The IgG Subclass Responses to Influenza Virus Haemagglutinin in the Mouse: Effect of Route of Inoculation

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SUMMARY

The influence of the route of infection, regime of inoculation and virus preparation on murine IgG subclass responses to the haemagglutinin of influenza A virus were examined. Virus preparations inoculated by the intraperitoneal, intravenous, intramuscular and intranasal routes were found to induce different IgG subclass profiles after a primary and secondary dose. IgG2a was found to be a major contributor to the responses elicited by all virus preparations irrespective of the route of inoculation. The magnitude of the response varied with the number of doses of virus and the route of inoculation; the intravenous and intramuscular routes produced the largest responses after two doses of virus. Evidence for the local production of antibody is presented and the influence of antigen presentation on the induction of different subclasses is discussed.

INTRODUCTION

Immunoglobulins of various subtypes, e.g. G, A and M have been shown to have neutralizing activity against animal viruses (Ogra et al., 1975). Taylor & Dimmock (1985a, b) have found large differences between the abilities of monomeric (IgG and IgA) and oligomeric (IgM and secretory IgA) antibody to neutralize virus. The levels of antibody isotypes stimulated by inoculation of influenza virus depends upon the route, dose and antigenic form of the virus (Webster, 1965, 1968; Scott & Sydiskis, 1976; Tannock et al., 1984). The generation of antibody in response to influenza virus infection or inoculation in the animal model plays a primary role in host protection (Schulman, 1975; Virelizier, 1975; Liew et al., 1984; Balkovic & Howard, 1986) and with its widespread occurrence in the bloodstream, lymphatic system and peripheral body fluids the antibody isotype IgG is likely to be the most important in neutralizing virus (Virelizier et al., 1979).

In the mouse, four isotypes of IgG designated IgG1, IgG2a, IgG2b and IgG3 have been identified (Fahey et al., 1964; Grey et al., 1971; Potter, 1972). All four vary in their biochemical and physiological properties (Spiegelberg, 1974; Dorrington, 1978; Winkelhake, 1978; Burton, 1985). The IgG subclass induced in response to different viral antigens, including influenza virus (Coutelier, 1987; Lemercier et al., 1977; Liew et al., 1984) is therefore important when considering the mechanisms by which antigen is neutralized (Dimmock, 1987).

In previous work we have examined the distribution of murine IgG subclasses induced in response to influenza virus preparations and found that IgG2a predominates (Hocart et al., 1988). However, the responses observed could be a reflection of the route of inoculation, the virus preparation used or the schedule of inoculation. Therefore, the aim of this study was to examine the effect that dose, route and virus preparation has on the IgG subclass responses induced by influenza A virus in a mouse model system.

METHODS

Viruses. Viruses used in this study were A/Queensland/6/72 (H3N2) wild-type (wt) and a cold-adapted (ca) derivative of the same virus (both were kindly supplied by Dr G. Tannock, Faculty of Medicine, University of
Purification of haemagglutinin (H3). H3 from wt was prepared by incubating virus at room temperature with 1 to 1.5 % (v/v) NP40 for 60 to 70 min. After centrifuging to clarify the solution, the resulting supernatant was layered onto continuous 5 to 20 % (w/v) sucrose gradients containing 0.1% (v/v) NP40. The gradients were centrifuged for 7.5 h at 260 000 g and then separated into 13 fractions. Those fractions shown to contain HA activity were further purified on an oxamic acid-agarose (Sigma) affinity column to remove any contaminating neuraminidase (Phelan et al., 1980). NP40 was then removed with Bio-Beads SM-2 beads (Bio-Rad) by the method of Holloway (1973) and the resultant sample was dialysed against PBS. The H3 was then analysed for purity by HA, neuraminidase assay and SDS-PAGE (as described previously by Hocart et al., 1988).

Neuraminidase assay. The neuraminidase activity of samples was determined using N-acetylneuraminic–lactose (Sigma) as substrate according to the method of Cuatrecasas and Iliani (1977).

Mouse inoculations. Ten-week-old CBA/CaH mice, obtained from the Animal Resource Centre of Western Australia, were used throughout these studies. At the time of inoculation every mouse was anaesthetized by an intraperitoneal (i.p.) injection of 0.15 to 0.2 ml of pentobarbitone sodium (Ceva Chemicals) in PBS. Three different virus inocula were used as follows: the wt virus and the ca virus were inoculated i.p., intramuscularly (i.m.) and intravenously (i.v.) and the purified H3 was inoculated i.p., i.v. and intranasally (i.n.). A dose of 100 HA units (0.3 μg protein) of wt and ca viruses and 100 HA units (0.075 μg protein) were given to individual mice. Virus inocula were instilled i.n. in a total volume of 0.05 ml using a calibrated 0.025 ml dropping pipette. All other inoculations were delivered using a 1 ml syringe in a total volume of 0.1 ml. Control mice received the appropriate volumes of PBS. All mice were reisoanesthetized 3 weeks after the primary injection of virus.

Preparation of lung, nasal and serum samples. Nasal and salivary secretions were stimulated by injecting anaesthetized mice i.m. with 0.1 ml of a 2 % (w/v) solution of pilocarpine hydrochloride solution in 0.5 % (v/v) hypromellose (Holt et al., 1987). The resulting secretions were collected over a 5 to 7 min period. The mice were then bled, killed and their lungs lavaged with 1 ml of PBS. Individual samples from five mice per time point were stored at −20 °C until analysed and the results were expressed as a mean and standard deviation.

Albumin content. The albumin content of lung lavages and pilocarpine-induced salivary secretions was assessed using plates coated with rabbit anti-mouse albumin (Nordic Immunological Laboratories) in an ELISA capture assay. The bound albumin was detected using a rabbit anti-mouse albumin–alkaline phosphatase conjugate (prepared by the method of Voller et al., 1976). Purified mouse albumin (Nordic Immunological Laboratories) was used as a positive control.
RESULTS

Serum IgG responses to i.p. and i.v. inoculations

Intraperitoneal

Intraperitoneal inoculation of wt virus lead to an IgG2a response that was at least 10-fold higher than those of the other three subclasses. A slight boosting of IgG2a levels was observed after a secondary inoculation of virus (Fig. 1a). The ca virus produced a similar response dominated by IgG2a except that initially an IgG1 component was absent and the levels of the other subclasses were 100-fold less than IgG2a. By 7 weeks after primary inoculation IgG1 was present and after a second dose of virus increased IgG1 levels were seen (Fig. 1b). The purified H3 (Fig. 1c) generated responses which were lower in magnitude than those due to the wt and ca viruses. Three weeks post-inoculation IgG2a and IgG2b constituted the IgG response and at 7 weeks post-inoculation all four subclasses were present. After a second dose of virus at 8 weeks, IgG1 predominated a response consisting of IgG1 and IgG2a.

Intravenous

Intravenous inoculation of wt virus produced an IgG subclass profile similar to that seen when inoculation was by the i.p. route, i.e. a response where the levels of IgG2a are at least 10 to 20-fold greater than other subclasses detected (Fig. 1d). A response with all four subclasses present occurred only after a second inoculation of virus, but the level of IgG2a was still 10 to 20-fold greater than that of any other subclass. At 3 and 7 weeks post-primary inoculation with the ca virus, a response involving all four subclasses all at equal levels was seen (Fig. 1e). Only after a second dose of virus did IgG2a show slightly elevated (five-fold greater) levels. This is in contrast to the response elicited when ca virus was given i.p. where clearly higher levels of IgG2a were observed. Purified H3 given i.v. produced an initial response consisting of IgG2a, IgG1 and IgG2b in that order of magnitude (Fig. 1f). By 7 weeks post-primary inoculation IgG3 was present and the levels of IgG1 and IgG2b were still less than IgG2a. After a secondary
inoculation, the magnitude of all subclass responses was boosted but the differences between the levels of IgG2a, IgG2b and IgG3 were increased whereas the level of IgG1 remained only two fold lower than that of IgG2a. This was in contrast to i.p. inoculation of purified H3 in which there was little boosting of the levels of IgG1 and IgG2a (the only components of the response).

**Lung IgG responses to i.p. and i.v. inoculations**

**Intraperitoneal**

Lung responses to the inoculation of wt were composed of all four subclasses (Fig. 2a) with IgG1 attaining the same levels as all the other three subclasses by 7 weeks post-inoculation. A secondary inoculation boosted levels of IgG2a and IgG2b but IgG1 and IgG3 disappeared from the response. The ca virus caused a response very similar to that due to the wt virus with all four subclasses present at equivalent levels (Fig. 2b). A second dose of ca virus boosted IgG2a levels to a value at least 10-fold higher than IgG1 and IgG2b, with IgG3 having decreased to undetectable levels. In contrast, the response of mice inoculated with purified H3 at 3 weeks post-primary inoculation involved IgG3 and IgG2a only, with higher levels of IgG3 than IgG2a (Fig. 2c). By 7 weeks post-primary inoculation all four subclasses were present at equivalent levels. Secondary inoculations produced a subclass response devoid of an IgG1 component and with lower levels of the other three subclasses compared with levels after primary inoculation.

**Intravenous**

The i.v. primary inoculation of wt virus produced a response at 3 weeks consisting of all four subclasses (Fig. 2d). By 7 weeks post-primary inoculation IgG2a and IgG2b at similar levels constituted the IgG response. After a second dose of virus all four subclasses were induced with the level of IgG2a being five-to-10-fold higher than those of IgG2b and IgG3. Intravenous inoculation with the ca virus elicited the production of all four subclasses at equal levels by 3 and 7 weeks after primary inoculation (Fig. 2e). A second dose of ca virus increased IgG2a levels by
IgG subclass responses

Fig. 3. Saliva IgG subclass responses to the haemagglutinin from i.p. (a, b, c) and i.v. (d, e, f) inoculated mice. Panels (a) and (d) describe the responses to wt virus, panels (b) and (e) to ca virus, and panels (c) and (f) to purified H3 haemagglutinin. All mice were reinoculated (↓) at 8 weeks post-primary inoculation. IgG1 (■), IgG2a (□), IgG2b (■) and IgG3 (□).

10-fold, retaining IgG2b at primary inoculation levels and decreasing IgG1 and IgG3 levels. Purified H3 induced all four subclasses (Fig. 2f) and by 7 weeks post-primary inoculation all four subclasses were still present at equal levels. A second dose of purified H3 had no effect on the composition of the IgG subclass response with levels of the IgG subclasses remaining at primary inoculation levels.

Saliva IgG responses to i.p. and i.v. inoculations

Intraperitoneal

Both the wt and ca viruses induced all four IgG subclasses after a primary inoculation, the only difference being that the IgG2b response induced by the wt virus was lower than for the ca virus-inoculated mice (Fig. 3a and b). All four subclasses were still present at 7 weeks post-primary inoculation for the ca virus but a second inoculation of virus produced a response consisting of only IgG2a and IgG2b at 14 weeks post primary inoculation. A similar phenomenon was seen for the wt virus where a second dose of virus elicited only two subclasses, IgG2a and IgG3. At 3 and 7 weeks after primary inoculation with purified H3, IgG2a and IgG2b were the only IgG subclasses present (Fig. 3c). After a second dose of purified H3 at 8 weeks post-primary inoculation, IgG1 and IgG3 appeared but the level of IgG2a fell and IgG2b was absent from the response.

Intravenous

Responses seen in the saliva of mice inoculated i.v. with wt virus had a subclass profile over time opposite to that seen in i.p., wt-inoculated mice (Fig. 3d and a respectively). A primary dose of wt virus inoculated i.v. produced IgG2a and IgG3 initially but by 7 weeks post-primary inoculation only IgG2a remained. A second dose of virus produced all four subclasses with IgG2b and IgG2a being the largest contributions. The ca virus in contrast, produced responses dominated by IgG2a (Fig. 3e). The other subclasses were induced at levels 10 to 100-fold less
Fig. 4. IgG subclass responses to the haemagglutinin from i.m. inoculation of wt (a, b, c) and ca (d, e, f) inoculated mice. Serum responses are shown in panels (a) and (d), lung responses in panels (b) and (e) and saliva responses in panels (c) and (f). All mice were reinoculated (↓) at 8 weeks post-primary inoculation. IgG1 (■), IgG2a (□), IgG2b (▲) and IgG3 (●).

Intramuscular inoculations

Serum responses

The response to wt virus was predominated by IgG2a (Fig. 4a) with levels which were 10 to 100-fold greater than those of the other subclasses detected at any time during the trial. IgG3 was absent from the response except at 7 weeks post-primary inoculation and levels of IgG1, IgG2a and IgG2b were boosted by a secondary inoculation of virus. The ca virus produced a similar pattern of responses except that levels of IgG2a had fallen to those of IgG1 and IgG2b by 7 weeks post-primary inoculation (Fig. 4d). However, upon reinoculation of ca virus levels of IgG2a were boosted to more than 100-fold greater than the levels of any other subclass, IgG3 was induced and IgG1 and IgG2b remained at primary inoculation levels.

Lung responses

All four subclasses were present in the response to ca virus seen in the lung at 3 and 7 weeks post-primary inoculation (Fig. 4e). A second inoculation of ca virus boosted IgG2a levels but IgG1 and IgG2b remained at primary inoculation levels with no IgG3 present. In contrast, wt virus induced IgG1, IgG2b and IgG3 initially but by 7 weeks post-inoculation IgG3 was undetectable (Fig. 4b). After a second dose of wt all four subclasses appeared in the response with IgG2a predominating at levels 10 to 20-fold higher than the other subclasses.

Saliva responses

All four subclasses were induced by wt and ca viruses at 3 weeks post-primary inoculation with the ca virus producing a response of higher magnitude (Fig. 4c and f respectively). At 7
weeks post-inoculation all four subclasses remained present in ca-inoculated mice but only IgG1 and IgG2b were detected in wt virus-inoculated mice. A secondary inoculation of virus induced all four subclasses in wt-inoculated mice with IgG2a and IgG2b at higher levels. Upon reinoculation with ca virus all subclass levels rose slightly except that of IgG3 which was five to 10-fold lower.

Intranasal inoculations

Serum

Purified H3 induced all four subclasses and these were present at equal levels by 7 weeks post-primary inoculation (Fig. 5a). A secondary inoculation eliminated IgG1 and IgG2a from the response leaving only IgG2b and IgG3.

Lung

As in the response detected in serum all four subclasses were present in the lung 3 and 7 weeks post-primary inoculation. The second dose of virus given at 8 weeks after primary inoculation maintained the response of all four subclasses (Fig. 5b).

Saliva

IgG2a was absent from the response detected at 3 weeks post-primary inoculation but by 7 weeks post-primary inoculation IgG2a was detectable at levels equivalent to those of the other three subclasses. Secondary inoculations of purified H3 failed to induce significantly higher levels of the four subclasses; the levels remained the same as at 7 weeks post-primary inoculation (Fig. 5c).

Albumin levels

Albumin levels in lung lavage fluids were 0.1 to 0.025 % of those found in serum indicating that the contamination of lung lavage samples with serum albumin was minimal. It was assumed therefore that damage to the lung during sampling was minimal and that contamination of lavage samples with serum antibodies was negligible.

DISCUSSION

This study was designed to determine if the route of inoculation, the schedule of inoculation, and the virus preparation used significantly influenced the influenza A/Queensland/6/72 H3 antigen-specific IgG subclass profiles detected in mice. The results obtained indicate the following. (i) IgG2a was the predominant subclass produced in response to the H3 antigen when mice were inoculated with wt or ca viruses. (ii) The maximum level obtained for any IgG subclass was dependent upon the route and schedule of inoculation as well as the virus preparation used. (iii) There was evidence for the local production of IgG subclasses in the lung.
and saliva of inoculated animals. In addition, local production of IgG subclasses was observed in a previous study when wt or ca viruses were inoculated i.n. (Hocart et al., 1988). (iv) The mechanisms for the control of IgG subclass production appear to be influenced by the virus preparation and route of inoculation.

Studies by Lemercier et al. (1977), Liew et al. (1984), Daeron et al. (1982), Randrupthomsen et al. (1985), Coutelier (1987) and Balkovic et al. (1987) all indicate the importance of IgG2a in mice subjected to viral infections including influenza, and in protection studies using monoclonal antibodies Tao et al. (1987) found that the most effective antibody against H3N2 viruses was of the IgG2a isotype. The importance of IgG2a in responses to influenza virus are undoubtedly related to its structure and effector functions such as its ability to activate complement, its binding to cells such as macrophages and its ability to induce Fc receptors on cells (Winkelhake, 1978; Spiegelberg, 1974; Ezekowitz et al., 1983; Burton, 1985). The route and schedule of inoculation were seen to affect the induction and levels of the four subclasses in serum, lung and saliva in this study and Webster (1965, 1968a, b) observed a similar phenomenon in rabbits using u.v.-inactivated virus and various inoculation regimes. In the same studies it was also found that the avidity of IgG antibodies varied with the virus preparation used and inoculation regime.

The predominance of IgG2a seen after the inoculation of wt and ca viruses could possibly point to the more efficient clearance of virus by this subclass in serum and on mucosal surfaces. It has been found that monoclonal antibodies of isotypes IgG1, IgG2a and IgG2b from BALB/c mice vary in their susceptibility to proteolysis by pepsin and other proteases (Parham, 1983). In BALB/c mice IgG1 was the most resistant to fragmentation followed closely by IgG2a and IgG2b. Therefore differing quantities of each IgG subclass may be secreted onto mucosal surfaces, where proteolytic enzymes abound and, depending upon the susceptibility of the different IgG subclasses to fragmentation and inactivation by these enzymes, are able to inactivate virus.

However, generalization of these results to other strains of mice should not be made as the subclass response may be strain-specific as was found by Perlmutter et al. (1978) and Moreno & Esdaile (1983). Also the efficiency of neutralization of virus by individual IgG subclasses has not been studied in the murine model and may not be affected by the subclass present in highest concentration in serum or on mucosal surfaces. In humans Mathiesen et al. (1988) have been able to correlate the neutralizing activity of individually purified IgG subclasses from the sera of herpes simplex virus type 1 (HSV-1) seropositive donors and the serum levels of each subclass. It was found that both HSV-1 ELISA titres and neutralization titres were highest for the IgG1 subclass. Additional studies in other inbred lines of mice are required along with neutralization studies using purified IgG subclasses and inoculation regimes before generalizations can be made about the role of IgG subclasses in the mouse model.

The appearance of antibody on mucosal surface can occur through local production and secretion of antibody (Jones & Ada, 1986; Owens et al., 1981) or by diffusion from serum (Wagner et al., 1987). The resultant level of antibody is probably a function of both of these mechanisms depending upon the ability, which is unknown at present, of the four subclasses to pass across mucosal membranes. Our results are indicative of local production of antibody in both lung and saliva with the appearance on mucosal surfaces of varying isotypes of IgG not present in serum at the time of sampling.

The mechanism by which cells located near mucosal surfaces or elsewhere are stimulated to begin production of a particular subclass is unknown. However, from our work in presenting antigen in various forms and by different routes it is apparent that this mechanism is influenced by antigen-specific stimulants as has been shown previously by Siskind et al. (1966), Liu et al. (1974), der Balian et al. (1980), Slack et al. (1980) and Moreno & Esdaile (1983).

REFERENCES


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