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Role of Different Lymphoid Tissues in the Initiation and Maintenance of DNA-Raised Antibody Responses to the Influenza Virus H1 Glycoprotein

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Antibody responses in mice immunized by a single gene gun inoculation of plasmid expressing the influenza virus H1 hemagglutinin and in mice immunized by a sublethal H1 influenza virus infection have been compared. Both immunizations raised long-lived serum responses that were associated with the localization of antibody-secreting cells (ASC) to the bone marrow. However, the kinetics of these responses were 4 to 8 weeks slower in the DNA-immunized than in the infection-primed mice. Following a gene gun booster, the presence of ASC in the inguinal lymph nodes, but not in other lymph nodes, revealed gene gun responses being initiated in the nodes that drain the skin target site. Both pre- and postchallenge, the DNA-immunized mice had 5- to 10-times-lower levels of antibody and ASC than the infection-primed mice.

Immunization with plasmid DNA by a variety of routes has been shown to elicit both cellular and humoral immune responses that are protective in a number of experimental models (10, 20, 23). DNA immunization, like viral infection, can raise long-lived responses, including persistent antibody responses (25, 26, 29, 30). The persistence of virus-specific antibody following infection is often critical for protection from a second challenge infection (1). Therefore, the ability of DNA immunization to raise long-lasting humoral responses has important implications for vaccine development.

In this study we have examined the role of lymphoid tissue in the initiation and maintenance of long-lived antibody responses raised by gene gun delivery of influenza virus hemagglutinin-expressing vaccine DNA. The initiation of antibody responses involves interactions between B cells, T cells, antigen-presenting cells, and antigen. These interactions take place in the microenvironment of the lymph nodes or spleen, resulting in the differentiation of naive antigen-specific B cells into memory and effector B cells (antibody-secreting cells [ASC]) (12, 21). The newly generated memory B cells recirculate, while effector ASC are found at sites of antigen presentation or in activated lymphoid compartments such as the spleen (2, 9). As antibody responses mature, the long-term maintenance of these responses is associated with the localization of ASC to the bone marrow (5, 9, 14, 27, 28). While only one viral challenge is sufficient for ASC to localize to the bone marrow (14, 27, 28), protein immunogens typically require a booster for ASC to traffic to this lymphoid compartment (6, 7, 13, 17).

Very limited research has been done on B-cell responses raised by DNA-based immunizations. Studies of short-term responses following an intramuscular immunization with a plasmid expressing the malaria circumsporozoite protein revealed ASC in the lymph nodes, spleen, and bone marrow (22). Analysis of short-term responses in BALB/c mice immunized by gene gun with an influenza virus H1 hemagglutinin-expressing plasmid showed very low levels of ASC in the spleen and bone marrow but no ASC in the lymph nodes (16). However, following an intranasal challenge ASC were detected in the cervical and mediastinal lymph nodes (16). Here, we have examined both the long-term antibody and ASC responses in gene gun-immunized mice and compared these with responses in mice sublethally infected with influenza virus. We have also used the postbooster localization of ASC to test for the distribution of DNA-expressed antigen in lymphoid tissues. Lastly, we have examined the effects of an influenza virus challenge on antibody and ASC responses in mice that had been primed for a least 3 months by gene gun immunization.

Time course of antibody and ASC responses in DNA-immunized and influenza virus-infected mice. To study the temporal appearance of antibody and ASC, 6- to 8-week-old female BALB/c mice were immunized by gene gun delivery of 0.4 μg of pJW4303/H1 plasmid DNA (a DNA expressing the influenza virus H1 hemagglutinin) (26) or by intranasal infection with a sublethal dose of A/PR/8/34 (H1N1) (20 PFU delivered to methoxyflurane [Metofane]-anesthetized mice). An enzyme-linked immunosorbent assay (ELISA) was used to quantitate the amount (in micrograms) of H1-specific immunoglobulin G (Ig) in serum by comparison to a standard curve for convalescent-phase mouse serum (from mice that had been immunized with pJW4303/H1 and challenged with a lethal dose of A/PR/8/34) for which total anti-A/PR/8/34-specific serum IgG levels had been determined with purified mouse IgG. An A/PR/8/34-specific ELISPOT assay (28) was used to examine lymphoid tissues for the frequency and temporal localization of A/PR/8/34-specific ASC.

Anti-influenza virus antibody and ASC responses exhibited similar longevities but slower kinetics of appearance in gene gun-immunized than in sublethally infected mice (Fig. 1). In gene gun-inoculated mice, 8 to 12 weeks was required to achieve plateau levels of influenza virus-specific antibody (≈100 μg/ml). At 2 weeks after gene gun immunization, very low numbers of H1-specific ASC were detected in the spleen (≈5 IgG-ASC per 10^6 cells), while only borderline levels of ASC were present in the bone marrow (≈3 IgG-ASC per 10^6

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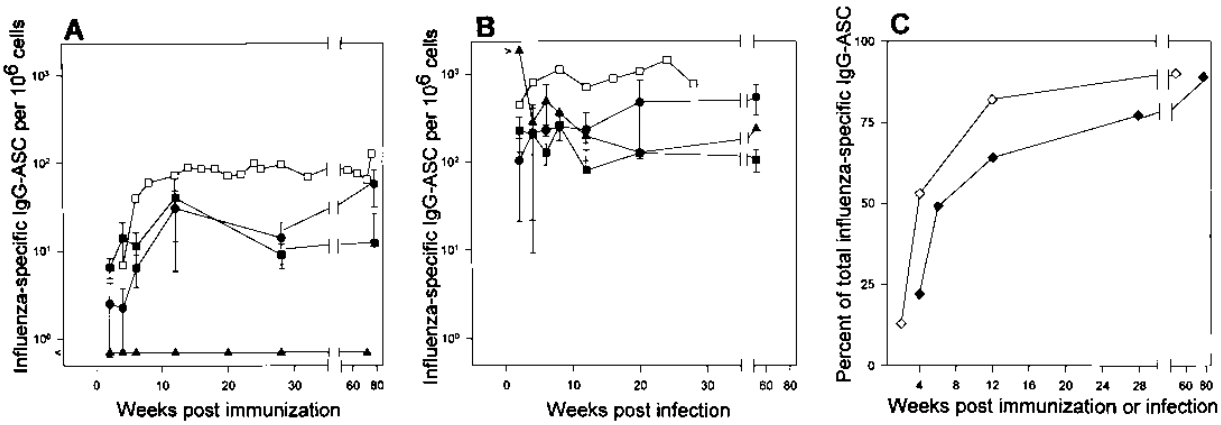

FIG. 1. Temporal anti-influenza virus IgG-ASC and IgG antibody responses raised by gene gun immunization and sublethal influenza virus infection. BALB/c mice were immunized with 0.4 μg of H1-expressing DNA by gene gun or infected intranasally with 20 PFU of A/PR/8/34. (A) Kinetics and localization of IgG-ASC and amount of anti-A/PR/8/34 IgG antibody with time in DNA-immunized mice. No ASC were detected in lymph nodes. (B) Kinetics and localization of IgG-ASC and amount of anti-A/PR/8/34 IgG with time in sublethally infected mice. (C) Localization of IgG-ASC to the bone marrow. Each point represents the mean frequency ± standard deviation for two to eight individual mice. Symbols (A and B): □, anti-A/PR/8/34 IgG; ●, bone marrow ASC; ■, spleen ASC; ▲, lymph node ASC (lymph nodes for DNA-immunized mice included axial, cervical, inguinal, mediastinal, mesenteric, and popliteal lymph nodes; lymph nodes for infection-primed mice were the mediastinal lymph nodes. Symbols (C): ◆, mice immunized with DNA via gene gun; ◇, infection-primed mice.

cells), and no ASC were detected in the lymph nodes (Fig. 1A). The frequency of ASC in both the spleen and bone marrow compartments increased for 8 to 12 weeks, at which time the frequencies in both compartments were similar (≈40 IgG-ASC per 10^6 cells).

In the sublethally infected mice, influenza virus-specific antibody and ASC rose to high levels within 2 to 4 weeks following infection. Postinfection levels of influenza virus-specific antibody were approximately 10 times higher (≈1 mg/ml) than in the DNA-immunized mice (≈100 μg/ml). At 2 weeks postinfection, the mediastinal lymph nodes draining the site of infection had a very high frequency of A/PR/8/34-specific IgG-ASC (≈2,000 per 10^6 cells). At this time lower frequencies of ASC were present in the spleen (≈226 IgG-ASC per 10^6 cells) and in the bone marrow (≈103 IgG-ASC per 10^6 cells). By 4 weeks the frequency of ASC in the mediastinal lymph nodes had decreased to levels comparable to those in the spleen and bone marrow. Our ELISA and ELISPOT assay detect antibodies and ASC specific for all influenza virus proteins. Therefore, the higher titers of antibody and frequencies of ASC in the sublethally infected mice are due, in part, to detection of responses to nonhemagglutinin proteins.

In both the DNA-immunized and sublethally infected mice the plateau levels of antibody responses persisted for more than 1 year (the duration of the experiment). These long-lived responses were associated with the movement of the influenza virus-specific ASC to the bone marrow (Fig. 1C). To compare the kinetics of the localization of ASC to the bone marrow, the contribution of IgG-ASC in this compartment as a percentage of total A/PR/8/34-specific ASC was plotted with time (Fig. 1C). The total number of ASC was calculated on the basis of the total number of cells harvested with each sample. For the calculation of total bone marrow ASC response, the ASC count for both femurs was multiplied by a factor of 7.9, as ^{59}Fe distribution studies have shown that 12.6% of the total bone marrow response is in both femurs (8). These data show a more rapid movement of influenza virus-specific ASC to the bone marrow in sublethally infected mice than in the DNA-immunized mice (Fig. 1C). By 4 weeks, the IgG-ASC response in the bone marrows of sublethally infected animals accounted for approximately 50% of the total influenza virus-specific ASC response. In contrast, 6 to 8 weeks was required for at least 50% of the total ASC response to localize in the bone marrows of the gene gun-immunized mice. By 1 year postimmunization, approximately 90% of the total influenza virus-specific response had localized to the bone marrow in both the DNA-immunized and the infection-primed mice.

ASC in the draining lymph nodes of gene gun-immunized mice. A single immunizing dose of plasmid DNA resulted in H1-specific IgG-ASC responses in the spleen and bone marrow, but no ASC were detected in any lymph nodes (Table 1). One possible explanation for this is that the priming immunization resulted in such low levels of antigen that the ASC response in the draining lymph nodes was below the sensitivity

TABLE 1. Postbooster localization of IgG-ASC in lymph nodes of DNA-immunized mice

Gene gun delivery[a]	Time post-immunization (wk)	Frequency of influenza virus-specific IgG-ASC/10^6 cells				
		Lymph nodes[b]			Spleen[c]	Bone marrow[c]
		Mediastinal	Inguinal	Other		
Single immunization	2	ND	ND	ND	5 ± 1	3 ± 2
	78	ND	ND	ND	13 ± 13	47 ± 19
Booster	1	ND	27 ± 12	ND	24 ± 23	58 ± 49

[a] Mice were gene gun immunized with a total of 1.25 μg of H1-expressing pJW4303/H1 for priming and booster immunization.
[b] Numbers are the means ± standard deviations for four pools of lymph nodes from two or three mice per pool. Other includes axial, cervical, mesenteric, and popliteal nodes. ND, not detected.
[c] Data are means ± standard deviations for 4 to 12 mice.

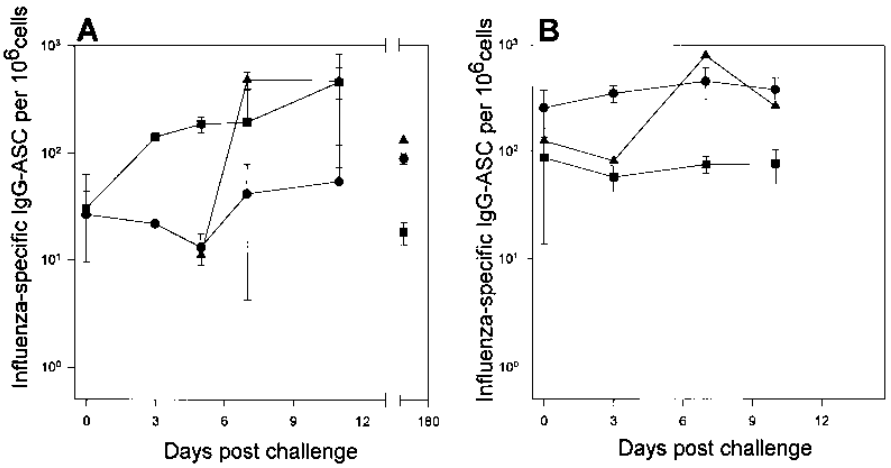

FIG. 2. Localization of IgG-ASC following lethal challenge. (A) Mice gene gun immunized with 0.4 μg of pJW4303/H1 DNA. (B) Mice infected with a sublethal dose of A/PR/8/34 (H1N1). At 3 months or greater following immunization, both groups of mice were anesthetized with methoxyflurane and given a lethal (450 to 500 PFU) intranasal challenge of A/PR/8/34 influenza virus. Symbols: ●, bone marrow ASC; ■, spleen ASC; ▲, mediastinal lymph node ASC. Each point represents data for at least two mice (mean ± standard deviation).

of the assay. Another possible explanation is that gene gun delivery of plasmid DNA to the abdominal epidermis resulted in the presence of antigen in the spleen but not the draining lymph nodes, and therefore responses were exclusively mounted in the spleen (Fig. 1A). The localization of B cells after booster immunization can serve as a functional marker for the presence of booster antigen (3, 4, 18, 24). Therefore, mice that had been immunized once with pJW4303/H1 were given boosters via gene gun in the same abdominal skin site. Boosters were given ≥3 months after primary immunization, when serum antibody responses to the primary immunization had reached plateau levels. The ELISPOT assay was then used to test for ASC localization in lymph nodes. These included pools of axial, cervical, inguinal, mediastinal, mesenteric, and popliteal lymph nodes.

Following the DNA booster immunization, H1-specific IgG-ASC appeared in the inguinal lymph nodes. All other lymph nodes were negative. At 1 week after the booster, A/PR/8/34-specific IgG-ASC were present in the inguinal lymph nodes at a frequency of 27 per 10^6 cells, with a range of 9 to 34 (Table 1). ASC in the spleen and bone marrow remained at pre-booster frequencies. These data suggest that DNA-expressed antigen was present in the inguinal nodes that drain the target site: the abdominal skin. Therefore, it is likely that DNA-generated immune responses are established, at least in part, in these lymph nodes.

Effect of viral challenge on frequency and localization of ASC in mice primed by DNA immunization or sublethal infection. In order to compare the effects of viral challenge on ASC localization and antibody levels in DNA-immunized and infection-primed mice, methoxyflurane-anesthetized mice were given a lethal dose of A/PR/8/34 (500 PFU) by intranasal inoculation. Challenge was administered 10 to 12 weeks after immunization, when plateau levels of serum antibody and protection had been achieved in both groups of mice (Fig. 1A and B).

During the acute postchallenge response in gene gun-immunized mice, A/PR/8/34-specific IgG-ASC appeared in the mediastinal lymph nodes, increased in frequency in the spleen, and remained fairly constant in the bone marrow (Fig. 2A). Influenza virus-specific IgG-ASC were detectable in the mediastinal lymph nodes by day 5, peaked by day 7 (>473 per 10^6 cells), and remained constant through day 10. In the spleen, the frequency of influenza virus-specific ASC had increased by day 3. This rise in ASC peaked on day 10, with an IgG-ASC frequency approximately 10 times higher (450 per 10^6 cells) than on the day of challenge (43 per 10^6 cells) (Fig. 2A). The bone marrow ASC showed little increase between day 2 and day 10 postchallenge.

More-limited postchallenge changes in influenza virus-specific ASC frequencies were found in animals primed with a sublethal dose of A/PR/8/34 (Fig. 2B). Both spleen and bone marrow showed only slight increases from day 0 through day 10 postchallenge. However, the frequency of influenza virus-specific IgG-ASC in the mediastinal lymph nodes increased by approximately 10-fold, peaking at day 7. In contrast to the postchallenge ASC response in the lymph nodes of DNA-immunized mice, this lymph node response had fallen by day 10 (Fig. 2B).

By 6 months postchallenge the distribution of influenza virus-specific IgG-ASC in DNA-immunized mice was similar to that in the infection-primed animals (Fig. 2). At this late time, the frequencies of A/PR/8/34-specific IgG-ASC had fallen from the acute-phase postchallenge levels.

Because an important parameter of the humoral response to respiratory viruses is the IgA response (19), the distribution of IgA-ASC was also examined following challenge. Little change was detected in the IgA-ASC frequencies in the spleens and bone marrows of DNA-immunized and sublethally infected mice. However, in the mediastinal lymph nodes of both groups of mice IgA-ASC had appeared or increased by day 7 postchallenge. The frequency of IgA-ASC in these lymph nodes (100 to 250 per 10^6 cells) was much higher than the frequency of IgA-ASC in either the spleen (2 to 4 per 10^6 cells) or bone marrow (10 to 63 per 10^6 cells) in both the DNA-immunized and infection-primed mice.

Effect of viral challenge on antibody in mice primed by DNA immunization or sublethal infection. The postchallenge anti-A/PR/8/34 antibody titers were not significantly changed in infection-primed mice. In contrast, the anti-A/PR/8/34 antibody titers of DNA-immunized mice increased approximately fivefold (32 to 165 μg/ml) (Table 2). This titer was maintained for the remainder of the experiment (6 months). Despite this increase, the postchallenge levels of serum antibody remained three- to fourfold lower than the influenza virus-specific anti-

TABLE 2. Influenza virus-specific IgG serum responses pre- and postchallenge

Time	Concn of IgG in mice			
	A/PR/8/34 specific (μg/ml)		HKx31 specific (U/ml)	
	DNA immunized	Sublethal A/PR/8/34 infection	DNA immunized	Sublethal A/PR/8/34 infection
Prechallenge[a]	32	743	ND[b]	274
Postchallenge[c]				
Day 10	68	753	13	182
6 mo	165	529	49	201

[a] Prechallenge, serum samples from at least 3 months postimmunization.
[b] ND, not detected.
[c] Postchallenge, methoxyflurane-anesthetized mice were given a lethal challenge of 450 to 500 PFU of A/PR/8/34 intranasally.

body response in the infection-primed mice (529 μg/ml) (Table 2).

The increased antibody response in DNA-immunized mice postchallenge included reactivity to other influenza virus proteins. To discriminate between antibodies specific for H1 or other influenza virus proteins, sera from postchallenge DNA-immunized mice were tested by ELISA for reactivity with the mouse-adapted influenza virus HKx31 (H3N2). Anti-H1 antibody does not cross-react with the H3 hemagglutinin of HKx31. In DNA-immunized mice, reactivity to HKx31 was not present at the time of challenge but did appear postchallenge. This anti-HKx31 IgG response appeared by day 7 postchallenge and reached a plateau by day 14 (Table 2). This plateau level was maintained for at least 6 months following challenge. As expected, sera from infection-primed mice reacted with HKx31. This anti-HKx31 IgG (approximately 250 U) was not increased by the challenge (Table 2). Like long-term antibody to A/PR/8/34, long-term antibody to HKx31 was present at approximately fourfold-lower levels in DNA-immunized and challenged mice than in infection-primed and challenged mice.

Discussion of findings. Our results demonstrate that the long-term maintenance of DNA-induced antibody is associated with the localization of ASC to the bone marrow (Fig. 1). While similar in longevity to antibody responses raised by sublethal influenza virus infection, the rise of antibody and the movement of ASC to the bone marrow were 4 to 8 weeks slower in the DNA-immunized mice. We suggest that this difference in kinetics may be linked to, and determined by, differences in the time course and dose of antigen expression following DNA immunization and a natural infection.

Our data also suggest that the initiation of responses following gene gun delivery of DNA is accomplished in part in the inguinal nodes that drain the skin target site (Table 1). At 1 week following a gene gun booster, H1-specific ASC were detected in the inguinal but not in other lymph nodes. This localization reveals that DNA-expressed antigen is present in the inguinal nodes and that DNA-initiated B-cell responses are, at least in part, established in these nodes. The inability to detect ASC in the inguinal lymph nodes following a single gene gun immunization is likely due to quantitative restrictions on ASC detection following low levels of antigen expression.

Previous work from our laboratory has demonstrated that a single immunization of H1-expressing DNA provides complete protection from a lethal influenza virus challenge (11). The current study shows that this protection is associated with the rapid appearance of effector B cells (ASC) in the mediastinal lymph nodes (Fig. 2). The appearance of IgG- and IgA-ASC in the mediastinal lymph nodes by day 7 postchallenge reflects the recruitment of B cells, as prior to challenge no ASC could be detected in these nodes (Fig. 1 and 2). Thus, the ability of gene gun immunization to establish B cells that efficiently traffic to and generate IgA in the mediastinal lymph nodes is an important attribute of this method of immunization (15, 16).

Finally, we show that protective DNA immunizations are associated with much lower specific-antibody levels and ASC frequencies than those that occur after natural influenza virus infection (Fig. 1 and Table 2). Despite the increases in antibody and ASC responses in gene gun-immunized mice following challenge, both of these responses remained substantially lower than those observed in infection-primed mice. This likely reflects the limited replication of the challenge virus due to efficient trafficking of effector cells to the sites of challenge and the presence of high levels of prechallenge neutralizing antibodies (26). The ability of DNA immunization to provide protection with a low level of immune responses should benefit the vaccinated host by decreasing the potential for postchallenge immunopathology.

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