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Antibody-Forming Cell Response to Virus Challenge in Mice Immunized with DNA Encoding the Influenza Virus Hemagglutinin

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Immunization of mice with DNA encoding the influenza virus hemagglutinin (HA) affords complete protection against lethal influenza virus infection and the means to investigate the mechanisms of B-cell responsiveness to virus challenge. Using a single-cell enzyme-linked immunospot assay, we sought to determine the localization of HA-specific antibody-forming cells (AFCs) during the development of humoral immunity in mice given HA DNA vaccine by gene gun. At 33 days postvaccination, populations of AFCs were maintained in the spleen and bone marrow. In response to lethal challenge with influenza virus, the AFCs became localized at the site of antigenic challenge, i.e., within the draining lymph nodes of the lung compartment. Immunoglobulin G (IgG)- and IgA-producing AFCs were detected in lymph nodes of the upper and lower respiratory tracts, underscoring their importance in clearing virus from the lungs. Response to challenge required competent $CD4^+$ T cells, without which no AFCs were generated, even those producing IgM. By contrast, in mice vaccinated with an HA-containing subunit vaccine, fewer AFCs were generated in response to challenge, and these animals were less capable of resisting infection. Our findings demonstrate the comparable localization of AFCs in response to challenge in mice vaccinated with either HA DNA or live virus. Moreover, the former strategy generates both IgG- and IgA-producing plasma cells.

The recently devised immunization strategy based on gene-gun inoculation of nucleic acids into the skin (5, 16, 30, 41, 44, 46) has clear advantages in that the skin and related lymphoid tissues efficiently process and present antigen to the immune system (15, 27, 38, 40). Immunization with a DNA-encoded vaccine that expresses the influenza virus hemagglutinin (HA) stimulates the development of protective antibodies and has been successfully used to generate immunity against pulmonary influenza virus infection in mice (16). These results raise the question of how HA DNA vaccination induces humoral immunity against influenza virus, in particular, whether the immune response is comparable to that generated by replicating virus or purified antigen. The immune response against influenza virus has been extensively studied (4, 6), and the importance of HA-specific antibodies and cognate help provided by virus-specific helper T cells is well established (10, 21, 22, 26, 32, 33). Two important aspects of protective immunity against live virus challenge are the presence of preexisting HA-specific antibodies and the rapid generation of antibody-forming cells (AFCs). Antiviral antibody production is often sustained for long periods in the bone marrow after resolution of an acute viral infection (19, 21, 36, 42). To understand the mechanism by which HA DNA induces immunity against influenza virus infection, we examined (i) the anatomical localization of AFCs following vaccination and (ii) the postchallenge AFC responsiveness of lymphoid tissues and the requirement of $CD4^+$ helper T cells for their development. Finally, we compared AFC responses before and after challenge of mice immunized with HA DNA with those having received subunit vaccine or live virus.

Here we show that AFCs are colocalized with challenge antigen in the lung compartment; however, the maintenance of B-cell memory appears to reflect long-term AFC activity in the bone marrow and spleen. The AFC response to challenge is characterized by rapid increases in the numbers of immunoglobulin G (IgG)- and IgA-secreting cells in the draining lymph nodes of the respiratory tract. Regardless of the form in which HA is presented to the immune system, DNA vaccination is not effective unless competent $CD4^+$ T cells are present.

MATERIALS AND METHODS

Vaccine DNA. The pJW4303/H1 vaccine DNA relies on the cytomegalovirus immediate-early promoter to express the HA gene from influenza virus A/PR/8/34 (H1N1) (29). The pJW4303 control plasmid is the expression vector without the HA gene. Plasmid DNA was affixed to gold particles and delivered with the Accell II electric discharge DNA gun (Agracetus, Middleton, Wis.) to the abdominal skin of 8- to 10-week-old BALB/c ($H\text{-}2^d$) mice (14, 16).

Virus and conventional vaccines. The A/PR/8/34 (H1N1) virus was propagated in the allantoic cavity of 10-day-old embryonated eggs and purified by sucrose gradient centrifugation (35).

Subunit vaccine was prepared by extracting the HA and neuraminidase (NA) proteins from influenza virus particles by treatment for 30 to 60 min with the nonionic detergent n-octyl-β-D-thioglucopyranoside in acetate buffer (final concentration, 7.5%), as described by Johansson et al. (20). The HA/NA-rich supernatant was separated by centrifugation of detergent-treated virus (15,000 rpm in a Beckman 50Ti rotor) and retained for study. Examination of this fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11) indicated that under nonreducing conditions at room temperature, more than 98% of the proteins assumed representative oligomeric forms. Dissociation of HA and NA oligomers to monomers upon exposure to 100°C followed by reduction permitted density scans of Coomassie blue-stained monomeric profiles (Sparc station 2 densitometer; Sun Systems, Foster City, Calif.) with Bioimaging Visage 110 software (Millipore, Bedford, Mass.). The estimated HA content of the subunit vaccine, expressed as micrograms of HA, was 55%, while that of the virus was 35%.

Vaccine trials. We studied two groups of HA DNA-vaccinated mice. One group received a dose of DNA on day 0 and a second dose 4 weeks later; the

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other group received a single DNA immunization on day 0. Control mice either received DNA that did not encode the viral HA or remained untreated. At 2 weeks after the second DNA inoculation, lethal PR8 virus challenge with 5 μg of HA in 30 μl of phosphate-buffered saline (PBS) (equivalent to 3 50% lethal doses [LD_{50}]) was administered intranasally (i.n.) into mice that were under light anesthesia with Metofane (Pitman-Moore, Mundelein, Ill.). In some mice, $CD4^+$ T cells were depleted by intraperitoneal (i.p.) injections on day −4 before challenge, on day 0, and every 2 to 3 days thereafter, with 0.5-ml aliquots of diluted mouse ascitic fluid containing the GK1.5 monoclonal antibody to CD4 as described previously (1). Some mice from each group were not challenged and were maintained for 58 days to assess the durability of immunity.

Another experimental group received an HA subunit vaccine. These mice were primed on day 0 and boosted 2 weeks later, each time with delivery of 50 μg of HA of subunit vaccine given i.p. The 2-week interval between priming and boosting favored the development of memory B cells responsive to virus challenge, because few or no memory AFCs are maintained after 1 month (21).

Sampling of mice. Anesthetized mice were bled from the eye vein on days 14, 33, 37, 44, 48, 52, and 58 postvaccination to assess antibody levels in serum by serologic techniques. Exsanguination by heart puncture was followed by harvesting of inguinofemoral, cervical, and mediastinal lymph nodes, spleen, and bone marrow. The specimens were processed individually, with care taken to remove erythrocytes and dead cells (1, 19). Single-cell suspensions were prepared fresh from lymphoid tissues and sampled from individual mice (1). Lungs taken for virus titer determination were not subjected to lavage. Leukocytes were resuspended in complete medium (Iscove's modified Dulbecco's medium or RPMI-1640 and 15% fetal calf serum) and used on the same day. Tissues from one to three mice per group were analyzed individually.

Serologic testing. Sera from mice were tested individually following treatment with receptor-destroying enzyme (45). Hemagglutination-inhibition (HI) titration of the serum was performed as previously described (16).

Enzyme-linked immunospot (ELISPOT) assays. Elements of HA-specific B-cell responsiveness, including antibody secretion, were assessed by a modification of the enzyme-linked immunospot (ELISPOT) assay (8, 18, 19, 21, 22, 31, 34). In brief, 96-well plates (Millipore) were coated overnight with isolated PR8 HA (0.5 μg of HA in 100 μl of PBS at 4°C). After three washes with PBS and a blocking step (2% fetal calf serum in PBS for 90 min at room temperature), single-cell suspensions of lymphoid tissues from individual mice (range, 32 to 10^6 cells per ml) were added and the cells were incubated for 4 h at 37°C. To plates that had been washed once with PBS, once with 0.1% Tween 20 in PBS, and three times with PBS were added phosphatase-labeled goat anti-mouse Ig isotype-specific reagents (in 5% bovine serum albumin in PBS) for 3 h. Antibody spots were developed with 5-bromo-4-chloro-3-indolyl phosphate (1 mg/ml) in diethanolamine buffer (pH 9.5). The number of antibody spots was normalized to the number of AFC per 10^5 total cells for any mouse tissue at all time points in the four replicate platings.

Lung virus titers. Lungs were processed and virus titers were determined as described previously (1, 23, 33).

Statistical analysis. Significance testing was limited to a comparison of the proportions of surviving mice in groups given one or two doses of HA DNA (by Fisher's two-tailed exact test).

RESULTS

We first determined the time course and Ig isotype profile of the AFC response before and after lethal virus challenge in mice vaccinated with HA DNA (pJW4303/H1). The results were then compared with the response in mice primed with an HA-containing subunit vaccine and the response to challenge in naive normal mice.

Capacity of DNA vaccination to induce HA-specific antibodies in serum. The pJW4303/H1 plasmid (0.4 μg) was administered by gene gun into the abdominal skin of BALB/c (*H-2*^{d}) mice. At 33 days later, blood was taken and concentrations of influenza virus-specific IgG antibodies capable of inhibiting hemagglutination were determined in serum (Fig. 1a). HI antibodies were detected in serum. Booster immunization with an additional dose of HA DNA did not substantially increase HI antibody titers, as both groups had comparably increasing levels within the next 2 weeks.

Expansion and localization of HA-specific AFCs in DNA-vaccinated mice. To determine the specific localization of AFCs after HA DNA inoculation, we measured their levels, beginning 33 days following initial vaccination, in inguinofemoral, cervical, and mediastinal lymph nodes, as well as bone marrow and spleen, using the ELISPOT assay. Low concentrations of AFCs were found only in spleen and bone marrow,

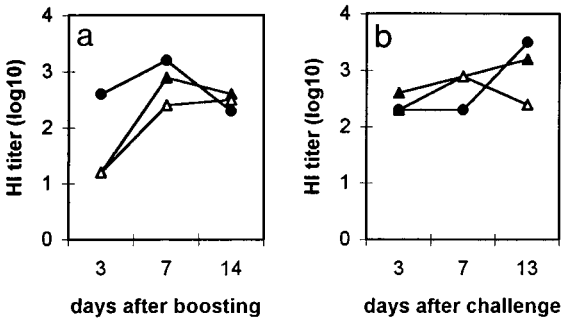

FIG. 1. Increase in HA-specific serum antibody titer in mice immunized with HA DNA or HA subunit vaccine. Mice were either given a single dose of vaccine (pJW4303/H1; open symbols) or boosted on day 30 (solid symbols) and were challenged i.n. with 3 LD_{50} of live PR8 virus on day 45; HI determinations (triangles) were begun on day 33 (3 days after boosting). On days 0 and 15, other mice were given i.p. injections of 50 μg of an HA glycoprotein preparation extracted from PR8 virus and were similarly challenged on day 30. HI (circles) titer determination was begun 18 days after the initial dose of vaccine (3 days postboosting). (a) Each symbol represents the mean titer for at least three mice at each time point prior to challenge. (b) A maximum of three mice were sampled at each time point after challenge. The postchallenge sampling times in HA DNA-vaccinated mice correspond to days 48, 52, and 58 overall; in HA subunit-vaccinated mice, the postchallenge sampling times correspond to days 33, 37, and 43 overall. The limit of detection of the HI test was 1 $\log_{10}$ unit. Antibody titers determined in HA DNA-vaccinated mice represent the same mice sampled in Tables 1 and 2; in HA subunit-vaccinated mice, the titers were determined for the same mice sampled in Table 4.

whether mice received one or two doses of the DNA vaccine (Table 1). Similar results were obtained when the AFC response was measured as the total number of AFCs per organ (results not shown). Although low, the measurements should be considered significant because they exceeded values in mice given control DNA (results not shown).

Serum antibody responses following virus challenge. Once- and twice-immunized mice were challenged i.n. on day 30 with a triple lethal dose of live PR8 virus. At 3 days later, serial determinations of titers of HI antibodies in serum were begun (Fig. 1b). By the end of the second week of challenge, antibody levels in serum had increased more than 1,000-fold above the levels seen after vaccination. Within the next 3 weeks, the serum had an HI titer of $1{:}1.6 \times 10^3$.

TABLE 1. Localization of AFCs in HA DNA-immunized mice prior to challenge

Tissue[a]	AFC detection on prechallenge day[b]:								
	IgM			IgG			IgA		
	33	37	44	33	37	44	33	37	44
Twice-immunized group									
MLN	−	−	−	−	−	−	−	−	−
BM	+	−	+	+	+	−	−	−	−
Spleen	+	−	+	−	−	−	−	−	−
Once-immunized group									
MLN	−	−	−	−	−	−	−	−	−
BM	+	−	−	+	−	−	−	−	−
Spleen	+	−	−	−	−	−	−	−	+

[a] Mice were immunized by gene gun with 0.4 μg of HA DNA. At 30 days later, a subset of mice were boosted with the same dose. Lymphoid tissues were then sampled by ELISPOT assay. MLN, mediastinal lymph nodes; BM, bone marrow.

[b] The findings were recorded as mean numbers of AFCs in the tissues of one or two mice per group, normalized to 10^5 total cells from four replicate platings. They are reported here as positive (1 to 4 AFCs per 10^5 cells) or negative. The inguinofemoral and cervical lymph nodes were also sampled, but no AFCs were found.

TABLE 2. Localization of AFCs and CD4$^+$ T-cell dependence of response to live virus challenge in mice immunized once or twice with HA DNA

Tissue[a]	No. of AFCs detected on postchallenge day[b]:								
	IgM			IgG			IgA		
	3	7	13	3	7	13	3	7	13
Once-immunized group									
CLN	0/0	1/0	5/0	0/0	52/23	40/24	0/0	8/5	7/7
MLN	0/0	4/4	3/0	2/0	295/275	60/55	0/0	33/19	4/9
BM	0/0	5/5	0/0	1/2	0/1	5/11	0/0	0/1	1/8
Spleen	0/0	21/23	18/20	9/10	20/18	41/44	0/1	11/9	9/7
Twice-immunized group									
Untreated									
CLN	0/0	9/10	1/3	0/0	58/237	25/44	0/0	85/65	7/10
MLN	9/0	1/1	1/1	0/0	275/575	34/70	3/1	7/8	2/11
BM	0/0	0/1	0/7	2/2	3/3	4/4	0/0	1/1	8/5
Spleen	13/5	31/38	1/10	10/10	30/32	13/19	1/1	26/18	16/23
CD4 depleted									
CLN	0	0	0	0	0	0	0	0	0
MLN	0	0	0	2	0	0	0	0	0
BM	0	0	0	2	0	0	0	0	0
Spleen	0	0	0	6	0	0	1	0	0

[a] Mice were immunized once or twice by gene gun with HA DNA. At 45 days later, they were challenged i.n. with 3 LD_{50} of live PR8 virus and monitored for 2 weeks by ELISPOT sampling of lymphoid tissues. CLN, cervical lymph nodes; MLN, mediastinal lymph nodes; BM, bone marrow. A subset of twice-immunized mice were depleted of CD4$^+$ T cells prior to challenge.

[b] Postchallenge sampling on days 3, 7, and 13 corresponds overall to days 48, 52, and 58, respectively. In twice-immunized mice, postchallenge sampling corresponds to days 18, 22, and 28 after boosting. Each finding for untreated mice is reported as the mean number of AFCs in tissues from two mice per group at each time point (mouse 1/mouse 2), normalized to 10^5 total cells from four replicate platings; in CD4 depletion experiments, data represent AFCs from a single mouse per time point similarly normalized from four replicate experiments. The inguinofemoral lymph nodes were also sampled, but no AFCs were detected. Data from unchallenged mice from the same group (<10 AFCs per 10^5 total cells per organ) differed from the background levels set by unchallenged naive and DNA control animals.

Localization and character of AFCs in response to virus challenge. To examine the capacity of transfected HA DNA to stimulate the generation of AFCs responsive to lethal challenge, we sampled several lymphoid tissues within and outside the respiratory tract. In once-immunized mice, challenge resulted in peak frequencies of IgG-producing AFCs in the mediastinal lymph nodes within 7 days (Table 2). These cells represented a maximum of about 0.4% of the total organ population. Other tissues contained substantially smaller populations of all isotypes. Boosting with a second dose did not yield increased numbers of AFCs in any tissue except the cervical nodes, where a 10-fold expansion of the IgA-producing cell population was seen on day 7 postchallenge (Table 2). The same profile of responsiveness was seen when the total number of AFCs per organ was calculated (Table 3). Concentrations of AFCs in the lower respiratory tract (mediastinal nodes) were three- to sixfold higher than in the nasopharynx (cervical nodes). Approximately 4.4×10^4 AFCs were found in respiratory tract lymphoid tissues (cervical and mediastinal nodes) in both once- and twice-immunized groups. On the basis of their dominant numbers (90% of the total body count), these cells were probably the greatest contributors to the postchallenge antibody levels in serum as seen in Fig. 1. This small number of AFCs can maintain a neutralizing serum antibody titer (2).

Role of CD4$^+$ helper cells in the generation of AFCs. It was possible to demonstrate the importance of CD4$^+$ T cells in the generation of AFCs by use of depletion monoclonal antibodies. Multiple i.p. injections of the anti-CD4 monoclonal antibody GK1.5 into mice immunized twice with HA DNA, before and after challenge (day 45 overall), eliminated AFCs from the inguinofemoral, mediastinal, and cervical lymph nodes and the bone marrow and spleen within 1 week (Table 2). Loss of the ability to elaborate AFCs resulted in more severe influenza (i.e., increased pneumonitis, infectious virus lung titers, piloerection, lethargy, and anorexia), but the effect on prevention could not be ascertained because the experiment was terminated on day 7.

Protection from virus challenge. Given the HA-specific AFC responses in the cervical and mediastinal nodes of DNA-vaccinated mice, we sought to determine whether DNA vaccination could confer protection against influenza virus. All 14 mice given two doses of HA DNA survived influenza virus infection, compared with 3 of the 9 mice given control DNA or no DNA. The level of protection achieved with a single injection of HA DNA was not as effective, with 4 of 16 (25%) dying of influenza. The difference in survival between twice- and once-immunized mice was significant at the $P < 0.05$ level by Fisher's two-tailed exact test. Several factors may have contributed to the improved survival of the twice-immunized group. For example, greater numbers of AFCs in the spleen and bone marrow before (Table 1) and after (Table 3) challenge, as well as in the mediastinal nodes (30% of total on day 3 versus $<10\%$ in the once-immunized group) and cervical nodes (about 40% of total on day 7 versus $<10\%$ in the once-immunized group), may have given rise to higher titers in serum by HI assay (Fig. 1b). The protection afforded by HA DNA vaccination was within the range previously described for this strategy (16).

Serum antibody responses in mice immunized with an HA subunit vaccine. Mice were given two i.p. injections, at a 2-week interval, of a high dose of an HA-containing vaccine (50 μg of HA) in the absence of adjuvant. Beginning on day 18

TABLE 3. Total AFCs in response to live virus challenge in mice immunized with HA DNA

Tissue[a]	AFC totals on postchallenge day[b]:		
	3	7	13
Twice-immunized group			
CLN	0	16,870	1,320
MLN	864	26,350	4,810
BM	80	152	600
Spleen	2,081	2,430	3,990
Once-immunized group			
CLN	120	3,050	1,320
MLN	0	33,200	4,440
BM	0	100	570
Spleen	1,530	5,980	6,210
DNA control group			
CLN	0	0	ND
MLN	7,840	8,000	ND
BM	0	0	ND
Spleen	1,650	200	ND

[a] Mice were either immunized once or twice by gene gun inoculation with HA DNA. Both groups as well as the DNA controls were challenged i.n. with 3 LD_{50} of live PR8 virus on day 45 after vaccination and examined for AFCs. CLN, cervical lymph nodes; MLN, mediastinal lymph nodes; BM, bone marrow.

[b] To estimate the total number of AFCs per organ, we multiplied the AFC frequencies in each tissue with the corresponding mean B-cell cellularity of that organ at each time point; for the mediastinal lymph nodes and cervical lymph nodes, 50% of total was assumed (41), while for the spleen and bone marrow, 30% was assumed (3). ND, not done.

TABLE 4. Pre- and postchallenge localization of AFCs in mice immunized twice with subunit vaccine

Tissue[a]	No. of AFCs on day[b]:								
	IgM			IgG			IgA		
	3	7	14	3	7	14	3	7	14
Twice-immunized group									
Prechallenge									
CLN	0	0	0	0	0	0	0	0	0
MLN	3	0	0	0	0	10	0	0	0
Spleen	15	35	15	275	23	4	1	0	0
Postchallenge									
CLN	1	3	ND	0	1	ND	0	1	ND
MLN	0	11	ND	0	125	ND	0	36	ND
Spleen	14	43	ND	9	28	ND	0	20	ND
Naive group									
CLN	8	20	5	1	4	32	0	0	18
MLN	2	21	11	0	20	45	0	0	2
Spleen	6	0	0	2	0	0	0	0	0

[a] Mice were immunized twice with subunit vaccine (50 μg of HA) given i.p. or remained untreated (naive). Two weeks later, vaccinated mice were challenged i.n. with 3 LD_{50} of live PR8 virus and monitored for 1 week by ELISPOT sampling of lymphoid tissues. CLN, cervical lymph nodes; MLN, mediastinal lymph nodes. Naive mice were infected i.n. with the same challenge dose.

[b] Prechallenge (post-secondary immunization) sampling on days 3, 7, and 14 corresponds overall to days 18, 22, and 29, respectively. Postchallenge sampling on days 3 and 7 corresponds overall to days 33 and 37, respectively. Each result is the mean number of AFCs per 10^5 total cells in a given tissue from one or two mice in four replicate platings at each sampling time. The mice were sampled simultaneously with the groups reported in Tables 1 and 2. Unchallenged vaccinated mice from the same group had about 30 IgM-producing AFCs in the spleen prior to challenge; cells secreting IgG and IgA were not present. The inguinofemoral lymph nodes and bone marrow were also sampled, but no AFCs were detected. ND, not done.

(3 days postboost), concentrations of HI antibodies in serum were measured (Fig. 1a). The resulting titers (about 1.4×10^2) remained elevated over the next 2 weeks. Although HI titers in serum increased progressively after live virus challenge (Fig. 1b), they were not fully protective against influenza (3 of 10 mice died).

Comparison of AFC responses in mice immunized with an HA subunit vaccine or live virus. The localization of AFCs in vaccinated mice was examined under the same conditions as described above for DNA-immunized mice in response to boost and challenge. Comparison was made with the results obtained with naive mice challenged with the same dose of live virus (Table 4). AFCs were found predominantly in the spleen in vaccinated mice after boosting. Peak levels of IgG-producing AFCs appeared by day 3, a reflection of their rapid response to high antigen concentrations (43). Increased AFC numbers were detected postchallenge in the mediastinal nodes; however, in contrast to the response to boosting, maximum levels were reduced and the kinetics of AFC development were delayed. By the end of the first week, total AFC numbers per mouse were distributed between the mediastinal nodes and the spleen. However, the AFC responses differed from and surpassed the responses to challenge in naive mice, which were equivalently distributed between the cervical and mediastinal nodes. These data indicate that high doses of HA subunit vaccine generate fewer AFCs in response to challenge than does the HA DNA vaccine. In an earlier study (20a), we found that lower doses of HA subunit vaccine were even less capable of providing adequate immunity to virus challenge.

DISCUSSION

AFC localization in response to DNA vaccination. Our study indicates that gene-gun delivery of very small amounts (0.4 μg) of HA DNA (pJW4303/H1) into the epidermis results in the development of immunologic memory that can be rapidly and efficiently reactivated by lethal virus challenge. As summarized in Table 5, AFCs producing IgG and IgA were localized predominantly in respiratory tract lymphoid tissues, at the site of challenge antigen deposition. The emergence of HA-specific AFCs in the cervical and mediastinal lymph nodes correlates well with the relative importance of these tissues in protection against virus challenge. The lag phase of 7 days may indicate the time needed for cognate T-cell help to be recruited for HA-specific memory B cells localized in skin or skin-associated lymphoid tissues or in other tissues (27, 37). Removal of the $CD4^+$ population by in vivo treatment with monoclonal antibodies eliminated all AFC responses, demonstrating the T-cell dependence of HA-specific AFCs induced by DNA immunization. The finding of durable AFC responses in the bone marrow agrees with the model of Bachmann et al. (3), in which long-lasting antibody production in the marrow is independent of antigen persistence in that tissue. Nonetheless, the increasing levels of antibody observed after vaccination and the maintenance of AFCs in the spleen and bone marrow suggest that DNA vaccination provides long-lasting immunity. Given the critical roles of epidermal (38) and splenic (25) dendritic cells

TABLE 5. Summary of AFC responses to live virus challenge after immunization with HA DNA, an HA subunit vaccine, or live virus

Immunogen	Route of delivery	Peak response and dominant Ig isotype[a]					HI titer[b]	Protection[c] (%)
		Regional and draining lymph nodes		Non draining IFLN	Bone marrow	Spleen		
		CLN	MLN					
HA DNA	Gene gun, two doses	+++ (day 7), IgG > IgA	++++ (day 7), IgG	−	+, IgM	+ (day 7), IgG/M/A	8×10^2	14/14 (100)
HA DNA	Gene gun, single dose	++ (day 7), IgG	+++ (day 7), IgG	−	+, IgM	+ (day 7), IgG/M/A	8×10^2	12/16 (75)
HA	i.p.	−	+++ (day 8), IgG	−	+, IgG	++ (day 8), IgM > IgG	2×10^2	7/10 (70)
Live virus[d]	i.n.	+ (day 3), IgG	++++ (day 3), IgG > IgA	−	+, IgG/M	+, IgG/M	1×10^3	10/10 (100)

[a] ++++, >400 AFC per 10^5 total cells; +++, >100 AFC per 10^5 cells; ++, <100 but >25 AFC per 10^5 cells; +, <25 AFC per 10^5 cells; −, no response. CLN, cervical lymph nodes; MLN, mediastinal lymph nodes; IFLN, inguinofemoral lymph nodes.

[b] Day 7 titers.

[c] Protection indicates the number of surviving animals divided by the total number.

[d] Summarized from reference 21: mice were primed i.n. with 0.0025 μg of HA presented as live PR8 and similarly challenged with 3 LD_{50} of virus.

in inducing T and B cells, it is possible that the spleen gives rise to AFCs that mature in the bone marrow.

AFC responsiveness to virus challenge. We would stress that the heightened AFC responsiveness took place in the appropriate immunologic compartment, the lungs. The HA-specific AFCs in lung-associated lymphoid tissue ranged from 0.4 to 0.6% of the total organ population of these cells and represented greater than 90% of the total number of AFCs in the mouse. This result reflects the immunogenicity of the HA provided by DNA vaccination, whether given in one or two doses. Appropriate localization is not necessarily the case with other forms of the HA molecule (22).

Relation of antigen persistence to AFC generation. It could be argued that the AFC localization patterns we describe simply reflect extensive HA distribution following inoculation and the overt persistence of antigen in lymphoid tissues, as in other systems (9, 24). This issue was not explicitly examined, but our findings suggest that it may not be the case. First, long-lasting AFC populations were restricted to the spleen and bone marrow, sites of long-term antibody production (3, 19, 31, 36, 42) following acute and nonpersisting infections such as influenza (13). Second, AFCs were absent in the inguinofemoral and mediastinal lymph nodes, even though these nodes probably drain the site of gene-gun inoculation.

Helper T-cell requirement in the generation and maintenance of AFCs. $CD4^+$ Th1 cells, which support an antiviral B-cell response (7, 32), are thought to play a major role in clearing virus from the lungs and promoting recovery from infection (17), even in the absence of functional $CD8^+$ T lymphocytes (12, 47). The findings reported here are consistent with this view. That AFC development in response to challenge depends on helper T cells is indicated by the results of anti-CD4 monoclonal antibody treatment of long-term primed and boosted mice, which prevented the development of HA-specific AFCs upon challenge, even in animals positive for IgM expression. Additional support for this requirement comes from the increased proportion of IgG- and IgA-producing AFCs collected from DNA-immunized mice, an isotype profile consistent with $CD4^+$ T-cell-mediated class switching.

Does HA DNA vaccination stimulate a different memory AFC response from that stimulated by HA subunit vaccine? Adequate humoral immunity to influenza virus would include not only preexisting antibodies but also rapidly responding memory B cells and AFC localization at appropriate sites of antigen entry. Memory $CD4^+$ T cells would provide the interleukin milieu needed to promote a more rapid virus-specific antibody response. The lymphoid tissues draining the respiratory tract would be responsible for generating the majority of the AFC response to challenge (21). Ideally, adequate defense against influenza virus infection would be achieved without regard to the priming antigen or the site of vaccination.

The AFC response induced by HA DNA is more closely aligned with i.n. priming by live virus than with delivery of high doses of HA glycoprotein by the i.p. route. Full protection of DNA-vaccinated mice against challenge is afforded by both preexisting antibodies and the rapid development of AFCs in the cervical and mediastinal lymph nodes, producing elevated levels of IgG and IgA. The slight delay in the generation of AFCs is possibly due to the initially high partitioning of B cells to memory cells instead of the effector subset.

How is memory initiated by DNA vaccination? The events that control initial B-cell activation (virgin-to-memory transition) by HA DNA remain undefined. Nonetheless, skin and associated lymphoid tissues provide highly efficient sites for antigen processing and presentation, resulting in long-lasting immunity not only to skin-associated antigen (27, 38, 39) but also to antigen targeted there by DNA vaccination (16, 28). Likely contributors to the important role of these sites in the generation of immunity include Langerhans cells, the cytokine-rich milieu generated during the primary immune response in the skin, and the distribution of antigen-specific memory cells back to extracutaneous sites as well as the skin (15, 27). The precise mechanisms of action of these candidate factors are under investigation.

Conclusions. In mice immunized with HA DNA, the localization of AFCs in response to influenza virus challenge primarily reflects the distribution of challenge antigen and the extent of T-cell help, much like the responses elicited by live virus. Gene-gun delivery of DNA into the skin thus provides an efficient means of transfection and antigen presentation to the immune system.

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