JOURNAL OF VIROLOGY, Sept. 1996, p. 6119–6125 Vol. 70, No. 9
0022-538X/96/$04.00+0

Influenza Virus Nucleoprotein-Specific Immunoglobulin G Subclass and Cytokine Responses Elicited by DNA Vaccination Are Dependent on the Route of Vector DNA Delivery

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Received 22 December 1995/Accepted 28 May 1996

Endpoint immunoglobulin G (IgG) titers and cytotoxic T-lymphocyte (CTL) activities were identical between mice immunized via the intramuscular and epidermal (gene gun) routes with 100 and 1 μg, respectively, of an influenza virus nucleoprotein (NP) expression vector. However, examination of the relative levels of two IgG subclasses demonstrated that muscle inoculation resulted in predominantly IgG2a responses, whereas gene gun immunization yielded a preponderance of IgG1 antibodies. Inasmuch as these data suggested that muscle inoculation and gene gun delivery elicited Th1-like and Th2-like responses, respectively, gamma interferon release profiles from antigen-stimulated splenocytes were remarkably similar between these groups. Interleukin-4 (IL-4) production assays, on the other hand, revealed qualitative differences that could be correlated with the divergent IgG subclass data. Waning gamma interferon production in gene gun-immunized animals was countered by a marked increase in IL-4 production following the third immunization, as was the case in control animals immunized with inactivated influenza virus formulated with Freund's adjuvant. In contrast, significant levels of IL-4 production were not observed in the intramuscular DNA inoculation group, despite similar decreases in gamma interferon production with increasing immunizations. These data show that intramuscular inoculation leads to Th1-like responses due to elevated IgG2a levels, production of gamma interferon, CTL activity, and lack of IL-4. However, gene gun responses are more difficult to categorize because of the presence of significant gamma interferon and CTL activity on the one hand and elevated IgG1 antibodies and increasing IL-4 production with successive immunizations on the other. In addition, there was a lack of correlation between IgG isotype ratios and cytokine production in all of the NP DNA-immunized animals, in that IgG subclass ratios remained fixed while cytokine production patterns fluctuated with successive immunizations. These data are consistent with the idea that the types of responses elicited following DNA immunization are dependent on both the identity of the antigen and the route of DNA administration.

DNA immunization offers a means of mimicking the important characteristics of live attenuated viral or bacterial vaccines since they induce the de novo production of microbial antigens leading to the presentation of correctly folded conformational determinants and the induction of major histocompatibility complex class I-restricted cytotoxic T-lymphocyte (CTL) responses. Because plasmid DNA-based vaccines are noninfectious and incapable of replication, they may be regarded as an attractive alternative to the use of live attenuated or live recombinant viruses that generally carry a finite risk of pathogenicity. Recent activity in the development of candidate DNA vaccines has involved two parallel tracks based on the method of delivery. While the first demonstration of a DNA or genetic vaccine involved the intracellular delivery of an antigen-encoding plasmid vector to the skin of mice by using a gene gun (26), a number of subsequent DNA vaccine reports were based on intramuscular or intradermal inoculation of naked plasmid DNA or RNA (2–6, 12, 14, 18, 19, 22, 27–29, 33, 34). Both methods elicit humoral, cellular, and protective immune responses and represent an attractive strategy for the development of a new generation of safe and effective vaccines for a variety of infectious diseases.

Gene gun-based candidate DNA vaccines differ considerably from those delivered by intramuscular or intradermal inoculation in that cutaneous administration using a gene gun is intended to target the intracellular delivery of DNA-coated gold particles to the epidermis (7, 9, 10, 17, 35). This tissue is continually sloughed and is composed of 3 to 4% epidermal Langerhans cells that play an important role in antigen presentation and immunocompetence of the skin (1, 24, 25). Penetration of DNA-coated gold particles beyond the epidermis was shown to result in a decrease in both the levels of antigen expression and resultant immune responses (7). More recent experiments are consistent with a temporal pattern of gene expression and transient maintenance of DNA in the skin following gene gun delivery of DNA to the epidermis, consistent with the regular turnover of this tissue (17a).

Intramuscular inoculation of naked DNA vaccines is based on the transfection of a limited number of muscle fibers, resulting in the expression of the transgene DNA for an extended period of time as a result of the lack of muscle fiber turnover (31). While the mechanism of DNA uptake into muscle fibers is unknown, some groups have succeeded in enhancing plasmid DNA uptake into muscle cells by either predelivering or codelivering certain "facilitators" that result in localized muscle damage and subsequent muscle fiber regeneration (5, 28, 30). The inoculation of DNA into regenerating muscle is correlated with elevated levels of expression and immune responses. Data on the duration of foreign gene expression following intramus-

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cular inoculation are inconsistent and may depend on the identity of the encoded antigen and on whether or not facilitators were used. Although the potential for long-term antigen expression in muscle fibers could play a role in the continued maintenance of specific immune responses following immunization, a transient presence of the immunizing DNAs following vaccination may be more acceptable because of safety concerns.

Direct comparisons between gene gun-based DNA vaccines and those delivered by intramuscular inoculation have been limited to dosage studies examining the strength of responses and levels of protection elicited following the administration of decreasing quantities of DNA (10, 17). These studies showed that the epidermal delivery route is more efficient in terms of dosage requirements, but there have been no reports describing qualitative differences in immune responses between different routes of DNA delivery. In this report, we present a comparative analysis of the types of responses elicited against the influenza virus nucleoprotein (NP) following administration of an NP DNA vaccine via the intramuscular and epidermal routes. The most striking difference was in the relative levels of immunoglobulin G1 (IgG1) and IgG2a antibody responses, in that intramuscular inoculation elicited a preponderance of IgG2a antibodies while gene gun delivery resulted in predominantly IgG1 responses. In addition, significant differences were also observed in the tendency to elicit interleukin-4 (IL-4) production, while gamma interferon (IFN-γ) production patterns were more similar. Finally, cross-boosting experiments demonstrated that neither route was dominant over the other and that the elicitation of predominantly IgG1 or IgG2a responses is determined solely by the route of the primary immunization.

MATERIALS AND METHODS

Immunizations. DNA immunizations via the intramuscular, intradermal, and gene gun routes, using the pCMV-NP vector encoding the production of influenza virus A/PR/8/34 nucleoprotein, were as previously described (17) and were administered to 7-week-old female BALB/c mice. Intramuscular and intradermal immunizations contained 100 μg of vector DNA in 50 μl of sterile saline, while gene gun immunizations contained 1.0 μg of the same DNA coupled to 0.5 mg of 0.95-μm-diameter gold particles. Gene gun immunizations were performed with the handheld, helium-powered Accell gene delivery system (13, 17). Mice immunized with inactivated influenza virus A/PR/8/34 received 100-μl subcutaneous inoculations containing 6,760 hemagglutinating units of formalin-inactivated virus. Freund's complete adjuvant was used for the primary immunization, while Freund's incomplete adjuvant was used for the booster immunizations.

Antibody titer and isotype analysis. Blood collection and NP-specific antibody endpoint titer determinations were as previously described following the coating of 96-well enzyme-linked immunosorbent assay (ELISA) plates with detergent-disrupted influenza virus (9, 17). For determination of the relative levels of NP-specific IgG1 and IgG2a responses, a goat anti-mouse IgG1–alkaline phosphatase conjugate and a goat anti-mouse IgG2a–alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, Ala.), respectively, were substituted for the goat anti-mouse IgG (heavy plus light chain) conjugate described previously. Determination of the relative sensitivities of the IgG1 and IgG2a ELISAs was performed by use of purified IgG1 and IgG2a standard curve samples that were either coated directly onto ELISA plates or captured with a precoated goat anti-mouse Ig capture antibody (Southern Biotechnology Associates). Similar results were observed with both methods. The IgG2a ELISA used for Fig. 1 was 3.1-fold more sensitive than the corresponding IgG1 ELISA, while the IgG2a ELISA used for Fig. 2 to 4 was 1.7-fold more sensitive than the corresponding IgG1 assay.

CTL assays. CTL responses to influenza virus NP were measured as previously described (9, 17) except that the synthetic NP peptide, TYQRTRALV (21), was substituted for the human immunodeficiency virus type 1 gp120 peptide in the target cell preparation step. In addition, the in vitro stimulation step used formalin-inactivated influenza A/PR/8/34 virus rather than the gp120 peptide.

Quantification of IFN-γ and IL-4 production from antigen-stimulated splenocytes. Splenocytes from immunized and control mice were washed twice in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (RPMI-10). Splenocytes were resuspended at a final concentration of 2×10^7 cells per ml in RPMI-10, and 50-μl aliquots were plated into individual wells of a standard 96-well culture plate. Then 150 μl of formalin-inactivated influenza virus (A/PR/8/34) in RPMI-10 was added to each well, for a final concentration of 3,200 hemagglutinating units per well. Plates were covered and incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Following stimulation, 100 μl of medium supernatant was removed from each well and stored at −80°C for subsequent analysis.

For measurement of IFN-γ and IL-4 levels in the supernatants of antigen-stimulated cells, purified rat anti-mouse IFN-γ and rat anti-mouse IL-4 (PharMingen, San Diego, Calif.) were diluted to 2 μg/ml in 0.1 M $NaHCO_3$ (pH 8.2) and used to coat 96-well ELISA plates overnight at 4°C at 50 μl per well. Plates were washed three times with phosphate-buffered saline–0.025% Tween 20 (PBS/T) and blocked by using 250 μl of 2% Carnation nonfat dry milk per well at 37°C for 90 min. Plates were then washed three times with PBS/T, after which samples and standards were added in 100-μl aliquots and incubated at 37°C for 2 h. Plates were then washed three times with PBS/T, and biotinylated second antibodies specific for murine IFN-γ or IL-4 (PharMingen) were added in PBS/T at a final concentration of 2 μg/ml. After 45 min at 37°C, plates were washed three times with PBS/T, and a 1:8,000 dilution of streptavidin-AP (Gibco BRL, Grand Island, N.Y.) in PBS/T was added at 100 μl per well. After 30 min at 37°C, the plates were again washed with PBS/T, developed, and read at 405 nm.

RESULTS

IgG subclass determination. To compare the subclasses of NP-specific IgG antibodies elicited following NP DNA immunization by various routes of delivery, two groups of three BALB/c mice each received primary and booster immunizations spaced 4 weeks apart via intramuscular and intradermal inoculation, respectively, while a third group of four mice received epidermal gene gun immunizations following the same schedule. The relative levels of IgG1 and IgG2a antibodies specific for NP were determined by ELISA analysis of 1:1,000 dilutions of serum samples collected 4 weeks following the primary immunization and 2 weeks following the booster immunization. Figure 1A shows the relative levels of NP-specific IgG1 antibodies in which the strongest responses were seen following gene gun delivery of 1 μg of vector DNA to the epidermis. Intramuscular inoculation of 100-fold more DNA resulted in much less IgG1 activity relative to the gene gun group. Intradermal inoculation of 100 μg of NP vector DNA resulted in responses that were not significantly different than those obtained via gene gun delivery.

Markedly different results were observed upon comparison of NP-specific IgG2a antibody responses between these groups (Fig. 1B). While the relative differences in IgG2a antibodies were not as striking as those observed for IgG1, the strongest IgG2a responses were detected in the intramuscularly inoculated animals. Animals immunized by intradermal inoculation were again more similar to those immunized via the epidermal route.

Given a 3.1-fold-greater sensitivity for the IgG2a ELISA relative to the IgG1 assay (see Materials and Methods), intramuscular inoculation elicited IgG responses that were predominantly of the IgG2a subclass, whereas the gene gun and intradermal routes elicited predominantly IgG1 antibodies. While these data were consistent with the premise that intramuscular DNA inoculation and the cutaneous delivery routes may have elicited Th1-like and Th2-like responses, respectively, additional immunizations were needed to confirm the original IgG subclass observations and to directly examine IFN-γ and IL-4 release profiles and CTL activity as a function of the route and number of immunizations. To this end, three groups of four mice each received one, two, and three gene gun immunizations, respectively, using 1.0-μg doses of the NP vector, while an additional three groups of mice received one, two, and three intramuscular immunizations, respectively, using 100-μg doses of the same DNA preparation. In those animals that were immunized multiple times, the primary, first booster, and second booster immunizations were administered at weeks 0, 4, and 6, respectively. Because of the similarities between the intradermal and gene gun groups shown above, the intrader-

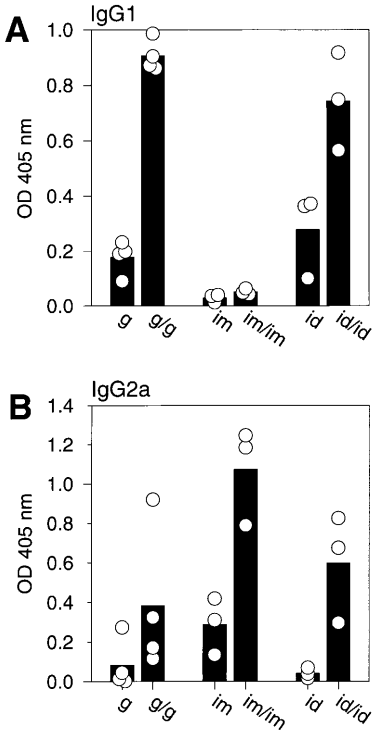

FIG. 1. Measurement of NP-specific IgG1 and IgG2a antibody responses in DNA-immunized mice. Three groups of three to four mice each received primary and booster immunizations with the pCMV-NP vector via the gene gun (g; 1 μg), intramuscular (im; 100 μg), and intradermal (id; 100 μg) routes, respectively. NP-specific IgG1 (A) and IgG2a (B) responses were measured 4 weeks following the primary immunization and 2 weeks following the booster immunization for each animal. Solid bars indicate the arithmetic means; open circles show datum points for individual animals. Repetition of "g", "im", and "id" in the group labels reflects the number of immunizations received. OD, optical density.

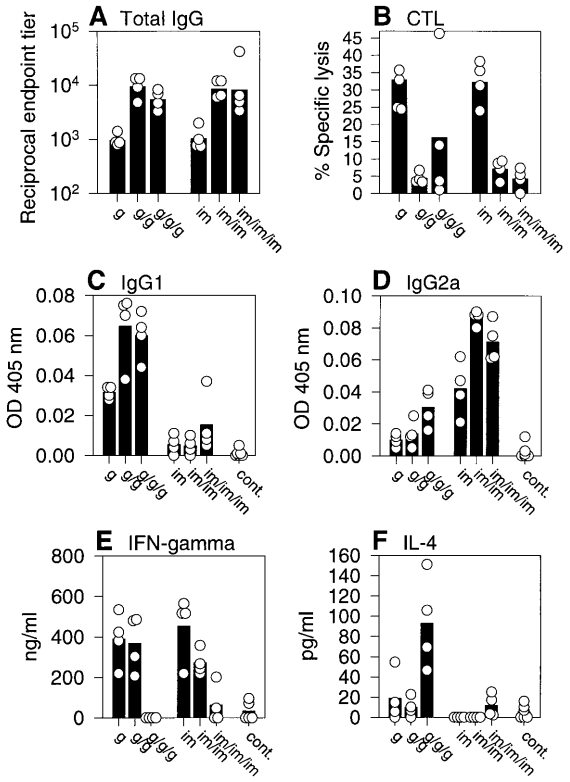

FIG. 2. Measurement of NP-specific antibody, CTL, and cytokine responses in DNA-immunized mice. Three groups of four mice each received one, two, and three immunizations, respectively, via gene gun inoculation of 1 μg of NP vector DNA to the epidermis (g), while an additional three groups of four mice each received one, two, and three immunizations, respectively, via intramuscular inoculation of 100 μg of the NP vector (im). Immunizations were administered at weeks 0, 4, and 6. Serum samples and splenocytes were harvested at weeks 4, 6, and 8 from animals that received one, two, and three immunizations, respectively. (A) Total IgG; (B) CTL activity following in vitro stimulation; (C) IgG1; (D) IgG2a; (E) IFN-γ; (F) IL-4. Solid bars show the arithmetic means except in panel A, where the solid bars indicate the geometric means; open circles show datum points for individual mice. Repetition of "g" and "im" in the group labels indicates the number of immunizations received. cont. represents control animals immunized with irrelevant DNA by a variety of routes. OD, optical density.

mal route was not included in this and subsequent experiments.

Figure 2A shows the total endpoint IgG titers in the six groups of animals. Similar geometric mean titers of approximately 1:1,000 were observed in both the gene gun and intramuscular groups following a primary immunization, with enhanced responses of approximately 1:10,000 being observed in the two groups that received a single booster immunization. Geometric mean titers did not vary significantly between the groups that received two and three immunizations by either route. As with the total IgG data, CTL activities between the gene gun and intramuscular groups were similar. In both cases, CTL activity was highest in those animals that received only a primary immunization and was significantly reduced in those groups that received additional immunizations (Fig. 2B). A similar decline in CTL activity was also reported following successive gp120 DNA immunizations in mice (9) and was correlated with decreasing IFN-γ production (see below).

While similar results were observed between the intramuscular and gene gun groups with respect to total IgG and CTL activities described above, examination of NP-specific IgG1 and IgG2a antibody levels showed marked differences between these groups, confirming the results presented in Fig. 1. Figures 2C and D show the relative NP-specific IgG1 and IgG2a activities, respectively, in the gene gun and intramuscular groups that received increasing numbers of immunizations. With respect to IgG1 induction, gene gun-immunized mice developed much stronger responses than corresponding intramuscularly inoculated animals, whereas IgG2a responses were strongest in the intramuscular groups. Given a 1.7-fold difference in the sensitivities between the IgG1 and IgG2a assays (see Materials and Methods), gene gun immunization again elicited predominantly IgG1 antibodies, while intramuscular inoculation resulted in predominantly IgG2a responses.

Cytokine production. Inasmuch as the IgG1 and IgG2a results shown in Fig. 1, 2C, and 2D are consistent with the elicitation of divergent T helper responses following gene gun and intramuscular inoculation, IFN-γ production patterns following in vitro stimulation of splenocytes were remarkably similar between these routes (Fig. 2E). In both cases, maximal IFN-γ production by antigen-stimulated splenocytes was observed in animals immunized only once, while insignificant levels of this cytokine were observed by the third immunization. This pattern of decreasing IFN-γ production may be related to the decline in CTL activity following the administration of booster immunizations observed previously for a gp120 DNA vaccine (9).

Despite the fact that IFN-γ production patterns were identical between the gene gun and intramuscular groups, IL-4

production patterns were markedly different (Fig. 2F). Significant levels of IL-4 production were observed only in the gene gun animals immunized a total of three times; the same group exhibiting the lowest levels of IFN-γ production. This observation is consistent with a reciprocal or antagonistic relationship that is documented for these cytokines (16). Interestingly, while IFN-γ production was also reduced in the animals immunized three times by intramuscular inoculation, only insignificant levels of IL-4 production were observed in these same animals.

From the observations presented above, it is tempting to classify the intramuscular responses as Th1-like because of the preponderance IgG2a antibodies at all times, initially strong CTL activity and IFN-γ production, and a lack of significant IL-4 production. However, immune responses elicited via gene gun delivery to the epidermis were more difficult to categorize because of the preponderance of IgG1 antibodies occurring simultaneously with CTL and IFN-γ responses and a tendency to switch to IL-4 production with increasing immunizations.

Similarities between gene gun and Freund's adjuvant immunizations. The NP-specific antibody and cytokine responses described above following DNA vaccine administration via the intramuscular and gene gun routes were interesting from the standpoint that the predominant IgG subclasses elicited were different between the two routes, and the identity of the predominant subclass did not change with increasing numbers of immunizations. However, the cytokine release profiles were more dynamic and varied considerably as a function of the number of immunizations. To determine if these patterns were unique to the methods of DNA immunization or could be elicited with a conventional protein-plus-adjuvant immunization regimen, NP-specific IgG and cytokine responses following immunization with inactivated influenza virus formulated with Freund's adjuvant were evaluated. In this case, Freund's complete adjuvant was used in the primary immunization only, while subsequent booster immunizations were formulated with Freund's incomplete adjuvant. Figures 3A and B show the relative levels of IgG1 and IgG2a activity elicited as a function of increasing immunizations, while the IFN-γ and IL-4 production patterns are depicted in Fig. 3C and D, respectively. As was seen with prior gene gun immunizations, the Freund's adjuvant immunizations elicited IgG responses that were predominantly of the IgG1 subclass (note that the IgG2a ELISA was 1.7-fold more sensitive than the IgG1 ELISA). In addition, the pattern of IFN-γ and IL-4 production was remarkably similar to the gene gun data, showing a marked decline in IFN-γ production and a corresponding increase in IL-4 production with increasing numbers of immunizations. The similarity between the Freund's adjuvant and gene gun data demonstrate that the cytokine fluctuation patterns observed with successive immunizations are not unique to the DNA immunization procedures used here. This similarity is also consistent with the possibility that gene gun-based DNA vaccines elicit responses similar to those observed following protein-plus-adjuvant immunization, with the addition of CTL activity.

Lack of correlation between IgG subclasses and cytokine production patterns. Examination of the data from the DNA and Freund's adjuvant immunizations depicted in Fig. 2 and 3 revealed little correlation between the IgG subclass and cytokine results. Following the primary immunization, the gene gun and intramuscular immunization groups responded with essentially identical IFN-γ production levels despite the marked differences in their IgG subclass ratios. Moreover, while the IFN-γ levels dropped markedly in all groups that received successive immunizations, the IgG1-to-IgG2a ratios established with the primary immunization remained relatively

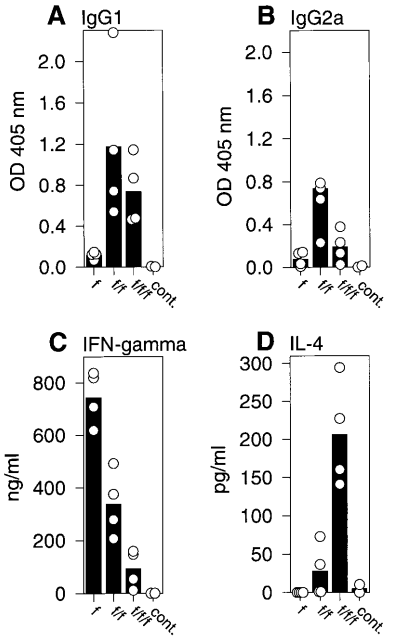

FIG. 3. Measurement of antibody and cytokine responses in mice immunized with inactivated influenza virus formulated with Freund's adjuvant. Three groups of four mice each received one, two, and three immunizations, respectively, with formalin-inactivated influenza virus at weeks 0, 4, and 6. Serum samples and splenocytes were harvested at weeks 4, 6, and 8 from animals that received one, two, and three immunizations, respectively. Solid bars show the arithmetic means; open circles show datum points for individual mice. Repetition of "f" in the group labels indicates the number of Freund's adjuvant immunizations received. cont. represents control animals immunized with Freund's adjuvant alone. OD, optical density.

constant. Even in the gene gun and Freund's adjuvant groups, in which pronounced reciprocity between IFN-γ and IL-4 production was observed with successive immunizations, the initial IgG subclass ratios remained largely unchanged. These observations are consistent with the idea that IgG subclass ratios are fixed by the primary immunization and that booster immunizations, which result in fluctuations in cytokine production patterns, have no effect on the original isotype patterns.

To confirm the above observations regarding the fixation of IgG subclass responses, a DNA immunization cross-boosting experiment was performed. Two groups of eight animals each that were primed via the gene gun and intramuscular routes, respectively, were boosted once or twice with the alternative route to determine if the initial subclass ratios were fixed or could be further modulated. These data, shown in Fig. 4, demonstrate that the initial IgG1 and IgG2a production patterns, established by the primary immunization, were indeed fixed. Animals that received a gene gun primary immunization and either one (g/im group) or two (g/im/im group) intramuscular boosts exhibited predominantly IgG1 responses after each immunization. Similarly, animals that received an intramuscular primary immunization and either one (im/g group) or two (im/g/g group) gene gun boosts developed much more IgG2a activity than IgG1 activity. The striking similarity between the cross-boosting data shown in Fig. 4 and those in Fig. 1 and 2 demonstrate that the isotype ratios established in the primary immunization could not be modulated by successive immunizations. Consistent with earlier observations, cytokine production data for IFN-γ and IL-4 from the cross-boosted animals did not correlate with the IgG subclass patterns, since similar cytokine profiles were seen in the four cross-boosted groups

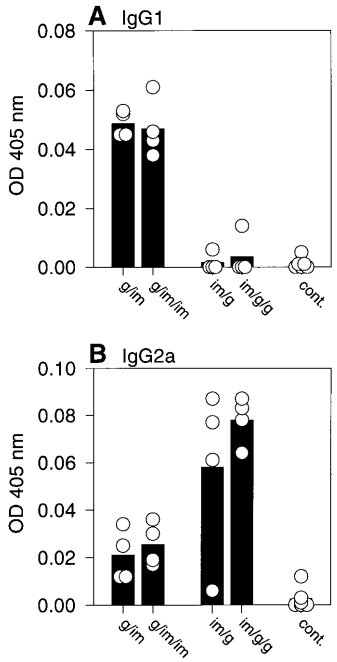

FIG. 4. Measurement of IgG1 and IgG2a antibody responses in mice that received DNA immunizations via both the gene gun (g) and intramuscular (im) routes. Two groups of four female BALB/c mice each received a primary immunization via gene gun delivery of 1 μg of NP vector DNA to the epidermis at week 0. These two groups then received one and two booster immunizations, respectively, via intramuscular injection of 100 μg of the same DNA. Likewise, two additional groups received an intramuscular primary immunization containing 100 μg of DNA and either one or two booster immunizations via the gene gun route containing 1 μg of the same vector. Solid bars indicate the arithmetic means; open circles indicate datum points for individual mice. Repetition of "g" and "im" in the group labels indicates the number and type of immunizations. cont. represents control animals immunized with irrelevant DNA via multiple routes. OD, optical density.

(high IFN-γ and low to nondetectable IL-4) despite a wide range of IgG subclass ratios (data not shown). These data demonstrate that IgG subclass production following DNA immunization using influenza NP vectors in mice is determined solely by the route of the primary immunization and cannot be modulated by subsequent booster immunizations or significant swings in the levels of IFN-γ or IL-4 production.

DISCUSSION

The administration of an influenza virus NP DNA expression vector by intramuscular inoculation of 100-μg doses or by gene gun delivery to the epidermis of 1-μg doses resulted in the elicitation of similar geometric mean endpoint titers and CTL activities following one, two, and three immunizations. These results are consistent with earlier findings demonstrating similar responses but the requirement for less DNA when a direct, intracellular delivery mechanism is used (10, 17). Despite the similarities in total IgG titers, considerable differences in IgG subclass profiles were observed between these two methods. While intramuscular inoculation elicited more IgG2a than IgG1 antibodies, epidermal immunizations resulted in the reverse scenario. Despite these marked differences, it was interesting that IFN-γ production profiles by antigen-stimulated splenocytes were remarkably similar between animals immunized by the two methods, a result that seemed inconsistent with the divergence in the IgG subclass results. Examination of IL-4 production patterns, however, did reveal a difference between the two DNA immunization routes in that gene gun delivery resulted in the production of considerable amounts of IL-4 by antigen-stimulated splenocytes, but only after the third immunization. Insignificant levels of IL-4 were observed by similar cells derived from intramuscularly inoculated mice at the same time point.

From these observations, it is tempting to conclude that intramuscular inoculation resulted in Th1-like responses due to the predominance of IgG2a antibodies, CTL activity, IFN-γ production, and lack of IL-4 release. In contrast, the gene gun results appeared mixed, given the early CTL activity and IFN-γ production on one hand and the prevalence of IgG1 antibodies at all times and IL-4 production replacing IFN-γ production after the third immunization on the other. The response patterns elicited following use of the gene gun were surprisingly similar to those observed following immunization with inactivated influenza virus formulated in Freund's adjuvant. Although the Freund's adjuvant antibody titers were stronger in general, and no CTL activity was elicited as expected, the preponderance of IgG1 and the shift from IFN-γ to IL-4 production were similar between the two immunization methods.

A striking observation from the intramuscular, gene gun, and Freund's adjuvant results was that IFN-γ production seemed independent of the method of immunization. IFN-γ production levels were highest following the primary immunization and were markedly reduced by the second boost. In addition, CTL activity in all of the DNA-immunized animals was highest following the primary immunization and reduced at later times, consistent with the IFN-γ results. In the gene gun and Freund's adjuvant groups, this drop in IFN-γ production was countered by a reciprocal increase in IL-4 production. In the intramuscular group, significant levels of IL-4 production were not observed. An IFN-γ-to-IL-4 shift in the gene gun and Freund's adjuvant animals is consistent with a switch from a Th1 to a Th2 response with successive immunizations. We previously reported a similar Th1-to-Th2 shift in mice that received successive gene gun immunizations with a human immunodeficiency virus type 1 gp120 DNA vaccine, demonstrating that the progression in cytokine production patterns is not unique to the present antigen system (9). Interestingly, a similar but reversed cytokine progression pattern was reported by Mor et al. (15) following intramuscular DNA immunization with a *Plasmodium yoelii* circumsporozoite (CS) expression construct. In this case, initial IL-4 production was replaced by IFN-γ production upon boosting. Divergent results of this nature are consistent with the idea that particular antigen-specific response patterns may be more a function of the identity of the antigen, and the strain of mice used, than of the method of immunization. Indeed, gene gun immunization studies of BALB/c mice, using a vector encoding the hepatitis B virus surface antigen, resulted in simultaneous IgG1, IgG2a, CTL, IFN-γ, and IL-4 responses that showed no evidence for shifting as a function of the number of immunizations and no clear bias in either the Th1 or Th2 direction (unpublished data).

Although the specific types of responses elicited following DNA immunization may be determined by the identity of the encoded antigen, the data presented here for NP demonstrate that IgG1 and IgG2a subclass ratios for a particular antigen, in a particular mouse strain, are still influenced by the route or method of DNA delivery. Additional data to support this idea come from recent experiments using expression vectors encoding the *Plasmodium berghei* CS antigen and the human carcinoembryonic antigen (CEA). In the CS model, BALB/c mice developed both IgG1 and IgG2a responses following intramuscular DNA immunization but only IgG1 responses following gene gun immunization (10a). Similarly, C57BL/6 mice devel-

oped both IgG1 and IgG2a responses to CEA following intramuscular DNA injection but only IgG1 responses following gene gun delivery (1a). While it may be tempting to conclude from the NP, CS, and CEA experiments that gene gun immunization is not effective in eliciting IgG2a subclass responses, this is clearly too broad a generalization since gene gun NP DNA immunizations in C3H mice yielded predominantly IgG2a antibodies (18a). Thus, a more accurate conclusion may be that reproducible differences in antibody isotype ratios can be seen following gene gun-mediated and intramuscular DNA immunization in a particular system, but the identity of the antigen and the genetic background of the animals are the most important factors in determining the ultimate responses generated.

The tendency of gene gun-mediated DNA immunization to elicit predominantly IgG1 subclass responses in certain systems, while intramuscular DNA inoculation yields mixed or predominantly IgG2a responses, could reflect significant differences in the amount of soluble antigen synthesized following DNA delivery using these two methods. The level of antigen load following DNA immunization could influence the role that B cells may play in antigen presentation and their tendency to shift the immune response to a Th2 phenotype (11, 20). Gene gun-based DNA transfer methods result in direct intracellular DNA delivery, which may lead to greater levels of antigen expression than obtained following passive DNA uptake in skeletal muscle and consequently influence the quality of the responses. However, recent data from a gene gun-based DNA immunization study using decreasing quantities of a vector encoding human growth hormone (hGH) suggest that this may not be the case. Decreasing the amount of hGH DNA delivered from 500 to 0.7 ng per immunization resulted in a 500-fold reduction in hGH expression in skin biopsies but only a 3-fold reduction in geometric mean IgG titers. More importantly, the IgG1-to-IgG2a subclass ratios remained steady at >10:1, demonstrating that dramatic reductions in both DNA dosage and antigen expression in the epidermis may have no effect on the tendency of gene gun immunizations to elicit predominantly IgG1 responses in certain systems (18a).

An interesting observation from the results reported here was that the preponderance of IgG1 and IgG2a responses observed following each immunization with the gene gun and intramuscular routes, respectively, were remarkably stable given the fluctuations in cytokine production with successive immunizations. In addition, the levels of IFN-γ production were identical between the gene gun and intramuscular groups following the first two immunizations, despite the reciprocal relationship between their respective IgG1 and IgG2a levels. These observations were surprising in view of the demonstrated interrelationship between cytokine production and Ig subclass switching (8, 23). One possibility is that the observed IgG subclass profiles were influenced more by IL-4 production than by IFN-γ production. Although the IL-4 production levels in the gene gun-immunized mice following the primary immunization were not significant, they were somewhat greater than those observed in the intramuscular group and could have been responsible for the differences in IgG subclass profiles. Nevertheless, with successive immunizations, the marked fluxes in the IFN-γ and IL-4 profiles were not enough to alter the original IgG subclass ratios established in the first immunization.

In the previous gp120 DNA vaccine study (9), successive immunizations also resulted in a shift from IFN-γ to IL-4 production as described above. Recent measurement of IgG isotypes in this system showed a 10-fold increase in the levels of IgG1, but not IgG2a, that was coincident with the increase in IL-4 production and decrease in IFN-γ production (unpublished data). Thus, the apparent lack of linkage between the fluctuating cytokine release patterns and the stable IgG subclass responses may be a feature unique to the NP system described here.

Data from the cross-boosting experiments were consistent with the observation that IgG isotype profiles appeared to be fixed following the primary immunization. While the gene gun and intramuscular routes exhibited differences in their tendency to elicit IgG1 and IgG2a responses, respectively, cross-boosting with the reciprocal route had no influence at all on the original IgG subclass ratios elicited. IFN-γ and IL-4 production patterns were essentially identical between the animals in the g/im and the im/g groups (data not shown), even though differences were observed in their IgG subclass ratios. Also, the IgG1-to-IgG2a ratios in the g/im/im mice (Fig. 4) were similar to those observed in the g/g/g animals (Fig. 2). The same was true regarding the IgG2a-to-IgG1 ratios in the im/g/g and im/im/im groups. Thus, the primary NP immunizations were dominant, leading to fixed Ig subclass ratios that did not change in response to successive immunizations or cytokine fluxes.

The data reported here are consistent with earlier reports describing the ability of various DNA immunization routes to result in the elicitation of vigorous humoral and cellular responses. However, it is apparent that the route of DNA vaccine administration will be important in determining the specific types of responses elicited. In addition, the quality of these responses may be further modulated by the codelivery of cytokine-encoding vectors as described by Xiang and Ertl (32) or by the inclusion of adjuvants. Preliminary data in our laboratory are consistent with the ability to modulate and augment responses to NP and human immunodeficiency virus type 1 gp120 DNA vaccines by adjuvantation (17b).

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