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Published in:
Journal of General Virology

Publication status and date:
Published: 01/04/2004

DOI (link to publisher):
10.1099/vir.0.19605-0

Document Version
Publisher's PDF, also known as Version of record

Citation for the published version (APA):
de Wit, M. C., Horzinek, M. C., Haagmans, B. L., & Schijns, V. E. J. C. (2004). Host-dependent type 1 cytokine responses driven by inactivated viruses may fail to default in the absence of IL-12 or IFN-α/β. *Journal of General Virology*, *85*(4), 795-803. https://doi.org/10.1099/vir.0.19605-0

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Erasmus University Rotterdam
Making Minds Matter

Host-dependent type 1 cytokine responses driven by inactivated viruses may fail to default in the absence of IL-12 or IFN-α/β

Marel C. de Wit,[1]† Marian C. Horzinek,[1] Bart L. Haagmans[2] and Virgil E. J. C. Schijns[3]

[1]Virology Unit, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, 3584 CL Utrecht, The Netherlands

[2]Institute of Virology, Erasmus MC Rotterdam, 3015 GE Rotterdam, The Netherlands

[3]Department of Vaccine Technology and Immunology, Intervet International BV, 5830 AA Boxmeer, The Netherlands

Correspondence
Marel C. de Wit
Marel.deWit@abbott.com

Received 27 August 2003
Accepted 10 December 2003

Replicating viruses generally induce type 1 immune responses, with high interferon (IFN)-γ levels and antibodies of the IgG2a isotype. In the present study we demonstrate the intrinsic ability of non-replicating virions to induce comparable immune responses in the notable absence of any adjuvant. Injection of inactivated pseudorabies virus, an alphaherpesvirus, by various routes into mice resulted in the generation of T helper (Th) 1 type immune response. Co-delivery of inactivated pseudorabies herpesvirus (iPRV) with protein redirected IgG1-dominated tetanus toxoid-specific responses towards an IgG1/IgG2a balanced response. Also inactivated preparations of viruses from the paramyxo- (Newcastle disease virus), rhabdo- (rabies virus), corona- (infectious bronchitis virus) and reovirus (avian reovirus) families led to IgG2a antibody responses; however, the genetic background of the host did result in considerable variation. Because disrupted virions also induced type 1 immune responses, we conclude that structural elements of virions inherently contribute to IFN-γ-dependent isotype switching by inactivated viruses. Strikingly, immunizations in gene-disrupted mice showed that a functional IFN-α/β, IFN-γ or interleukin (IL)-12 pathway was not required for the generation of a polarized Th1 type immune response initiated by inactivated virus particles. These findings have a bearing on the understanding of immune responsiveness to virus structures and the design of vaccines containing virus components.

INTRODUCTION

A hallmark of immune responses to virus infections is the activation of several interferon (IFN) genes, including expression of IFN-γ. IFN-γ can exert antivirus effects by directly inhibiting replication or through the stimulation of innate immune effector cells like natural killer (NK) cells, macrophages and neutrophils (Samuel, 2001). IFN-γ also regulates adaptive immune responses by directing T helper (Th) cell polarization and antibody isotype switching (Coffman *et al.*, 1991; Snapper & Paul, 1987). In mice it stimulates IgG2a, in contrast to IL-4, which is involved in IgE and IgG1 assembly (Coffman *et al.*, 1986). Immune complexes containing IgG2a molecules activate the complement system and enhance phagocytosis by macrophages and antibody-dependent cell-mediated cytotoxicity; mechanisms involved in antivirus immunity (Kipps *et al.*, 1985; Spiegelberg, 1974). Although antibodies are important in the early defence against e.g. vesicular stomatitis virus and polyoma virus (Bachmann *et al.*, 1997; Szomolanyi-Tsuda & Welsh, 1996), their major role is in the protection against reinfections (Bachmann & Kopf, 1999). Consistent with the characteristic IFN-γ production during virus infections, immunoglobulins of the IgG2a isotype predominate in virus-infected mice (Coutelier *et al.*, 1987).

Production of IFN-γ by NK cell and various T-cell populations is triggered either directly, through the recognition of infected cells, or indirectly, involving other cytokines. Especially, early interleukin (IL)-12 production is considered crucial for subsequent IFN-γ synthesis (Hsieh *et al.*, 1993; Magram *et al.*, 1996). However, the mechanism underlying the preference for the Th1 pathway, including the induction of IgG2a antibodies, during virus infections has not been fully elucidated. In fact, this polarization of the T cell responses may occur independently from IL-12 function during virus infection (Oxenius *et al.*, 1999; Schijns

†Present address: Abbott BV, Siriusdreef 51, 2132 WT Hoofddorp, The Netherlands.

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et al., 1998), in contrast to most parasitic and bacterial infections. This is plausible because IL-12 expression is inhibited by IFN-α (Cousens *et al.*, 1997). IFN-α is induced both by virus replication intermediates like double-stranded RNA and by dendritic cells (DCs) pulsed with inactivated viruses (Milone & Fitzgerald-Bocarsly, 1998); also, it polarizes human T cells to the Th1 phenotype (Rogge *et al.*, 1998). In mice, however, its regulatory role is unclear; although it fails to induce Th1 development (Wenner *et al.*, 1996) IFN-α may stimulate IFN-γ production during virus infections (Nguyen *et al.*, 2002).

In contrast to replicating viruses, inactivated viruses are usually adjuvanted in order to enhance the immune response, which results in an isotypic bias for IgG1 type antibodies in most cases (Brett *et al.*, 1993; Katayama *et al.*, 1999; Katz *et al.*, 1991), even following co-administration in complete Freund's adjuvant. However, adjuvants may mask the intrinsic capacity of virus antigens to induce antibody responses of a certain quality. Remarkably, some inactivated viruses, when given without adjuvant, induce IgG2a antibodies comparable to live virus-induced responses (Brett *et al.*, 1993; Hocart *et al.*, 1989; Huang *et al.*, 1993; Schijns *et al.*, 1998). The present study has been set up to define host factors and conditions that play a role in the *in vivo* regulation of virus-specific IgG2a antibody responses induced by non-replicating virions. We used inactivated pseudorabies herpesvirus (iPRV) particles as immunizing antigens in order 1) to avoid virus persistence, 2) to allow standardization of antigenic load, 3) to exclude potential replication-associated immune escape mechanisms and 4) to prevent destruction of target cells by cytopathogenicity. For comparison we studied responses to live attenuated PRV.

We show that in addition to iPRV, inactivated viruses of different families characteristically induce IFN-γ-dependent IgG2a antibody production. The quality of the antibody profile is influenced by the genetic background of the host, but is independent of the route of administration and endogenous IL-12 or IFN-α/β function.

METHODS

Mice. Wild-type (WT) and mutant 129/Sv mice, the latter deficient in their expression of IL-12 or IFN receptors, were bred at the Central Animal Laboratory, Utrecht University. IL-12-deficient (IL-$12^{-/-}$), IFNα/β-deficient (IFN$\alpha/\beta^{-/-}$) and IFN-γ receptor-deficient (IFN-γR$^{-/-}$) mice were generated by gene targeting in murine embryonic stem cells (Huang *et al.*, 1993; Magram *et al.*, 1996; Muller *et al.*, 1994). BALB/c and C57BL/6 mice were obtained from Harlan. All animals were housed in filtertop cages.

Unless indicated otherwise, 8- to 12-week-old female mice were immunized by intraperitoneal (i.p.) injection of 100 µl containing approximately 2×10^7 inactivated plaque-forming units (p.f.u.) of the respective virus preparations and boosted 4 weeks later by the same route. Mice were bled from the retro-orbital plexus at the indicated points in time. Approval for the animal experiments had been obtained from the Institutional Animal Welfare Committee.

Viruses and antigens. Because of the high virulence of WT PRV in mice, an attenuated gE$^-$, TK$^-$ mutant served as live replicating PRV control (1×10^6 p.f.u. per dose; Visser & Lütticken, 1989). A gE$^-$, TK$^+$ derivative of field isolate NIA-3 (Quint *et al.*, 1987) was used to prepare iPRV. Inactivation of the virus was performed using a final concentration of 0·01 M 2-bromoethylamine hydrobromide (BEA) pH 7·4 and NaOH for 24 h at 37 °C. BEA was neutralized by adding 20 % sodium thiosulphate to a final concentration of 0·67 % (v/v). No residual live virus was found, as assessed by inoculation of BHK cell cultures (>1500 cm^2 monolayer surface) with the preparation and checking for cytopathic effects (CPE). Disintegrated PRV particles were prepared by treatment of the inactivated virus with 1 % sodium deoxycholate (NaDOC) at 37 °C for 1 h (Balkovic *et al.*, 1987). After dialysis against PBS, 10 µg and 50 µg of virus were used in the immunization studies.

Newcastle disease virus (NDV) clone 30 (Romer-Oberdorfer *et al.*, 1999) and infectious bronchitis virus (IBV) strain M41 (Cavanagh, 1983) were grown in embryonated eggs and inactivated by incubation with formaldehyde (final concentration 0·075 %) at 4 °C for 21 days. No residual infectivity was found after inoculation of 0·1 ml of the preparations into the allantoic cavity of embryonated eggs and assessing embryo mortality and haemagglutination of chicken red blood cells after 6–7 days.

Reovirus (REO) strain 2408 (Rosenberger *et al.*, 1989) grown in cultures of chicken embryo fibroblasts (CEF) was inactivated using formaldehyde (final concentration 0·2 %; incubation at 37 °C for 40 h). The preparation was neutralized using sodium metabisulphite and assayed for residual infectivity on a 500 cm^2 CEF monolayer surface. After three to four passages, the cells were inspected for CPE. BHK (baby hamster kidney) cell-adapted rabies virus strain Pasteur (Consales *et al.*, 1988) was inactivated by incubation with β-propriolactone (final concentration 0·025 %) at 37 °C during 2 h.

BHK cell cultures were inoculated with the inactivated virus preparations and scrutinized for CPE for 14 days; also, mice were injected intracerebrally with 0·03 ml of the inactivated virus suspensions and observed for neurological symptoms for 21 days. No residual infectivity was found. Tetanus toxoid (TT; SVM) was administered at a concentration of 0·5 LF per dose.

Determination of antibody titres. Antibody levels and subtypes were determined using ELISA as described by Schijns *et al.* (Schijns *et al.*, 1994). Briefly, 96-well flat-bottom plates (Greiner) were incubated with 110 µl of the inactivated virus or TT diluted in carbonate buffer (0·05 M, pH 9·6) at 37 °C (or at 4 °C for TT) for 16 h and rinsed with tap water. Subsequently, two-fold serum dilutions (starting at 1/64) in 0·04 M PBS containing 0·1 % Tween 80 and 0·1 % BSA (Kordia, Leiden, The Netherlands) were added. After 1 h at 37 °C, the plates were rinsed again and incubated with IgG isotype-specific antibody/horseradish peroxidase conjugates (Southern Biotechnology) for 30 min. After another rinse, tetramethylbenzidine was allowed to react for 15 to 30 min at room temperature. The colour reaction was stopped with 2 M sulphuric acid and the result (absorbance) read at 450 nm. Antibody titres were defined as the reciprocal of the highest dilution with an absorbance 1·5 times that of the background, observed in naïve, non-immunized control groups; titres below a 2 log titre of 5 were regarded negative. No cross-reactivity between TT and PRV-specific antibodies was observed.

Analysis of cytokine production. For the analysis of IL-4, IL-5 and IFN-γ production, spleens were isolated 2 weeks after the booster immunization. Erythrocyte-depleted splenocytes (3×10^6 cells ml^{-1}) were cultured in 24-well plates (Nunc) in the presence of 10 µg dialysed iPRV ml^{-1}. Cell culture supernatants were harvested 2, 5 or

7 days afterwards and stored at −20 °C until use. Concentrations of IL-4, IL-5 and IFN-γ were measured using sandwich ELISA with mass-calibrated standards (R&D Systems).

Statistical analysis. If applicable, group means of cytokine responses and antibody titres were compared using Student's *t*-test. Values of $P<0\cdot05$ were considered significant.

RESULTS

Inactivated PRV induces IgG2a antibody responses irrespective of the route of immunization

To assess the quality of specific antivirus antibodies we determined the ratios of isotype-specific serum antibody titres by ELISA in groups of immunized mice. When immunized i.p. with iPRV, 129/Sv mice were found to preferentially mount IgG2a antibody responses (Fig. 1). In contrast, immunization with tetanus toxoid (TT) resulted in responses merely of the IgG1 isotype (Fig. 2, left panel). Immunization routes have been shown to influence the polarization of immune responses (Rogers & Croft, 1999), which we could not confirm: intramuscular, intraperitoneal and intradermal vaccination of 129/Sv mice all resulted in dominant IgG2a production (Fig. 1). The quality of the antibody response following iPRV immunization therefore proved independent of the route of administration.

Modulation of the antibody response directed against unrelated antigens

Virus infections may assist in the induction of IgG2a antibodies directed against co-administered non-virus antigens such as keyhole limpet haemocyanin and TT (Coutelier *et al.*, 1988; Markine-Goriaynoff *et al.*, 2000). To investigate the role of an inactivated virus, mice were immunized with TT in combination with live or iPRV, and with TT alone serving as a control. The total amount of antibodies directed against TT was not significantly influenced by co-administration of live or iPRV (Fig. 2), nor were the IgG1 titres affected. However, co-administration of iPRV enhanced TT-specific IgG2a levels ($P=0\cdot02$), which indicates a shift of the TT-specific reaction towards a type 1 immune response. The antibody isotype profiles to PRV in these animals were similar to those after PRV administration alone; no cross-reactivity for TT or PRV antigens could be detected in sera of co-immunized animals (data not shown). These results indicate that iPRV can act as a type 1 subclass selective adjuvant.

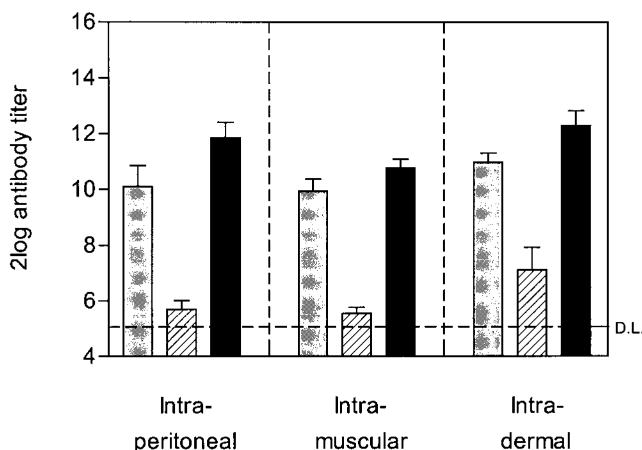

Fig. 1. PRV-specific IgG antibody titres after immunization with iPRV via different routes. Sera from 129/Sv mice ($n=5$) were tested by ELISA 4 weeks after primary vaccination for IgG heavy and light (H+L) chains (grey columns), IgG1 (hatched columns) and IgG2a (solid columns), using PRV antigen. Mean titres ± SEM are shown, the detection limit (D.L.) is indicated by the dotted line.

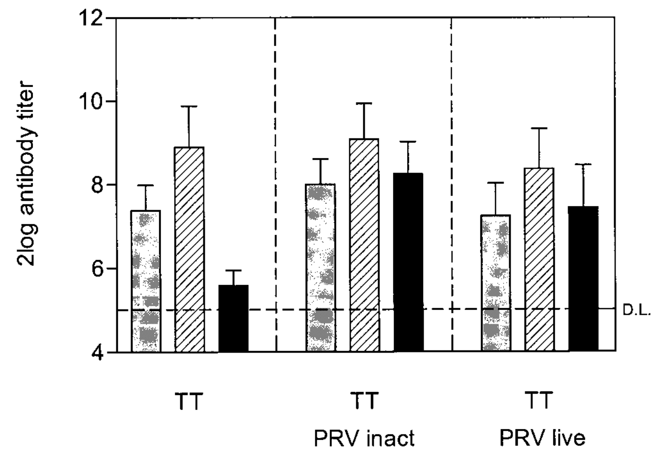

Fig. 2. Influence of inactivated and live PRV on the antibody levels of IgG subclasses directed against TT. Groups of at least four 129/Sv mice were immunized i.p. with TT alone or in the presence of live or inactivated PRV. TT-specific IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns) antibody titres in sera taken 4 weeks after immunization were measured using ELISA. Mean titres ± SEM are shown. Theoretical cross-reactivity between anti-PRV and anti-TT antibodies could be excluded because of negative reactions to each specific antigen in sera of animals immunized with only one of the two non-related antigens. The dotted line indicates the detection limit (D.L.).

Endogenous IFN-γ but not IFN-α/β or IL-12 is required for IgG2a isotype switching

To assess the contribution of IFN-γ in isotype switching to IgG2a, antibody levels were determined in IFN-γR$^{-/-}$ mice after immunization with iPRV. As shown in Fig. 3(B), these animals attained lower antivirus IgG2a antibody levels than the controls ($P=0\cdot003$), indicating that IFN-γ is largely responsible for the preferential IgG2a isotype response. We then looked for IFN-γ synthesis by PRV-specific T cells. Splenocytes isolated 14 days after the second immunization were stimulated *in vitro* with iPRV

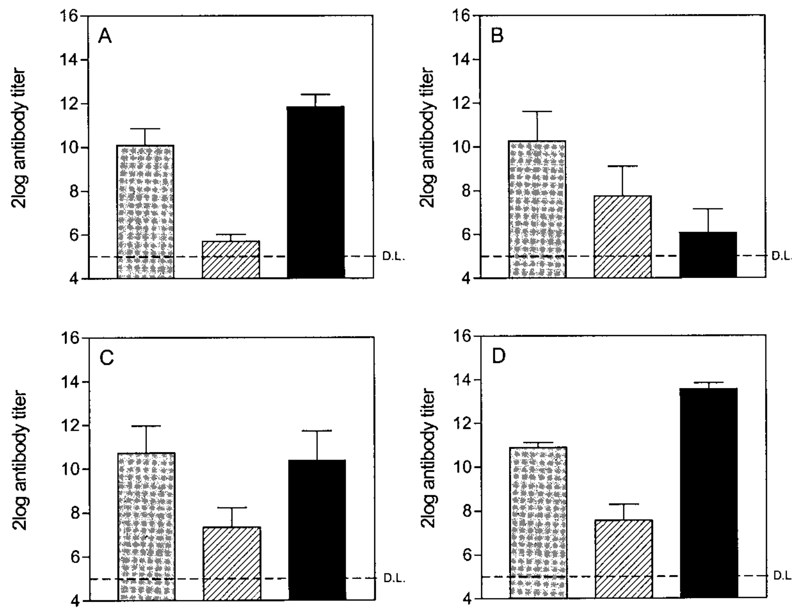

Fig. 3. Pseudorabies herpesvirus (PRV)-specific antibody responses in wild-type (WT) (A), IFN-γ receptor-deficient (IFN-$\gamma R^{-/-}$) (B), IFN-α/β receptor-deficient (IFN-$\alpha/\beta R^{-/-}$) (C) and IL-12-deficient (IL-$12^{-/-}$) (D) 129/Sv mice. Sera of five animals per group, taken 4 weeks after a single i.p. immunization with iPRV, were analysed for specific IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns). Mean titres ± SEM are shown. The dotted line indicates the detection limit (D.L.).

antigen. As shown in Fig. 4, high levels of IFN-γ could indeed be demonstrated in the supernatant of these splenocytes, whereas IL-5 levels were below the detection limit, similar to levels of IL-4 (data not shown). In contrast to immunization with replicating attenuated PRV (Schijns *et al.*, 1994) we noted that detectable cytokine levels (IFN-γ, but no IL-4 or IL-5) could be measured only after 7 days of antigenic stimulation, indicating that the frequency of antigen-specific cytokine-producing cells was rather low using non-replicating PRV.

Many inactivated viruses are potent inducers of IFN-α/β (Isaacs & Lindenman, 1957), which may polarize T cells towards the type 1 phenotype. To address the role of these cytokines in IgG2a antibody regulation after immunization with non-replicating alphaherpesvirus particles, we injected iPRV into WT and IFN-$\alpha/\beta R^{-/-}$ mice and determined antivirus antibody subclass titres. As Fig. 3(C) shows, there were no conspicuous differences between the groups. It would appear therefore that class I IFNs do not play a key role in the polarization of the antibody response after vaccination with iPRV.

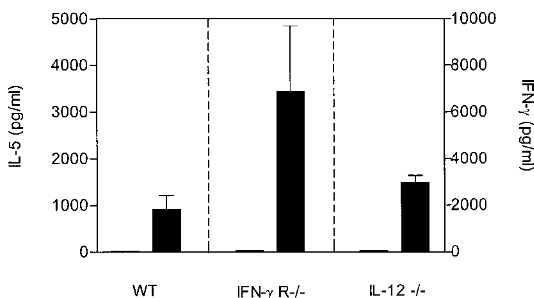

Fig. 4. Cytokine responses of splenocytes from wild-type (WT), IFN-γ receptor-deficient (IFN-$\gamma R^{-/-}$) and IL-12-deficient (IL-$12^{-/-}$) mice, isolated 2 weeks after a booster immunization with inactivated (i) PRV and restimulated with iPRV. After 7 days IL-5 (open columns) and IFN-γ (solid columns) concentrations in the cell culture supernatant were measured using ELISA. Mean concentrations of five mice per group are shown ± SEM. IFN-γ levels in splenocytes cultures from naïve mice proved lower than 100 pg ml^{-1}, while IL-5 levels were non-detectable (ND < 30 pg ml^{-1}).

Surprisingly IL-12 seemed to be dispensable for Th1 development following iPRV immunization as well. IL-$12^{-/-}$ mice, like WT animals, responded with IgG2a-dominated antibody titres (Fig. 3D) as well as with IFN-γ production upon restimulation of their splenocytes (Fig. 4).

Induction of responses after immunization with disrupted inactivated PRV particles

We needed to exclude trivial explanations for the immunogenicity of inactivated virions and the observed IgG2a responsiveness. Contamination of the preparations by endotoxin or by inflammatory substances comes to mind. Endotoxin was undetectable (< 10 IU ml^{-1}), as measured by the *Limulus* amebocyte lysate (LAL) test, and dialysed inactivated virus also induced type 1 antibody responses (not shown). Moreover, NaDOC disrupted virions also induced IgG2a-dominated antibody responses (Fig. 5A). This conclusion is corroborated by the results of the cytokine analysis after *in vitro* stimulation of splenocytes with PRV, depicted in Fig. 5(B).

IgG2a antibody responses after immunization with other inactivated viruses

To test whether the observed phenomena are exclusive for iPRV, the study was expanded to include other viruses.

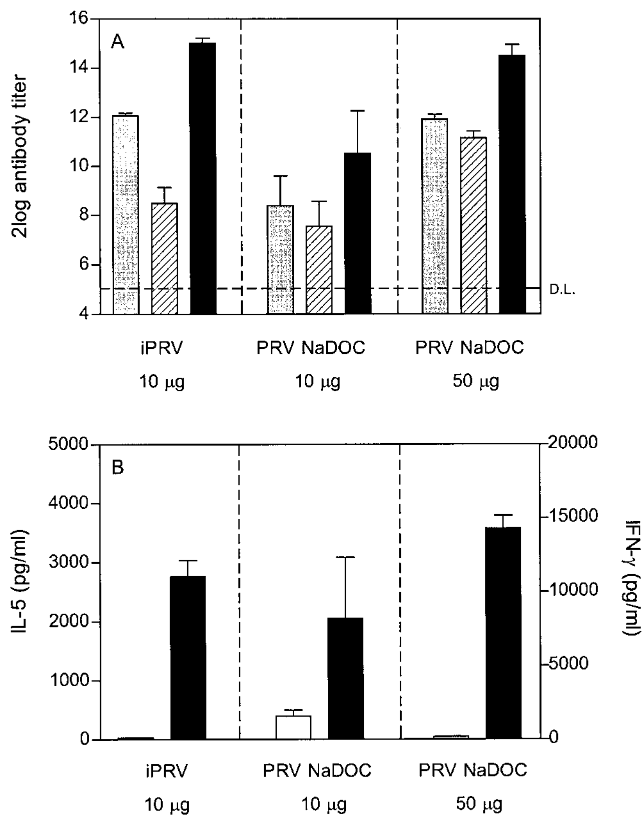

Fig. 5. PRV-specific immunoglobulin isotype (A) and cytokine responses (B) following immunization with iPRV particles or NaDOC disrupted virions. ELISA measured antibody titres in sera of five mice per group taken 4 weeks after primary immunization (A). Means ± SEM above the detection limit (D.L.) of IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns) are shown. Splenocytes were isolated 2 weeks after booster immunization and restimulated with iPRV (B). Supernatants of 7-day cell cultures were tested using ELISA for IL-5 (open columns) and IFN-γ (solid columns). Mean concentrations ± SEM are shown. IFN-γ levels in splenocytes cultures from naïve mice proved lower than 100 pg ml^{-1}, and IL-5 levels were below the detection limit (<30 pg ml^{-1}).

Mice were immunized with inactivated preparations of enveloped and non-enveloped, RNA and DNA viruses (paramyxo-, rhabdo-, corona- and reovirus) and their sera assayed as before. In all cases, the IgG2a antibody subclass was most abundant (Fig. 6), although titres varied per virus. This indicates that structural virion differences are not essential and that the underlying mechanism may be of a general nature.

Influence of host genotype on IgG2a titres

The experiments described thus far have been performed in WT and gene knockout mice on a 129/Sv background. To examine the influence of the host genotype, live and iPRV preparations were used to immunize mice of the inbred strains 129/Sv, C57BL/6 and BALB/c. Immunization with TT, an inducer of IgG1-dominated humoral responses, was used as a control. Live PRV immunization was found to induce type 1 responses in all mouse strains, although variations in the IgG2a/IgG1 ratios were observed (Fig. 7A). Upon immunization with iPRV, IgG2a antibody responses were lower in C57BL/6 ($P<0{\cdot}0001$) and BALB/c ($P=0{\cdot}0003$) mice than in the 129/Sv strain (Fig. 7B). Th2 isotype immune responses with relatively low levels of IgG2a were observed in the TT-immunized mice of all strains tested (Fig. 7C). Hence, host genetic factors seem to control the phenotype of immune responses after immunization with inactivated virus more noticeably than after infection. We finally asked the question whether the genetic background of the host is important also for viruses other than PRV, and immunized 129/Sv, C57BL/6 and BALB/c mice with inactivated rabies virus and NDV. C57BL/6 mice produced IgG2a antibodies against both viruses, although the ratios with respect to IgG1 were lower than that observed in 129/Sv mice (Fig. 8). On the other hand, BALB/c mice responded to NDV with a dominant IgG2a antibody reaction, with higher IgG2a/IgG1 ratios when compared to the TT response. This finding illustrates that in addition to the host's genetic background, viruses differ in their capacity to induce IgG2a antibodies.

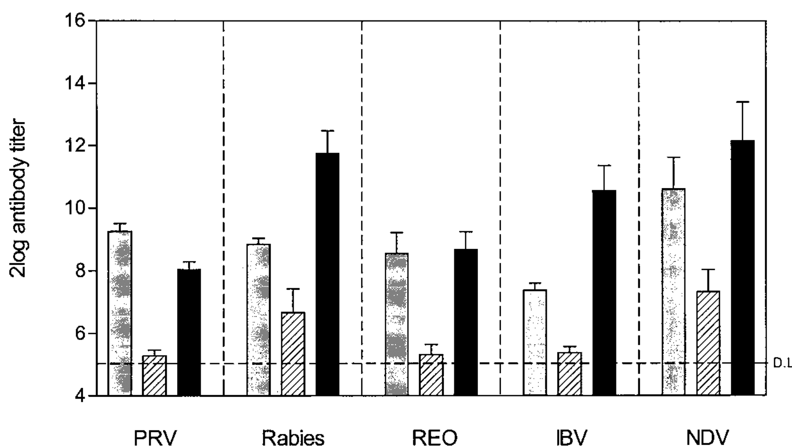

Fig. 6. Serum levels of immunoglobulin isotypes 2 (NDV) or 4 (iPRV, rabies virus, IBV) weeks after primary i.p. immunization or 3 weeks after a booster vaccination [reovirus (REO)] with inactivated virus preparations. Groups of four to seven mice (129/Sv) were used, and their individual sera analysed by ELISA for virus-specific IgG (H+L) chains (grey columns), IgG1 (hatched columns) and IgG2a (solid columns). Antibody titres are expressed as group means ± SEM. The detection limit (D.L.) is indicated by the dotted line.

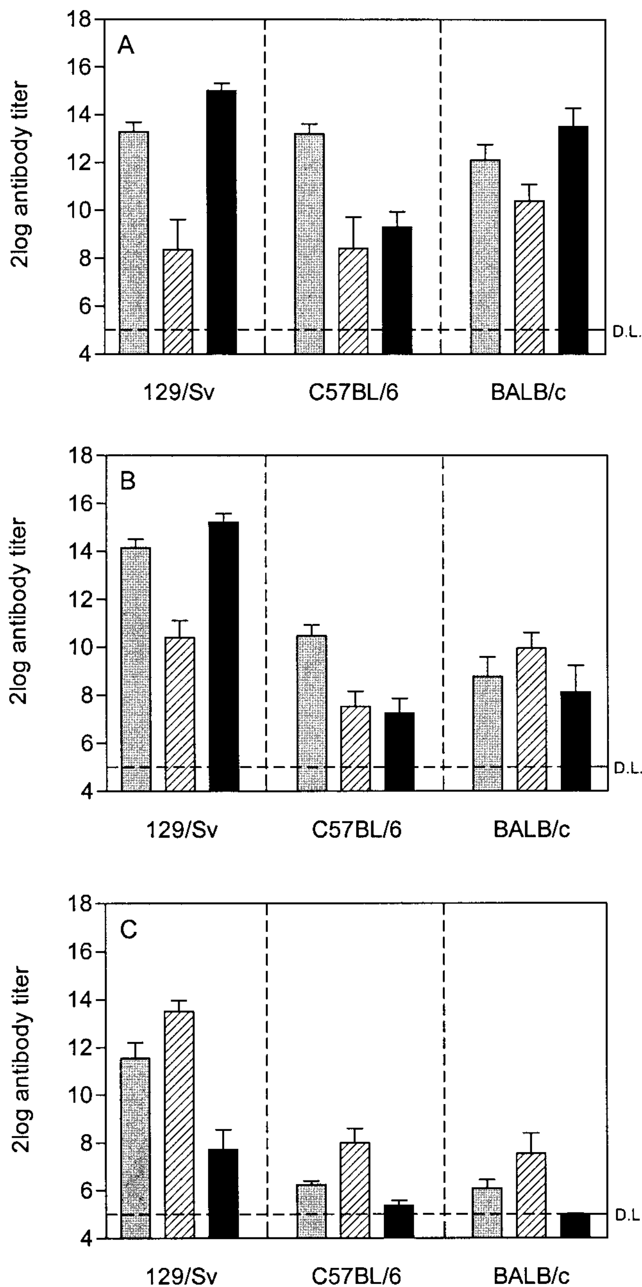

Fig. 7. Antibody titres in mice with different genetic backgrounds. Mice ($n=5$) of the 129/Sv, C57BL/6 and BALB/c strains were injected by the i.p. route with live PRV (A), iPRV (B) or TT (C). Group means ± SEM above the detection limit (D.L.) of specific IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns) antibodies detected in serum 3 weeks after secondary immunization are shown. Secondary responses are depicted because of low primary antibodies in C57BL/6 and BALB/c mice, which, however, showed a similar profile.

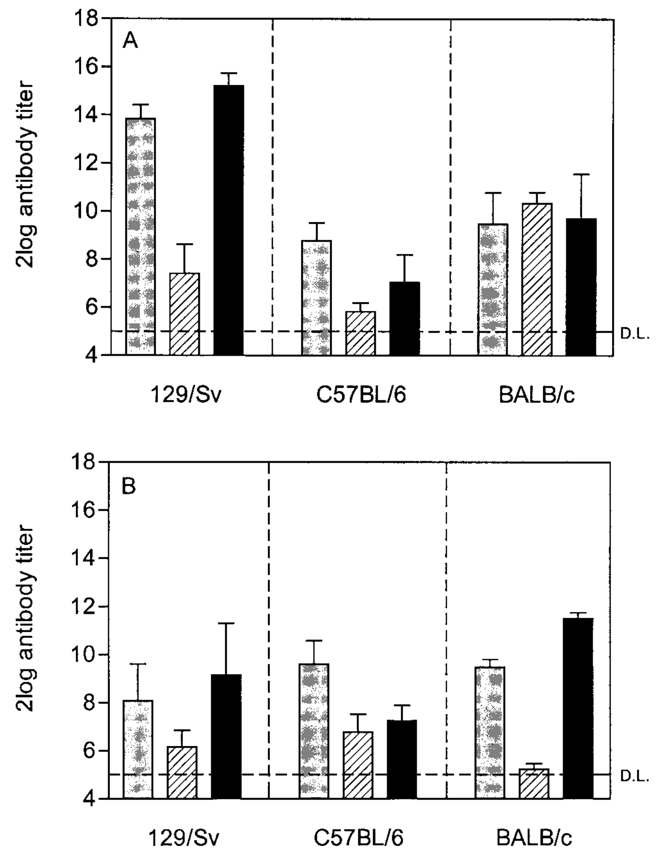

Fig. 8. Influence of the genetic background on the antibody response induced by inactivated rabies virus (A) or NDV (B). Serum levels of IgG subtypes 2 weeks after primary (NDV) or booster (rabies) vaccination were measured using specific ELISA systems and are shown as means of five mice ± SEM. The secondary responses to rabies virus are depicted because of low primary antibodies in C57BL/6 and BALB/c mice, which, however, showed a similar profile. Grey columns represent IgG (H+L), hatched columns IgG1 and solid columns IgG2a. The detection limit (D.L.) is indicated by the dotted line.

DISCUSSION

Virions may fulfil the structural requirements for IgM antibody responses in the absence of an adjuvant. Highly ordered, densely packed surface epitopes found on virions are assumed to cross-link surface Ig receptors on B cells and thereby evoke IgM responses, often in the absence of T cells (Fehr *et al.*, 1996; Ochsenbein *et al.*, 2000). In the presence of activated T cells, IgM-producing B cells may readily switch to the production of IgG. Immunizations with inactivated preparations like mouse adenovirus or lactate dehydrogenase virus (Markine-Goriaynoff *et al.*, 2000) evoke IgG responses, often showing an IgG1 isotypic bias, even when complete Freund's adjuvant was co-administered (Oxenius *et al.*, 1999). In our study, immunizations with iPRV, a herpes simplex-related α-herpesvirus, led to IgG2a-dominated immune responses, especially in 129/Sv mice. In C57BL/6 and BALB/c mice a more balanced IgG2a/IgG1 ratio was noted. Virions of other virus families, including the enveloped rhabdo-, corona- and paramyxoviridae, as well as the non-enveloped reoviruses, equally exhibit this Th1 polarization. Also, the observation that iPRV, similar

to replicating PRV, enhances IgG2a production to the co-administered non-virus TT antigen, suggests involvement of non-cognate, bystander immune modulation by IFN-γ in 129/Sv mice. This phenomenon has been noted for replicating viruses before (Coutelier *et al.*, 1988; Monteyne *et al.*, 1993), but not for inactivated virions.

Disintegrated PRV particles were also found to evoke IgG2a-dominated responses, although the IgG2a/IgG1 ratio was somewhat lower than when intact PRV virions had been used. This may indicate that Th1 type immune responses are not entirely dependent on the particulate form of the antigen, though it contributes to the isotype bias. Other virus features, like the genomic material, can also influence the outcome of an immune reaction. The double stranded DNA present in herpes virions, is able to induce the expression of co-stimulatory molecules and cytokine genes upon transfection in non-immune thyroid cells (Suzuki *et al.*, 1999). Such signal molecules could provide the critical immune activating and response directing molecules necessary for Th1 proliferation and down-stream IgG2a production.

Although modest IgG2a synthesis occurs in the absence of IFN-γ function (Markine-Goriaynoff *et al.*, 2000; Muller *et al.*, 1994), our studies demonstrate that the functional presence of IFN-γ substantially increases IgG2a isotype synthesis in response to iPRV. Notably, restimulation of IFN-$\gamma R^{-/-}$ splenocytes with virus antigen resulted in high IFN-γ and no detectable IL-5 production. This suggests that IFN-γ function is not essential to drive virion-induced IFN-γ production by T cells. Similar to earlier studies (Schijns *et al.*, 1994) IFN-$\gamma R^{-/-}$ splenocytes produced higher levels of IFN-γ than WT splenocytes. We speculate on less IFN-γ-mediated feedback inhibition or less cytokine consumption in the absence of a functional IFN-γ receptor.

Surprisingly, iPRV-induced responses fail to default to type 2 immune reactions in the absence of IFN-α/β or IL-12 gene function. IFN-α/β, historically discovered using inactivated influenza virus (Isaacs & Lindenman, 1957), is characteristically produced by plasmacytoid DCs (Hochrein *et al.*, 2001). Like IL-12, it is a key regulator of innate and adaptive immune responses after infections (Finkelman *et al.*, 1991). IFN-α/β, IL-18 and IL-12-independent type 1 immune responses have been described in particular for virus infections (Grob *et al.*, 1999; Oxenius *et al.*, 1999; Schijns *et al.*, 1998; van den Broek *et al.*, 1995; Xing *et al.*, 2000), but not for immune responses following exposure to non-replicating virus particles. We can however not rule out that in the absence of one of these two cytokines the other, or alternative Th1-associated cytokines, like IL-18, IL-23 or IL-27, compensates the deficiency (Okamura *et al.*, 1995; Oppmann *et al.*, 2000; Pflanz *et al.*, 2002; Xing *et al.*, 2000). Double depletion experiments should answer this possibility.

Apart from typical cytokines correlated with Th1 development, the virion-associated IFN-γ-driven IgG2a production may be triggered by very early recognition events in T cell polarizing DCs or B cells. However, limited information is available on the interaction between virions and immune cells. Recently, a few virus-related pathogen-recognition receptors have been defined, such as for the G protein of respiratory syncytial virus, which is involved in Toll-like receptor (TLR) 4-dependent responses (Haynes *et al.*, 2001; Kurt-Jones *et al.*, 2000). Immune-stimulatory activity has been noted also for the rabies virus nucleocapsid protein (Lafon *et al.*, 1992), without knowledge of its putative recognition receptor. It can therefore not be excluded that the induction of Th1 development upon recognition of inactivated viruses depends on a pathogen-recognition receptor, as shown for an increasing number of non-virus microbial antigens (Jankovic *et al.*, 2002).

Since the genetic background of the host did influence the outcome of the immune reaction (Hocart *et al.*, 1989; Raj *et al.*, 1992), we are led to conclude that the observed Th1 pathway may be activated differently in genetically different hosts. This could be the result of expression differences of pattern-recognition receptors on immune cells, or down-stream response modifying genes; a topic for further investigation.

ACKNOWLEDGEMENTS

We thank Dr M. Aguet (ETH, Zurich, Switzerland) for providing us with the IFN-α/β and IFN-$\gamma R^{-/-}$ mice, and Dr H. Mossmann (Max-Planck Institut, Freiburg, Germany) and Dr G. Alber (University of Leipzig, Leipzig, Germany) for the provision of IL-$12^{-/-}$ mice. We also acknowledge Karel van Stokkom for his assistance in performing the animal experiments, Hanneke van Zuilekom and Nicolette Scholtes for their excellent technical help and Winfried Degen for helpful discussions.

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