions resulting in high antibody titres may protect by immediate neutralization of the viral inoculum. Since combined treatment with IFN-γ and repeated daily injections of IL-2 further stimulated VNAb production this regimen may be especially valuable to increase the efficacy of vaccination by preventing systemic spread in those infections which depend upon viraemia during pathogenesis, which is not the case for rabies virus infections.

Previous studies have demonstrated the beneficial effects of type I IFN injection in mice and monkeys previously infected with rabies virus at the same site (Harmon *et al.*, 1975; Baer *et al.*, 1977). Further studies to evaluate whether the observed cytokine modulation also improves systemic post-exposure immunization in rabies infection are in progress. If so, this application would be of major importance for the protection of humans after accidental exposure.

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