

Effect of inactivation method on the cross-protective immunity induced by whole ‘killed’ influenza A viruses and commercial vaccine preparations

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We have recently shown that intranasal (i.n.) administration of γ -irradiated A/PR/8 [A/Puerto Rico/8/34 (H1N1)] protects mice against lethal avian influenza A/Vietnam/1203/2004 (H5N1) and other heterosubtypic influenza A infections. Here, we used γ -irradiated, formalin- and UV-inactivated A/PC [A/Port Chalmers/1/73 (H3N2)] virus preparations and compared their ability to induce both homologous and heterosubtypic protective immunity. Our data show that, in contrast to i.n. vaccination with formalin- or UV-inactivated virus, or the present commercially available trivalent influenza vaccine, a single dose of γ -ray-inactivated A/PC (γ -A/PC) conferred significant protection in mice against both homologous and heterosubtypic virus challenges. A multiple immunization regime was required for formalin-inactivated virus preparations to induce protective immunity against a homotypic virus challenge, but did not induce influenza A strain cross-protective immunity. The highly immunogenic γ -A/PC, but not formalin- or UV-inactivated A/PC, nor the currently available subvirion vaccine, elicited cytotoxic T-cell responses that are most likely responsible for the cross-protective and long-lasting immunity against highly lethal influenza A infections in mice. Finally, freeze-drying of γ -A/PC did not affect the ability to induce cross-protective immunity.

Received 14 November 2009

Accepted 7 February 2010

INTRODUCTION

Infectious diseases, such as influenza, remain one of the leading causes of death and morbidity in the human population and vaccination represents the most cost-effective and efficient defence against virus-induced diseases (WHO, 2005). Live attenuated virus-based vaccines have been the most successful for a number of infectious diseases including polio, yellow fever, measles and smallpox (Plotkin *et al.*, 2008). In regard to influenza, whole virus killed vaccines are associated with adverse reactions, especially in children, and consequently are little used (Quilligan *et al.*, 1949; Salk, 1948). To reduce reactogenicity, most influenza vaccines used today are split-product vaccines or surface-antigen vaccines from chemically inactivated viruses, containing only purified haemagglutinin and neuraminidase, the outer surface

proteins of influenza virus. These vaccines, however, are less immunogenic than whole virus vaccines (Barry *et al.*, 1974, 1976; Ortals & Liebhaver, 1978), most likely because their antigenic structures have been altered by chemical treatment and they fail to induce T-cell memory.

The protective efficacy of current subunit or split-influenza vaccines relies exclusively on the induction of neutralizing antibodies against the viral surface proteins, haemagglutinin and neuraminidase (Palese, 2006). These neutralizing antibodies are generally not cross-reactive among different subtypes (Ada & Jones, 1986). Thus, the influenza vaccine is only effective against viruses whose antigenicity is the same or at least very similar to the vaccine strains. Accordingly, the composition of influenza vaccines needs to be updated each year to cover the prevailing variants. A cold-adaptive, live influenza vaccine was developed to overcome the poor T-cell immunogenicity of subunit or split vaccines (Chen *et al.*, 2010).

In general, preserving the integrity of the immunological epitopes is important for vaccine efficacy. A number of

A supplementary table comparing the haemagglutination activity of live and inactivated influenza virus is available with the online version of this paper.

inactivation methods have been applied for influenza virus, including chemical treatment (Budowsky *et al.*, 1991, 1993; Goldstein & Tauraso, 1970; Redfield *et al.*, 1981; Takada *et al.*, 2003), UV irradiation (Budowsky *et al.*, 1981; Goldstein & Tauraso, 1970; Kantorovich-Prokudina *et al.*, 1978; Polianskaia, 1979; Zheleznova, 1982), ionizing radiation (Fridman *et al.*, 1979; Kulevich & Kosiakov, 1974; Lidbury *et al.*, 1997; Lowy *et al.*, 2001; Migunov *et al.*, 1986; Noack *et al.*, 1986; Pang *et al.*, 1992) and heat treatment (Blazevic *et al.*, 2000; De Flora & Badolati, 1973a, b). The currently used inactivation method for influenza vaccines is chemical treatment with formalin or β -propiolactone (Knipe & Howley, 2001). Chemical treatment induces extensive interaction and cross-linking of viral proteins (Jackson, 1978; Martinson *et al.*, 1979). In this process, the viral suspensions are kept unfrozen and physical conditions as well as chemical agents degrade the antigenic viral proteins. In contrast, γ -irradiation can be applied to frozen viral samples, preventing unwanted protein degradation. The primary target by which ionizing radiation brings about virus inactivation is via strand breaks of viral nucleic acids, single-stranded RNA in the case of influenza virus, rather than denaturation of virion structure (De Benedictis *et al.*, 2007). Therefore, γ -irradiation inactivates viruses without having a detrimental impact on the antigenic structure and biological integrity of proteins (Lowy *et al.*, 2001). This represents a major advantage over traditional methods of sterilization, particularly in regard to vaccine preparation.

We have previously suggested the use of γ -ray-inactivated influenza A virus as a vaccine candidate (Alsharifi & Müllbacher, 2010; Müllbacher *et al.*, 1988, 2006) and have found that a single intranasal (i.n.) administration of γ -irradiated A/PR/8 (H1N1) induces cross-protective immunity against lethal heterosubtypic infections, including an avian H5N1 strain (Alsharifi *et al.*, 2009). Thus, γ -irradiated influenza virus preparations represent a new technique for a cross-protective influenza vaccine. Here, we compare the efficacy of γ -ray-, formalin- and UV-inactivated virus preparations to induce immunity against homologous and heterologous viral challenges. We show that γ -irradiated influenza virus is superior to other forms of inactivated virus preparations, including a currently used trivalent influenza vaccine (TIV), in providing cross-protective immunity.

RESULTS

Effect of virus inactivation on haemagglutination activity

Haemagglutination activity after virus inactivation provides one indicator as to the denaturing effect of the sterilization treatment. Purified influenza stock was aliquoted into batches and treated with either formalin, UV or γ -irradiation. Following the complete loss of infectivity as verified by the absence of virus growth in embryonated eggs and lack of plaque formation, we

compared the haemagglutination activity of live and inactivated viruses (Supplementary Table S1, available in JGV Online). Haemagglutination activity was reduced threefold for γ -irradiated viruses, whereas formalin and UV inactivation resulted in a ninefold reduction in haemagglutination titres. These results provide evidence that γ -irradiation, in contrast to other sterilization methods, causes the least denaturation of viral protein structure.

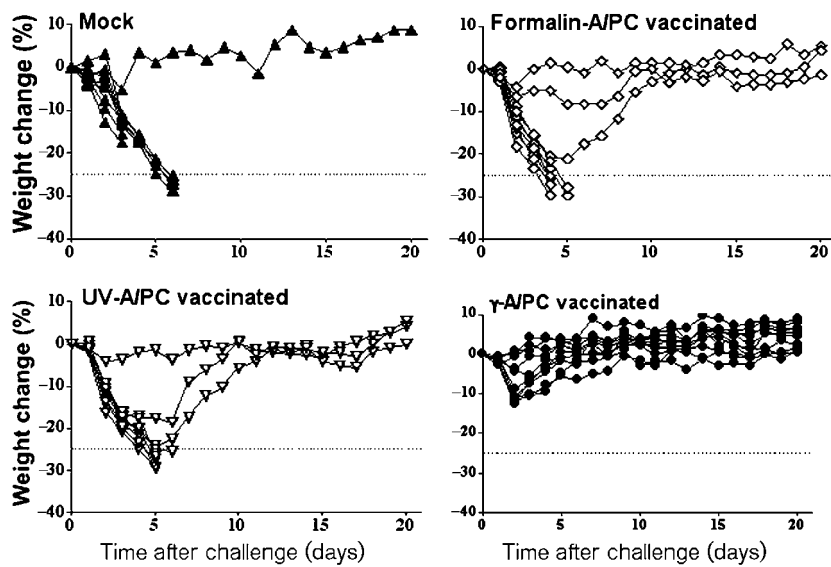
γ -Ray-inactivated A/PC (γ -A/PC), but not formalin- or UV-inactivated A/PC, induces heterosubtypic immunity

We compared the protective efficacy of γ -irradiated-, formalin- or UV-inactivated influenza virus preparations against homo- and heterosubtypic live virus challenges. Ten-week-old BALB/c mice (10 mice per group) were immunized i.n. with a single dose of inactivated A/PC. Three weeks later, mice were challenged i.n. with a lethal dose of homologous (A/PC, 3.2×10^5 p.f.u.) or heterosubtypic (A/PR/8, 7×10^2 p.f.u.) influenza A virus. Mice were monitored for 20 days for mortality and weight loss. As shown in Fig. 1, i.n. infection of naïve mice with A/PC or A/PR/8 caused a rapid weight loss with 90–100% mortality (based on 25% weight loss as an end point). Importantly, mice vaccinated i.n. with either formalin-inactivated or UV-inactivated A/PC developed significant weight loss and resulted in 70% mortality when challenged with live homologous virus (Fig. 1a). When similarly vaccinated mice were challenged with the heterosubtypic strain A/PR/8, the animals lost substantial body weight with 90–100% mortality (Fig. 1b). In either challenge, homologous or heterosubtypic, both formalin- and UV-inactivated preparations did not induce adequate protection (P -value >0.05 , Fisher's exact test). In contrast, mice immunized with a single dose of γ -A/PC were not only protected against homologous virus challenge (Fig. 1a), but also against heterosubtypic challenge (Fig. 1b). Therefore, i.n.-administered γ -irradiated influenza virus is the most effective vaccine preparation to induce protective immunity against homotypic and heterosubtypic influenza virus challenges.

Multiple doses of formalin-A/PC are required to enhance the protective effect

Previous studies have shown that multiple i.n. administrations of formalin-inactivated influenza vaccine can provide protection against a lethal heterosubtypic challenge (Takada *et al.*, 2003). Although γ -A/PC was clearly more effective after only one dose, we tested whether the weak protective efficacy of formalin-A/PC can be enhanced by boosted vaccination. At 1 week intervals, mice were immunized i.n. either once, twice or three times with formalin-A/PC. Three doses of formalin-A/PC give a collective dose equivalent to one dose of γ -A/PC based on haemagglutinin units (HAU). The mice were challenged

(a) Homologous challenge with A/PC



(b) Heterosubtypic challenge with A/PR8

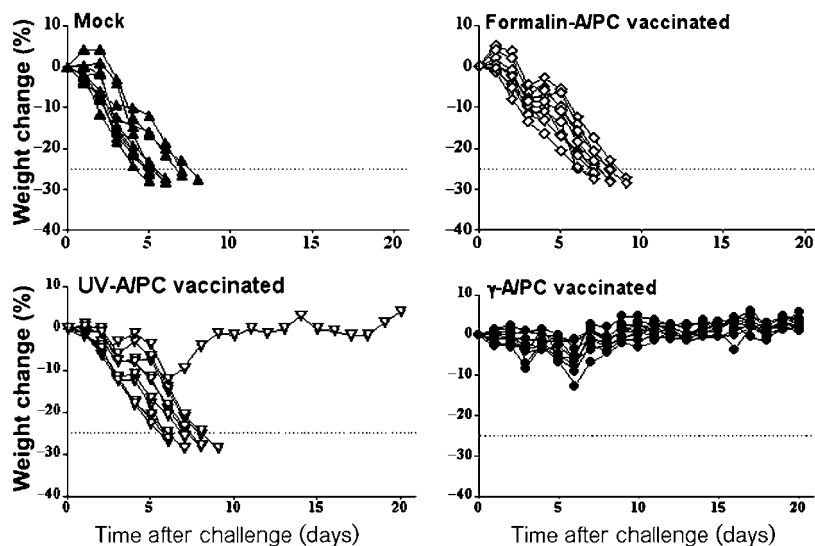


Fig. 1. Vaccination with γ -A/PC protects mice against both homologous and heterosubtypic influenza virus challenge. Groups of 9–10 BALB/c mice were mock treated or immunized with formalin-, UV- or γ -A/PC. Three weeks post-vaccination, mice were infected i.n. with A/PC as a homologous challenge (a) or A/PR/8 as a heterosubtypic challenge (b). Mice were monitored for 20 days for body weight loss. A loss of 25% of total body weight was used as the end point as required by the local animal ethics committee.

with the homologous virus either after 3 weeks of initial immunization for mice that received a single dose, or at weeks 2 or 1 post-immunization for mice that received two or three doses, respectively. As shown in Fig. 2(a), mice that received a single immunization dose had no improved survival rate compared to that of unvaccinated mice. Mice that received two doses of formalin-A/PC have a survival rate of 60% ($P < 0.05$ Fisher's exact test), although the majority of mice showed a significant body weight loss prior to recovery. Mice that received three doses of formalin-A/PC were completely protected and experienced little weight loss (Fig. 2a). Despite the enhanced protection against the homologous virus obtained with three doses of formalin-A/PC, only marginal protection (40%) (not significant $P > 0.05$ Fisher's exact test), against a heterosubtypic challenge with A/PR/8 (Fig. 2b) was obtained.

Thus, formalin-inactivated A/PC requires more doses to induce homologous protection than γ -ray-inactivated virus and fails to elicit cross-protection against heterosubtypic challenge. This is evidence that the immunity induced is not only quantitatively, but also qualitatively, inferior to that induced by γ -irradiated A/PC.

TIV is ineffective against drifted strains

Current subunit vaccines are administered using intramuscular (i.m.) injection. In contrast, our γ -irradiated influenza preparation is more effective when administered i.n. (Alsharifi *et al.*, 2009). Therefore, we tested the protective efficacy of i.n.- or i.m.-administered commercially available TIV using our mouse model of protection against influenza virus infection. Mice were immunized

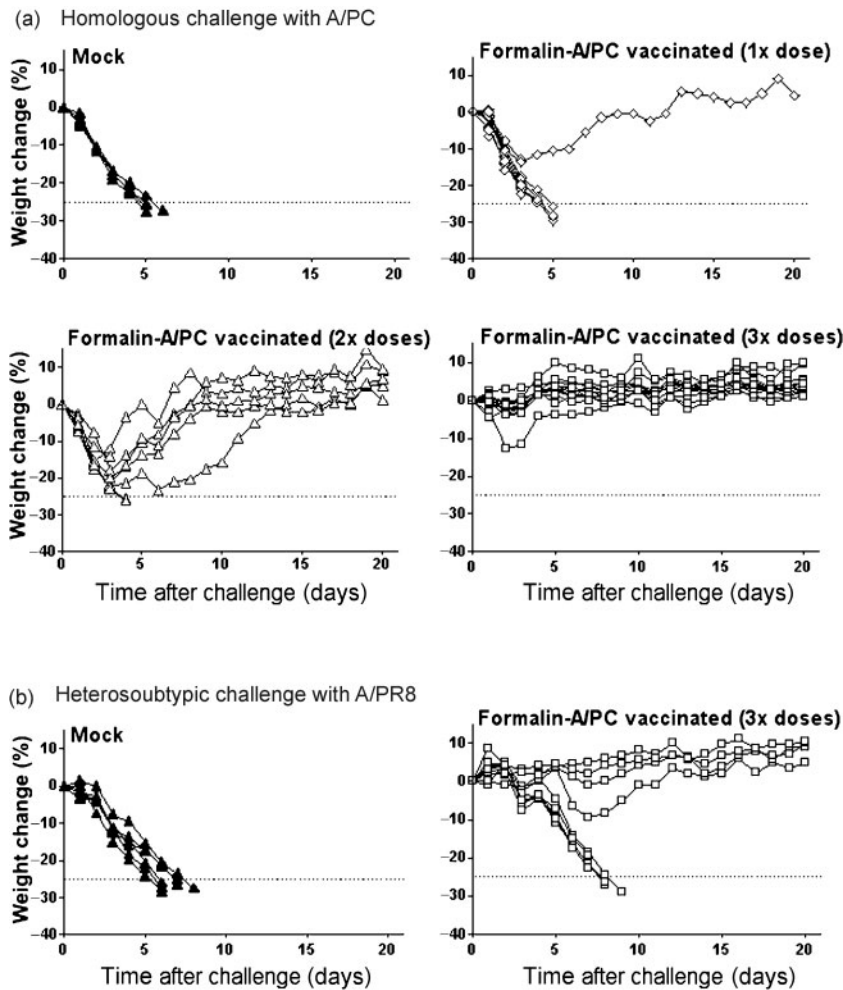


Fig. 2. Multiple immunizations with formalin-A/PC are required to induce homologous protection. Groups of 9–10 BALB/c mice were mock treated or immunized either once, twice or three times with formalin-A/PC. One week after the last immunization, mice were i.n. infected with either A/PC as a homologous infection (a) or A/PR/8 as a heterologous infection (b). Mice were monitored for 20 days for body weight and a loss of 25% of total body weight was used as the end point.

with TIV (CSL Fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 3 μ g haemagglutinin). Three weeks post-immunization, mice were challenged with A/PC or A/PR/8. As shown in Fig. 3, a single i.n. or i.m. immunization did not confer protection against either challenge. This clearly shows that i.n. administration of currently available subvirion influenza vaccine does not confer appreciable cross-protection, even against strains within the same subtype.

Effect of vaccination on lung viral loads

We evaluated the effect of our vaccination regimes on the pulmonary viral load at days 3 and 6 after heterosubtypic challenge with A/PR/8. I.n. vaccinated mice were challenged with live A/PR/8 3 weeks later. Lungs were harvested and virus titres were determined by plaque assay using Madin–Darby canine kidney (MDCK) cells. Virus titres of 10^7 and 10^6 p.f.u. per lung were detected in unvaccinated mice at days 3 and 6 post-infection (p.i.), respectively (Fig. 4). Virus titres in the lungs of formalin- and UV-A/PC immunized mice were comparable to those

of unvaccinated control mice. In contrast, the γ -A/PC vaccinated group had a 2 log reduction of A/PR/8 lung virus titres at both days 3 and 6 post-challenge ($P < 0.05$ using Student's *t*-test) when compared with unvaccinated control mice.

Effect of virus inactivation on the ability to elicit cytotoxic T (Tc)-cell immunity

We compared the ability of inactivated A/PC virus preparations to induce influenza virus-immune Tc-cell responses. BALB/c mice were intravenously (i.v.) immunized with live (10^7 p.f.u.) or inactivated (10^8 p.f.u. equivalent of γ -, formalin- or UV-) A/PC, and splenocytes were harvested 7 days post-immunization and tested for their killing activity against A/PC-infected P815 target cells. Our data show that effector splenocytes harvested from mice immunized with live or γ -A/PC resulted in lysis of A/PC-infected target cells, whereas splenocytes from formalin- or UV-A/PC immunized mice did not (Fig. 5a). For i.n. vaccination, we used a sublethal dose of A/PC to test Tc-cell responses in the lung. Similar results were obtained

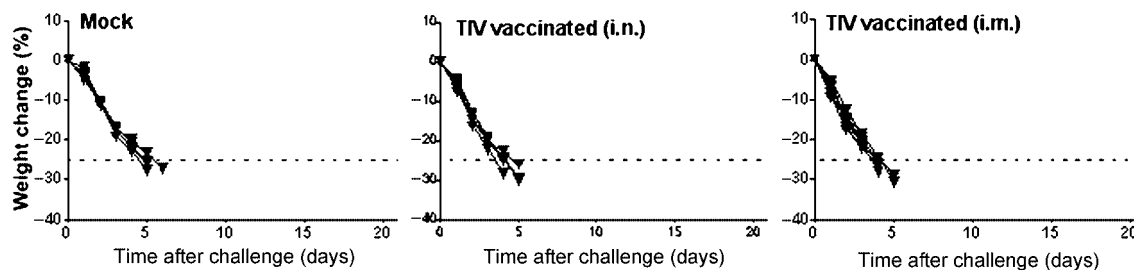
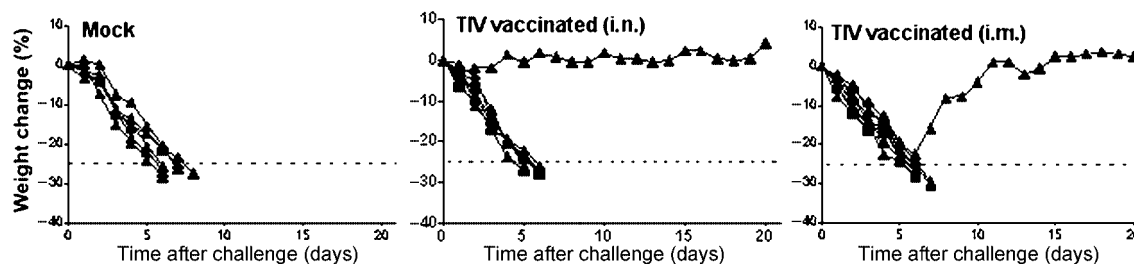
(a) Challenge with A/PC**(b) Challenge with A/PR8**

Fig. 3. TIV fails to provide protection against drifted strains of influenza virus. Mice were immunized once i.n. or i.m. with TIV (CSL Fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; 3 μ g haemagglutinin). Three weeks post-immunization, naïve and immunized mice were i.n. challenged with either A/PC (a) or A/PR/8 (b). Mice were monitored for 20 days for body weight and a loss of 25% of total body weight was used as the end point.

following i.n. vaccination (Fig. 5b). Therefore, γ -irradiated-, but not formalin- or UV-inactivated, virus induces Tc-cell immunity.

Protection against high doses of the heterosubtypic challenges

Given the potent protective efficacy of γ -A/PC against heterosubtypic challenge with a lethal dose of A/PR/8 (7×10^2 p.f.u.), we tested the potency of this protective immunity by challenging with doses of up to 50 times a lethal dose of A/PR/8 (3.5×10^4 p.f.u.) (Fig. 6). Immunized mice receiving heterosubtypic challenge of 1 times a lethal dose all survived and there was little or no weight loss (Fig. 6c). Some of the immunized mice showed an initial weight loss when challenged with 5 times or 50 times a lethal dose. However, all vaccinated mice, regained their body weight and fully recovered (Fig. 6d, e). Naïve mice receiving 1 or 5 times a lethal dose progressively lost weight and were sacrificed when they had lost 25% of their body weight (Fig. 6a, b). Thus, i.n. vaccination with γ -A/PC confers potent protection against high doses.

Long-lived heterosubtypic protection

A critical requirement for any effective influenza vaccine is the induction of long-lasting immunological memory. We tested whether mice vaccinated with γ -A/PC maintain

long-lasting heterosubtypic immunity. Mice were vaccinated with γ -A/PC and challenged 3 months later with a lethal dose of A/PR/8. As shown in Fig. 7, most of the age-matched unvaccinated mice lost substantial weight and 7/10 reached the end point of 25% body weight loss at around day 7 p.i. In contrast, only two of the γ -A/PC vaccinated mice showed substantial weight loss, but even these fully recovered from the heterosubtypic challenge.

Freeze-drying does not destroy the immunogenicity of γ -A/PC

A known shortcoming of the current liquid-based influenza vaccines is the requirement for refrigerated storage that imposes problems for vaccine distribution, particularly in developing countries. In an attempt to overcome the stringent storage requirement of the current influenza vaccines, we assessed freeze-drying γ -ray-inactivated influenza virus as a means to curtail refrigeration requirements. Freeze-dried γ -A/PC was reconstituted with distilled water prior to i.n. vaccination. All mice vaccinated with such reconstituted freeze-dried influenza preparations survived a heterosubtypic challenge with A/PR/8 as compared with 10% survival in naïve control mice (Fig. 8). In addition, the majority of vaccinated mice lost less than 10% of their body weight and only two mice lost >10% of their body weight and showed only mild symptoms of morbidity prior to full recovery. These data

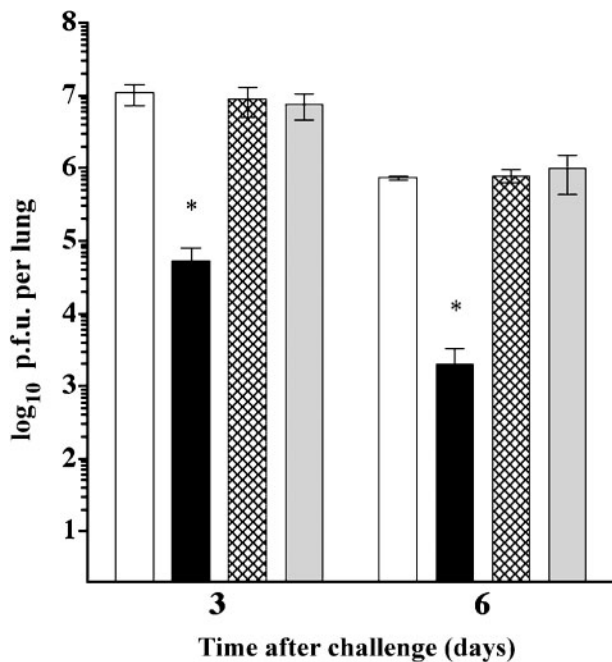


Fig. 4. Vaccination with γ -A/PC leads to early clearance of heterosubtypic viruses. Three weeks following i.n. vaccination with formalin- (diagonally hatched bars), UV- (grey bars) and γ -A/PC (black bars), vaccinated and mock-control (empty bars) mice were i.n. infected with A/PR/8. On days 3 and 6 p.i., three mice per group were sacrificed and the viral titres in lungs determined by the plaque assay. * $P < 0.05$ Student's *t*-test.

provide evidence that freeze-dried γ -influenza-A-virus preparations are suitable for an effective vaccine.

DISCUSSION

Previously, we have reported on the use of γ -irradiation as a means to prepare inactivated influenza vaccines and that such vaccines are highly effective against not only human heterosubtypic strains (Müllbacher *et al.*, 1988) but also against avian H5N1 (Alsharifi *et al.*, 2009). The current study evaluated, in a comparative setting, the protective efficacy of three types of inactivation regimens (γ -irradiation, formalin and UV inactivation) to assess whether the currently used chemical inactivation method, used since 1945, is the most suitable choice for influenza vaccine manufacture.

We have shown that γ -A/PC has superior immunogenicity compared with all the other preparations, including a presently commercially available subvirion vaccine, and confers a high level of protection against both homotypic and heterosubtypic infections. This superior protection was reflected in 100% survival and lower weight loss in γ -A/PC-vaccinated mice when compared with formalin- or UV-A/PC-vaccinated and naïve mice. This protection manifested itself with reduced lung inflammation and

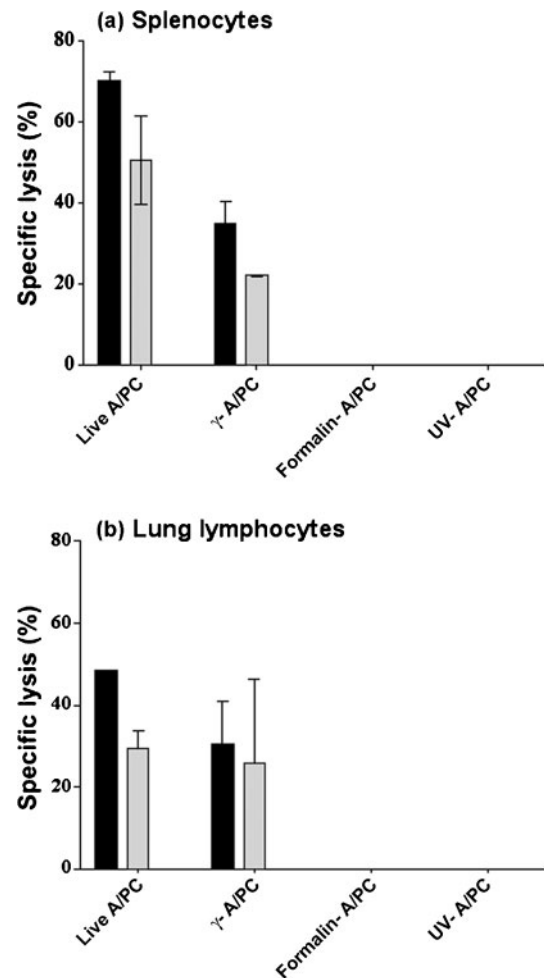


Fig. 5. Tc-cell responses induced by live and inactivated A/PC. Mice were immunized i.v. (a) or i.n. (b) with live, γ -irradiated, formalin- or UV-inactivated A/PC. Splenocytes (a) or lung-derived lymphocytes (b) were harvested 7 days post-immunization and were used as effector cells against A/PC-infected (black bars) and A/PR/8-infected (grey bars) PB15 target cells (empty bars are mock target cells). Mean values \pm SD of two mice per group are shown. Specific lysis values were interpolated from regression curves at an effector : target ratio of 50 : 1.

reduced lung viral loads. In addition, single doses of a currently used TIV provided no protection against A/PC or A/PR/8 challenges irrespective of whether the vaccine was given by i.n. or i.m. injection as at present given to humans. This is despite the fact that the currently used TIV contains the surface glycoproteins of antigenic variants to what might have been the parental strains.

High levels of protection similar to that afforded by γ -A/PC can be induced with formalin-A/PC when threefold higher doses (9.6×10^6 p.f.u. equivalent or 2300 HAU) and multiple immunization are given. However, even under such severe regimes, protection is conferred only against homologous but not against heterosubtypic virus challenge.

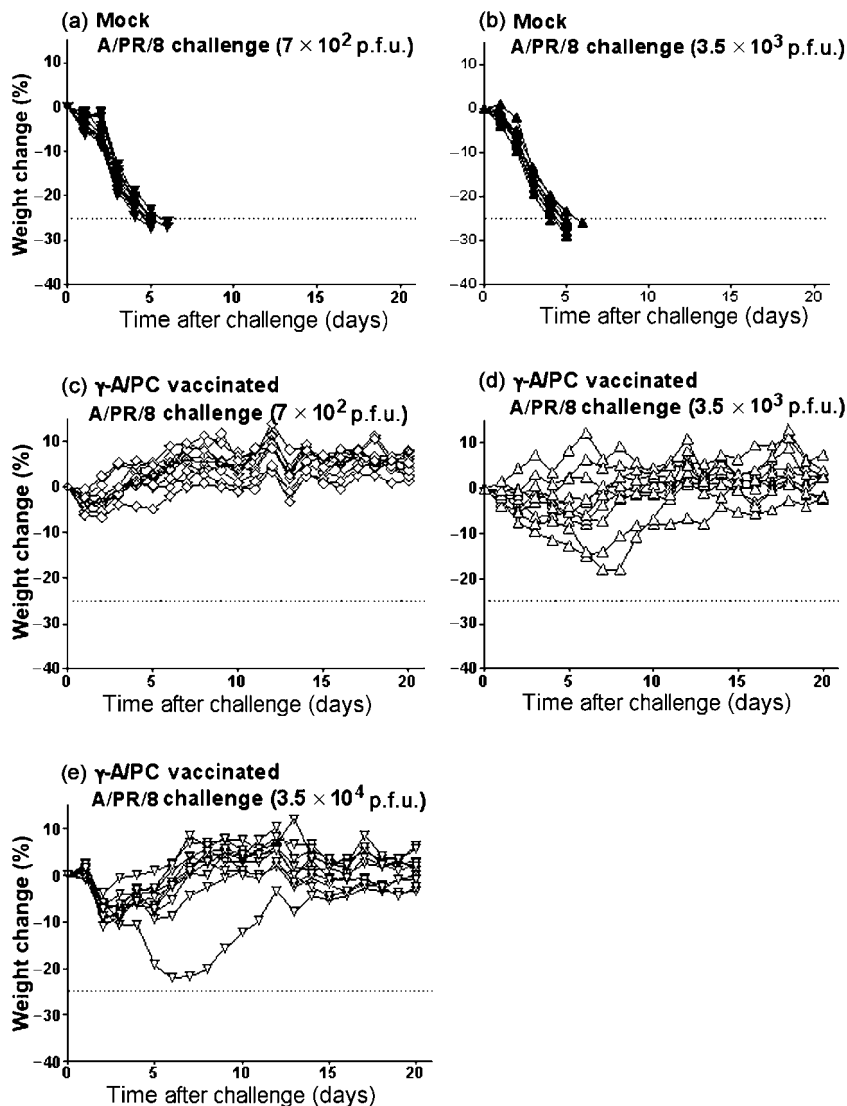


Fig. 6. I.n. immunization with γ -A/PC provides protection against lethal challenges with high doses of A/PR/8. BALB/c mice were mock treated (a, b) or immunized i.n. with γ -A/PC (c–e). Three weeks post-immunization, mice were i.n. challenged with A/PR/8 either 7×10^2 p.f.u. (a, c), 3.5×10^3 p.f.u. (b, d) or 3.5×10^4 p.f.u. (e). Mice were monitored for 20 days for their body weight and a loss of 25% of total body weight was used as an end point.

Therefore, increased dose and/or frequency of immunization does only improve the strain-specific immunity of formalin-A/PC preparations. Importantly, our data indicate that γ -ray-inactivated virus is far more immunogenic than formalin-inactivated virus per virus particle since formalin-inactivated virus preparation required three times the p.f.u.-equivalent dose for a comparable HAU dose and triple immunizations, compared with a single dose of γ -irradiated A/PC, to obtain strain-specific immunity. These findings provide evidence that γ -irradiated virus retains superior antigenicity and immunogenicity relative to the other inactivation procedures. Thus γ -ray-inactivated virus induced immunity that was not only quantitatively but also qualitatively superior to virus preparations inactivated by formalin treatment or UV-irradiation.

It is noteworthy that others have reported cross-protective efficacy of formalin-inactivated influenza vaccines (Takada *et al.*, 1999, 2003; Tumpey *et al.*, 2001) and conventional TIV preparations (Ichinohe *et al.*, 2007). However,

multiple, high-dose, immunization regimens (with or without adjuvants) were used in those studies. In the event of a pandemic, a single-dose regimen, as indicated to be achievable by γ -irradiated virus, would be highly desirable. Moreover, the fact that i.n. administration of γ -ray-inactivated influenza virus in the absence of adjuvants elicits potent protection suggests that reactogenicity may not be a problem. Furthermore, studies that reported cross-protection using chemically inactivated virus preparations did not test the duration of the cross-protective immunological memory nor the immunological mechanism responsible for it. The efficacy of γ -irradiated influenza virus is highlighted by the fact that after a single dose of i.n. administration, mice were able to resist heterosubtypic challenge doses for up to 50 times a lethal dose, and after time lags for at least 3 months, underscoring the robust immunity induced.

In our previous study, we highlighted the importance of the anatomical priming site for effective induction of

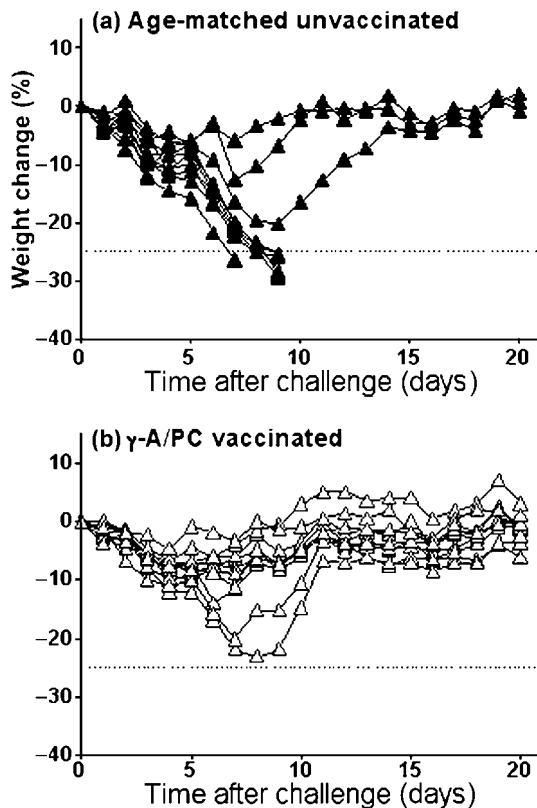


Fig. 7. Vaccination with γ -A/PC induces a long-lasting cross-protective immunity. BALB/c mice were either mock treated (a) or immunized i.n. with γ -A/PC (b). Three months post-immunization, mice were challenged i.n. with A/PR/8. Mice were monitored for 20 days for body weight and a loss of 25% of total body weight was used as the end point.

heterosubtypic protection (Alsharifi *et al.*, 2009). While, some secretory IgA antibodies are capable of intracellular neutralization of influenza virus during transcytosis into the infected epithelial cells (Mazanec *et al.*, 1992, 1995a, b), we speculated that this cross-protection is mediated by mucosal Tc-cell responses (Alsharifi *et al.*, 2009). The i.n. or mucosal route is known to be the only route by which cross-reactive Tc cells can be generated in the mucosa-associated lymphoid tissues from which Tc cells are recruited to the lungs during infections (Nguyen *et al.*, 1999). Our data clearly demonstrate that i.n. vaccination with γ -irradiated influenza induces a strong local influenza immune Tc-cell response cross-reactive on heterosubtypic virus-infected targets (Fig. 5). This shows that antigens from γ -ray-inactivated viruses do enter the class-I major histocompatibility complex antigen presentation pathway and elicit a Tc-cell response and that these cross-reactive Tc cells may be responsible for the cross-protection observed here. All other forms of inactivated influenza viruses are unable to prime for influenza-immune Tc-cell responses (Braciale & Yap, 1978; Müllbacher *et al.*, 1988). Moreover, in line with previous reports (IAEA, 1973; Lowy *et al.*,

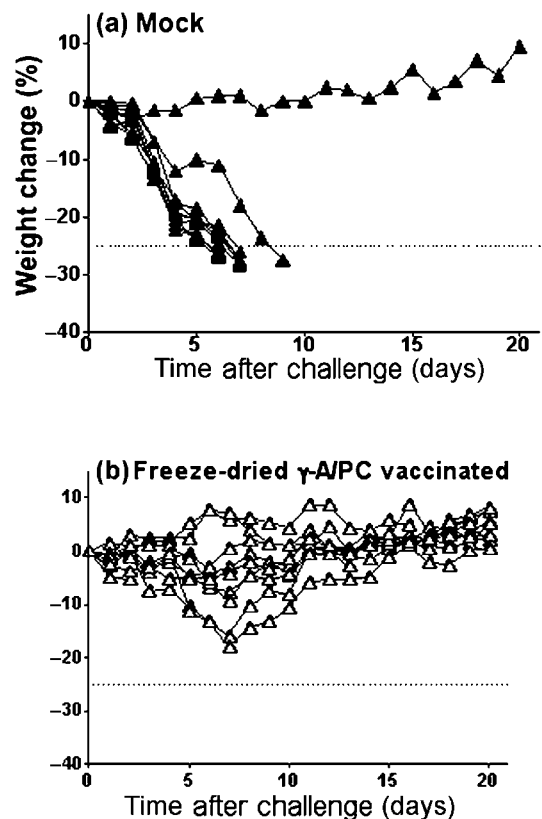


Fig. 8. Freeze-drying of γ -A/PC does not affect the induction of cross-protective immunity. γ -A/PC stock was freeze-dried and resuspended in distilled water immediately prior to i.n. administration. BALB/c mice were either mock treated (a) or immunized with freeze-dried γ -A/PC (b) and challenged with heterosubtypic strain A/PR/8 at 3 weeks post-immunization. Mice were monitored for 20 days for their body weight and a loss of 25% of total body weight was used as the end point.

2001), γ -ray inactivation has less impact on haemagglutination activity than formalin or UV inactivation. This in itself is of considerable importance as it significantly increases vaccine dose yield during vaccine production. Thus, γ -irradiated viruses retain antigens similar to their native forms, which may partially account for its superior immunogenicity. We are currently pursuing further studies to fully elucidate the mechanism by which γ -ray-inactivated influenza A virus provides protection.

Apart from the observed potent protective efficacy, several additional factors contribute to the attractiveness of the use of γ -irradiation for the production of an influenza vaccine. First, freeze-dried γ -A/PC maintained its ability to induce cross-protective immunity. Since dry powder formulations have been shown to have improved stability compared with liquid formulations under various storage conditions (Ada & Jones, 1986), this would enormously facilitate stable distribution of the vaccine in an event of a pandemic. Second, the i.n. route of delivery, which requires little

training for administration or medically qualified personnel, would provide additional advantages for developing countries. Third, γ -ray-inactivated influenza vaccine would be easy and inexpensive to manufacture when compared with other, presently employed, vaccine production processes. Fourth, unlike live attenuated vaccines, γ -irradiated virus is fully inactivated thereby eliminating the risk of reassortment with newly emerging influenza strain(s) or reversion to a virulent form. Most importantly with regards to manufacturing considerations and availability, the robust heterosubtypic protection induced by γ -ray-inactivated influenza may render annual reformulation of influenza vaccines obsolete.

We have characterized a vaccine candidate that provides long-lasting, cross-protective immunity in mice against all influenza A strains tested. This study demonstrated that sterilization by γ -rays is more effective than the currently used vaccine preparation methods, chemical inactivation, in preserving immunogenicity of the virus and induction of cross-protective immunity.

METHODS

Mice. Nine- to ten-week-old female BALB/c mice were routinely used in these studies. Mice were obtained and housed in Biosecurity Level 2 containment facilities at the John Curtin School of Medical Research, the Australian National University, ACT, Australia. All experimental procedures were approved by the institutional Animal Ethics Committees.

Viruses and cells. P815 mastocytoma, Madin–Darby canine kidney (MDCK) and baby hamster kidney cells were grown and maintained in Eagle's minimal essential medium (EMEM; Gibco) plus 5% fetal calf serum (Bovogen) at 37 °C in a humidified atmosphere with 5% CO₂.

The influenza type A viruses, A/PR/8 [A/Puerto Rico/8/34 (H1N1)] and A/PC [A/Port Chalmers/1/73 (H3N2)] were grown in 10-day-old embryonated chicken eggs (HiChick). Each egg was injected with 0.1 ml normal saline containing 1 HAU of virus, incubated for 48 h at 37 °C, and held at 4 °C overnight. Allantoic fluid was harvested, pooled and stored at –80 °C. Viral titres were 10⁷ p.f.u. ml⁻¹ (A/PC) and 2 × 10⁸ p.f.u. ml⁻¹ (A/PR/8) by using plaque assays on MDCK cells. Viruses were purified using chick erythrocytes as previously described (Sheffield *et al.*, 1954). Briefly, infectious allantoic fluid was incubated with red blood cells (RBCs) for 45 min at 4 °C allowing the haemagglutinin to bind to erythrocytes, and then centrifuged to remove the allantoic fluid supernatant. The pellets were resuspended in normal saline, incubated for 1 h at 37 °C to release the RBCs from the virus and then centrifuged to remove the erythrocytes and the supernatant containing the virus collected. Purified A/PC stock with a titre of 5 × 10⁸ p.f.u. ml⁻¹ was used for virus inactivation.

Virus inactivation. For formalin inactivation, virus stocks were incubated with 0.2% formalin (Merck) at 4 °C for a week (Takada *et al.*, 2003). The formalin was then removed by pressure dialysis using normal saline for 24 h at 4 °C. The dialysis method was adapted from Current Protocols in Immunology (Andrew *et al.*, 2001). For UV inactivation, the virus stocks were placed in 60 mm Petri dishes to a fluid depth of 10 mm and exposed to 4000 ergs cm⁻² from a UV source for 45 min at 4 °C. For γ -ray inactivation, influenza viruses

received a dose of 10 kGy from a ⁶⁰Co source (Australian Nuclear Science and Technology Organization – ANSTO). The virus stocks were kept frozen on dry ice during γ -irradiation. Loss of viral infectivity was confirmed by titration of inactivated virus preparations in embryonated eggs. For freeze-drying, one vial containing 0.5 ml of γ -A/PC was placed in a Manifold Freeze-Dryer (Dura-Dry MP; FTS Systems).

Haemagglutination assay. Live and inactivated virus preparations were serially twofold-diluted in a 100 μ l volume on a 96-well U-bottom microtitre plate. A 0.5% chicken erythrocyte suspension was added to all wells and plates were incubated for 30 min on ice. This method was adapted from Current Protocols in Microbiology (Szretter *et al.*, 2006).

Protection experiment. BALB/c mice were immunized i.n. with 32 μ l per mouse of inactivated virus or inactivated subvirion TIV (CSL Fluvax vaccine; A/Solomon Islands/3/2006 H1N1, A/Brisbane/10/2007 H3N2, B/Florida/4/2006; a total dose of 3 μ g haemagglutinin). Vaccination with formalin-inactivated A/PC involved three doses at weekly intervals. Mice were challenged i.n. at 1–3 weeks post-immunization with a lethal dose of either A/PC (3.2 × 10⁵ p.f.u.) or A/PR/8 (7 × 10² p.f.u.). Mice were monitored for body weight for 20 days post-challenge and a loss of 25% of total body weight was used as an end point. To estimate lung virus titres, three mice per group were humanely culled on days 3 and 6 post-challenge and lung tissue harvested.

Plaque assays. Lung tissue samples were collected at 3 or 6 days after i.n. challenge and homogenized in normal saline. Homogenates were centrifuged at 1500 r.p.m. for 5 min (Eppendorf 5810 R). Supernatants were stored at –70 °C for plaque assays.

Serial dilutions of lung samples were pipetted on MDCK cells monolayers in six-well tissue culture plates. After 1 h adsorption, the monolayers were overlaid with EMEM containing 1.8% Bacto-Agar (BD Biosciences) and incubated for 2–3 days. Cell monolayers were stained with 2.5% crystal violet solution (BDH) and the plaques enumerated.

Tc lymphocyte (Tc cell) assays. BALB/c mice were immunized i.v. (200 μ l per mouse) or i.n. (32 μ l per mouse) with live or inactivated (γ -irradiated, formalin- or UV-inactivated) A/PC. Seven days post-immunization, RBC-depleted splenocytes or Percoll-purified pulmonary lymphocytes were used as effector cells in ⁵¹Cr-release assays, as described previously (Müllbacher *et al.*, 1991). P815 target cells (1 × 10⁶ cells) were infected with 1 p.f.u. per cell of live A/PC or A/PR/8 and incubated for 1 h in the presence of 100 μ Ci (3.7 MBq) ⁵¹Cr ml⁻¹. Targets were washed twice and incubated with effector cells at different ratios in a 8 h ⁵¹Cr-release assay. The level of radioactivity in the supernatant was measured using Top Counter (Perkin Elmer). Specific lysis is given as the mean percentage lysis of triplicate wells and values were calculated using the formula: (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

ACKNOWLEDGEMENTS

We would like to thank Connie Banos and Justin B. Davies from ANSTO for providing excellent services. We would also like to thank Dr Tuckweng Kok at the Institute of Medical and Veterinary Science, Adelaide, Australia for providing CSL-made TIV (Fluvax). This work was supported in part by a grant from the NH and MRC (grant no. 410227). The granting agency had no role in study design, data collection and analysis, design to publish or preparation of the manuscript.

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