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Comparison of Humoral Immune Responses Elicited by DNA and Protein Vaccines Based on Merozoite Surface Protein-1 from *Plasmodium yoelii*, a Rodent Malaria Parasite

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Immunization with DNA vaccines encoding relevant Ags can induce not only cell-mediated immune response but also humoral immune responses against pathogenic microorganisms in several animal models. Our previous results demonstrated that, when the C terminus (PyC2) of *Plasmodium yoelii* merozoite surface protein-1 (MSP-1), a leading vaccine candidate against erythrocytic stages of malaria, was expressed as a fusion protein (GST-PyC2) with glutathione S-transferase (GST), it elicited Ab-mediated protective immune responses in BALB/c mice. In our present study, we wished to examine the humoral responses to a DNA vaccine (V3) encoding GST-PyC2. The GST-PyC2 expressed in V3-transfected Cos 7 cells was recognized by a protective monoclonal Ab to PyC2 (mAb302), although the secreted product had undergone N-linked glycosylation. When BALB/c mice were immunized with V3 plasmid, anti-PyC2 Abs were successfully induced. These Abs immunoprecipitated native PyMSP-1 protein and competed with mAb302 for binding to its epitope at a level similar to those elicited by GST-PyC2 protein immunization. However, these Abs had significantly lower titers and avidities, and different isotype profiles and protective capacities against a lethal erythrocytic stage challenge, than those resulting from immunization with GST-PyC2 protein. Most surprising was the finding that, in contrast to protein immunization, there was no significant increase in the avidity of either GST-specific or PyC2-specific IgG Abs during the course of DNA immunization. This suggests that there may be little or no affinity maturation of specific Abs during DNA immunization in this system. *The Journal of Immunology*, 1998, 161: 4211–4219.

Vaccination with DNA represents a novel means of expressing Ags in vivo for the generation of protective immune responses (1, 2). Direct administration of plasmid DNA not only induces potent cell-mediated immune responses but also elicits humoral immune responses against many infectious diseases in several animal models (3–19). The protective role of Abs induced by DNA vaccines was clearly demonstrated in a rodent influenza model (3, 5, 11, 18, 19) in which immunization with a DNA vaccine encoding hemagglutinin (HA) elicited high levels of anti-HA Abs that inhibited agglutination of RBC by influenza virus in vitro and protected mice against an otherwise lethal influenza challenge (19). However, humoral immune responses elicited by DNA vaccines have not been well characterized.

With the widespread development of drug resistance in the parasite and insecticide resistance in the mosquito vectors, malaria remains a major public health problem in the world (20–22). It is estimated that there are 2 to 3 million deaths due to malaria and at least 300 million cases of clinical malaria each year. However, there are no effective malaria vaccines available. Since malarial parasites change both antigenically and biochemically during their life cycle, an effective malarial vaccine should ideally induce different types of immune responses against multiple targets from different stages in the parasite’s life cycle. DNA vaccines seem to be well suited for this purpose (23–25) because a DNA vaccine against malaria could be designed to express Ags from both preerythrocytic and erythrocytic stages and thereby elicit immune responses against multiple life cycle stages. Recently, there has been significant progress in DNA vaccine development against preerythrocytic stages of malaria (8, 23–25). DNA vaccines encoding preerythrocytic Ags from *Plasmodium yoelii*, including PyCSP, PyHEP17, and PySSP2, have been shown to induce protective CD8+ T cell responses against sporozoite challenge, but not blood-stage challenge, in a genetically restricted manner in rodent models (23–25). Moreover, combination of the individual DNA vaccines circumvented the genetic restriction of protection against *P. yoelii* sporozoite challenge (26). In addition, Ag-specific CTL and humoral responses have also been induced in nonhuman primates following immunization with plasmid DNA encoding four *Plasmodium falciparum* preerythrocytic stage Ags (24). However, DNA vaccination has not yet been successful in inducing humoral immune responses required for protective immunity against a blood-stage challenge with malaria parasites.

One of the most promising vaccine candidates against blood stages of malaria is the merozoite surface protein-1 (MSP-1). Immunization with intact, affinity-purified MSP-1 protein can induce protective responses against challenge in nonhuman primate and rodent models of malarial infection (27–30), and these responses are primarily directed to the C-terminal region of MSP-1 (31). The C-terminal 19-kDa fragment contains a series of cysteine residues...
that are conserved among different species of plasmodia infecting humans, primates, and rodents (32), as well as between different isolates of P. falciparum (33–35). It has been suggested that these cysteine residues are arranged as two putative epidermal growth factor-like domains (36). We have previously shown that, when expressed as a fusion protein with glutathione S-transferase (GST) of Schistosoma japonicum, the C-terminal region of the P. yoelii MSP-1 (designated PyC2) can be recognized by a protective mAb (mAb302) (37) and can induce protective immune responses against another otherwise lethal blood-stage challenge infection in several adjuvant systems (38, 39). These results were confirmed by others (40, 41) and have been extended to the homologous region of P. falciparum in Aotus monkeys (42, 43). In rodents, we have shown that these protective effector functions are predominantly mediated by Abs because the passive transfer of immunized sera or purified Ig can protect naive mice against lethal blood-stage challenge with P. yoelii (39). In addition, no effector role for CD8+ T cells could be identified after challenge infection in immune mice selectively depleted of these cells (39).

In the present study, we have characterized the malaria-specific humoral immune responses induced by a DNA vaccine that contains the DNA sequence encoding GST-PyC2 and compared them to the corresponding protein vaccine, GST-PyC2.

Materials and Methods

Experimental infections

Six- to eight-wk-old, male BALB/c ByJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in our American Association for the Accreditation of Laboratory Animal Care-approved facilities. The lethal P. y. yoelii 17XL strain was originally obtained from Dr. John Finerty (National Institutes of Health, Bethesda, MD) and maintained as cloned cryopreserved stabilates. Blood-stage infections were initiated by i.v. injection of 108 P. yoelii 17XL parasitized erythrocytes (E). Resulting parasitemias were monitored by enumerating parasitized RBC in thin tail-blood smears stained with Leukostat (Fisher Diagnostic, Pittsburgh, PA).

Fusion protein construct and protein isolation

The fusion protein GST-PyC2 was described previously (39). Briefly, the C-terminal region of the P. yoelii 17XL MSP-1 gene was PCR amplified and joined in frame to the 3′ end of the S. japonicum GST gene within the pGEX/2T vector. The resultant fusion protein, designated GST-PyC2, was expressed in recombinant Escherichia coli and isolated by affinity chromatography.

Construction of PyMSP-1 based DNA vaccine

The DNA fragment encoding GST-PyC2 was PCR amplified from the pGEX/2T construct, and BamHI sites were added to each end of the PCR product. This PCR product was cloned into a BamHI site in the expression vector VR1020 (Vical), which contains a tissue-type plasminogen activator (TPA) leader sequence, a CMV promoter, a bovine growth hormone poly(A) terminus, and a kanamycin resistance marker. The sequences of the fusion region and the insert DNA encoding GST-PyC2 were confirmed by DNA sequencing. This DNA vaccine was designated as V3 and used to immunize mice. The V3 plasmid was prepared in large scale with the Endo-Mega kit (Qiagen, Hilden, Germany). The endotoxin level in the plasmid DNA was <3 EU/ml as measured by the E-TOXATE (Limulus amebocyte lysate) kit (Sigma, St. Louis, MO). Plasmid DNA was then analyzed by agarose gel electrophoresis and quantified by spectrophotometry (OD260/OD280 ratio > 1.8). The isolated DNA was diluted in endotoxin-free PBS and was then ready for immunization.

Transient transfection of Cos 7 cells in vitro

Protein expression from the V3 plasmid was evaluated in vitro by transiently transfecting Cos 7 cells (American Type Culture Collection, ATCC), Manassas, VA) using the LIPOFECTIN Reagent (Life Technologies, Grand Island, NY) following the instructions from the manufacturer. Briefly, Cos 7 cells were grown in DMEM supplemented with 10% FBS overnight. In a 6-well (35-mm) tissue culture plate, 1 106 Cos 7 cells in 2 ml complete DMEM were seeded and incubated in a 10% CO2 incubator at 37°C overnight until the cells were 40 to 60% confluent. For each transfection, 4 μg of V3 plasmid DNA was diluted in 100 μl of serum-free medium (OPTI-MEM I reduced serum medium (OPTI-MEM); Life Technologies), and 10 μl of LIPOFECTIN reagent was diluted in OPTI-MEM. The diluted DNA and diluted LIPOFECTIN reagent were combined, mixed gently, and incubated at room temperature for 15 min. The mixture was then added to 0.8 ml OPTI-MEM, mixed gently, and overlaid onto the attached Cos 7 cells. After the cells were incubated in a 10% CO2 incubator for 24 h, 1 ml of complete DMEM containing 20% FBS was added to each well, and the cells were incubated for an additional 36 h. The cells were then washed once with methionine-free DMEM medium (Life Technologies) and incubated in 1 ml of methionine-free DMEM containing 500 μCi [35S]Protein Labeling Mix (NEN Life Science Products, Boston, MA) for another 8 h. Following the labeling, the medium from each of the wells was collected for immunoprecipitation experiments, and the cell pellet was resuspended in solubilization buffer containing 20 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Brij58, 0.2 mM tosylphenylchloromethylketone, 0.2 mM 1, 10 phenanthalene, 2 mM PMSF, 2 mM tosyllysylchloromethylketone, and 1 mM iodoacetamide. The suspension was incubated on ice for 30 min with gentle mixing. Following the incubation, the mixture was centrifuged at 85,000 g for 1 h. The supernatant fluids were collected and used as soluble Abs from the pellet of transfected cells. TCA-precipitable proteins (1 106 cpm) from the supernatant fluids or cell pellets from transfected cells were used for each reaction in the immunoprecipitation experiments.

Treatment of transfected COS 7 cells with tunicamycin

Eight hours after transfection of COS 7 cells with V3 plasmid as above, 1 ml of complete DMEM supplemented with 20% FBS was added to each well, and the cells were incubated for 12 h. Tunicamycin was added to each well to a final concentration 1 μg/ml, and the cells were incubated for another 24 h (44). Following tunicamycin treatment, the cells were radio-labeled, and supernatants and the cell pellets were collected and treated as described above. Both supernatant and cell extracts were then used as Abs in immunoprecipitation experiments with antisera.

Immunoprecipitation assay

Mice were infected with P. yoelii 17XL, and blood was collected when parasitemia reached approximately 30%. Plasmodial proteins were then metabolically labeled with [35S]Protein Labeling Mix as described (37). Immunoprecipitation assays were then performed as described previously (45). Briefly, 2 μl of sera from pooled GST-PyC2 protein-immunized mice, V3 DNA-immunized mice, or 25 μl of mAb302 hybridoma tissue culture fluid were mixed with approximately 1 106 cpm of TCA-precipitable soluble parasite proteins or soluble Abs generated from transfected cell cultures as described above. The mixtures were then incubated for 1 h on ice. One million of rabbit anti-mouse IgG, IgM, IgA secondary Abs (Zymed Immunochemicals, San Francisco, CA) was added to the tube, mixed, and incubated for 30 min on ice. Twenty microliters of a 10% suspension of heat-killed Staphylococcus aureus Protein A (Boehringer Mannheim, Indianapolis, IN) were added to each tube, mixed thoroughly, and incubated on ice for 30 min. The mixture was then underlaid with 200 μl of 1 M sucrose in precipitation buffer and centrifuged at 12,000 rpm for 3 min. The supernatant was carefully removed and discarded, and the pellet was washed three times with precipitation buffer. Finally the pellet was resuspended in 20 μl of 2× SDS-PAGE sample buffer. Samples were boiled and subjected to analysis by SDS-PAGE.

Immunization, sera collection, and challenge infection

Groups of four 8- to 10-wk-old BALB/c mice were immunized with V3 plasmid DNA five times at 3-wk intervals either i.m. in the anterior tibialis muscles (two sites) or intradermally (i.d.) on the back skin of mice (three sites). The dosage for each immunization was 200 μg for the first and 100 μg for all subsequent doses in a volume of 100 μl. The negative control groups were immunized with the parental plasmid (VR1020) without insert (designated as W) either i.m. or i.d. Another group of 4 BALB/c mice was immunized with GST-PyC2 recombinant fusion protein in Ribi adjuvant system-GRAS; Ribi Immunocytex, Hamilton, Montana (46). Briefly, 8- to 10-week-old BALB/c mice were immunized with 60 μg of GST-PyC2 (providing 20 μg of PyC2). Fusion protein was first administered s.c. in 200 μl (two sites, 100 μl each site) of RAS adjuvant, suspended in 20 mM Na2HPO4/NaH2PO4-15 mM NaCl, pH 7.4 (PBS). This dose was repeated s.c. after 3 wk and again at 6 wk after the initial injection, but administered i.p. Mice were bled through the tail vein one day before each immunization to obtain sera for analysis. Two weeks after the final immunization, each group was challenged by i.v. injection of
ELISA for measurement of Ab titers and isotypes

ELISAs were performed as described (38). Briefly, GST-PyC2 fusion protein was isolated by affinity chromatography, cleaved with thrombin, and purified such that free GST and uncleaved fusion protein were removed from the preparations. The C-terminal MSP-1 portion of the fusion protein PyC2) was used to coat the wells of Maxi-sorb immunoplates (Nunc, Naperville, IL) at 0.5 μg/ml in carbonate buffer, pH 9.6. Wells were then blocked with 0.2% Tween 20 (Sigma) in 25 mM Tris-HCl (pH 8.0)-150 mM NaCl (Tris-buffered saline, TBS). Antisera collected from individual mice from each group were serially diluted in 0.2% Tween 20 in TBS. Ab dilutions were added to the appropriate wells in duplicate and followed by biotinylated rabbit anti-mouse Abs, including anti-IgG, IgM, IgA, Heavy and Light chain (Zymed), streptavidin-alkaline phosphatase, and p-nitrophenyl phosphate substrate (Sigma 104). The reaction was terminated by adding 50 μl of 5 N NaOH, and OD was measured at 405 nm using a Flow TiterTek Multiscan Plus plate reader. For isotype analysis, the bound Abs were detected with affinity-purified, biotinylated, rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, or IgM Abs (Zymed), avidin-alkaline phosphatase (Zymed), and p-nitrophenyl phosphate (Sigma 104), and the OD value was measured at 405 nm as described above.

mAb302 epitope competition assay

This assay was utilized to assess the ability of immune sera to compete with protective mAb302 for binding to MSP-1 (P. A. Calvo and C. A. Long, unpublished observations). Briefly, sera were serially diluted in 0.2% Tween 20 in TBS and incubated in individual wells of Maxi-immunoplates coated with PyC2 Ag. The Ab dilutions were incubated for 45 min at room temperature. Following incubation, approximately 1 × 10^7 TCA-precipitable counts of metabolically labeled mAb302 tissue culture fluid was added to each well and allowed to incubate for another 45 min. Following the second incubation, the supernatants were discarded, and wells were washed 3 times with 0.2% Tween in TBS and two times with TBS alone. Subsequently, 100 μl of 1% SDS was added to individual wells and incubated for 30 min. After incubation, the SDS solution in each well was carefully removed from the individual wells, transferred to scintillation vials containing 3 ml of Scintiverse II (Fisher Scientific, Fair Lawn, NJ), and radioactivity was measured using a Beckman LS7000 Scintillation Counter.

Ab avidity analysis

An ELISA-based Ab avidity assay was performed as described previously (47). PyC2 was used to coat the wells of Maxi-sorb immunoplates, and the tested sera were diluted in 0.2% Tween 20 in TBS and added to each well as described for ELISA. Following incubation at room temperature for 1 h, the individual wells were washed 3 times with 0.2% Tween in TBS. The Ag-Ab complexes bound to the plate were then subjected to incubation with 0.2% Tween 20 in TBS or increasing concentrations of ammonium thiocyanate for 30 min at room temperature. The concentrations of NH4SCN used in this experiment were 0 M, 1 M, 3 M, 4 M, and 6 M. Following extensive washes, the wells were incubated with biotinylated rabbit anti-mouse IgA, IgG, and IgM, streptavidin-alkaline phosphatase, and p-nitrophenyl phosphate substrate, and OD value was determined at 405 nm.

Results

Construction of a PyC2-based DNA vaccine for immunization and its expression in Cos 7 cells in vitro

The DNA fragment encoding GST-PyC2 was cloned into a BamHI site downstream from the TPA leader sequence in the VR1020 vector, which also contains a CMV promoter, a bovine growth hormone poly(A) terminus, and a kanamycin resistance marker. The sequences of the junction region and the insert were confirmed by DNA sequencing. This DNA construct encoding GST-PyC2 and its parental plasmid VR1020 without insert were designated as V3 and W, respectively. To investigate expression in mammalian cells, Cos 7 cells were transiently transfected with either V3 or W plasmids in vitro and labeled metabolically with [35S]methionine. An amount equal to 1 × 10^5 cpm of TCA-precipitable soluble Ags from both supernatants and cell pellets was immunoprecipitated with mAb302, which recognizes a conformational epitope in the PyC2 region, polygonal mouse anti-GST-PyC2, or control sera. The precipitates were analyzed by SDS-PAGE. While normal mouse sera (NMS) did not recognize any proteins, both anti-GST-PyC2 and mAb302 recognized two polypeptides from both supernatants (Fig. 1) and pellets (data not shown) of the transfected Cos 7 cells. The bands from the pellets precipitated by mAb302 were much weaker than those precipitated by anti-GST-PyC2 sera (data not shown), suggesting that not all of the intracellular GST-PyC2 expressed the epitope recognized by mAb302. However, the two polypeptides in the supernatant fluids were equally recognized by the two Abs, indicating that they carried the conformational mAb302 epitope. No proteins were recognized by either anti-GST-PyC2 or mAb302 from W-transfected cell cultures (Fig. 1), establishing that the precipitated polypeptides in V3-transfected cell cultures were products expressed from the insert in V3, not from the vector itself.

Interestingly, two radioactive bands of apparent molecular mass 39 kDa and 36 kDa were resolved during SDS-PAGE of each precipitate of V3-transfected cells using both anti-GST-PyC2 and mAb302, suggesting that the polypeptides expressed in Cos 7 cells may undergo secondary modifications. Considering the fact that there are two potential N-glycosylation sites in the PyC2 region of GST-PyC2, tunicamycin, an inhibitor of N-linked glycosylation, was added to the transfection cultures. After treatment with tunicamycin, only the 36-kDa protein remained after immunoprecipitation with mAb302 or anti-GST-PyC2 sera (Fig. 1). These results suggest that a proportion (about half) of the protein expressed in V3-transfected Cos 7 cells undergoes N-glycosylation. However, both the glycosylated and the nonglycosylated polypeptides expressed in V3-transfected Cos 7 cells maintained the conformational epitope recognized by mAb302.

Immunization of mice with V3 vaccine elicits specific Abs against PyC2

Two groups of 8- to 10-wk-old BALB/c mice were immunized either i.m. or i.d. with the V3 plasmid DNA in PBS. The W plasmid was used as a negative DNA control. Five immunizations were given to each group of mice at 3-wk intervals. In addition, another group of BALB/c mice was immunized with the corresponding recombinant protein GST-PyC2 in RAS three times at 3-wk intervals as described previously (46). Blood samples were collected 1 day before the next immunization or parasite challenge, and anti-PyC2 Abs were examined by ELISA using PyC2 cleaved from GST-PyC2 as the target Ag.
IgG3 and IgM Abs. However, sera from DNA-immunized mice showed a narrower distribution of isotypes, predominantly IgG1 and IgG2a for V3 i.m. group and almost exclusively IgG1 for the V3 i.d. group. In addition, the ratio of IgG1 to IgG2a for V3 i.d. group (5.58) was significantly higher than that of the V3 i.m. group (1.20) (p < 0.01). This suggests that V3 i.d. and V3 i.m. immunizations may elicit different Th-type immune responses, with V3 i.d. predominantly of the Th2 type while V3 i.m. may elicit both Th1- and Th2-type immune responses.

**DNA immunization elicits anti-PyC2 Abs with different isotype profiles from those of protein immunization**

The isotype distribution of anti-PyC2 Abs in prechallenge sera from DNA- and protein-immunized mice was determined by ELISA. As shown in Figure 3, GST-PyC2 immune sera (P-3) contained predominantly IgG1, IgG2a, and IgG2b, and lower levels of IgG3 and IgM Abs. However, sera from DNA-immunized mice showed a narrower distribution of isotypes, predominantly IgG1 and IgG2a for V3 i.m. group and almost exclusively IgG1 for the V3 i.d. group. In addition, the ratio of IgG1 to IgG2a for V3 i.d. group (5.58) was significantly higher than that of the V3 i.m. group (1.20) (p < 0.01). This suggests that V3 i.d. and V3 i.m. immunizations may elicit different Th-type immune responses, with V3 i.d. predominantly of the Th2 type while V3 i.m. may elicit both Th1- and Th2-type immune responses.

**FIGURE 2.** Concentrations of anti-PyC2 Abs from V3 plasmid- and GST-PyC2 protein-immunized mice by ELISA. A. Titration of anti-PyC2 Abs in prechallenge sera from DNA- and protein-immunized mice. Vm-5, Vd-5, P-3, and NMS indicate prechallenge sera from i.m. V3-, i.d. V3-, and GST-PyC2 protein-immunized mice, and normal mouse sera, respectively. B. Relative concentrations of anti-PyC2 Abs from DNA- and protein-immunized mice after each immunization. BALB/c mice were immunized five times with V3 plasmid i.m. and i.d., respectively, and three times with GST-PyC2 protein at 3-wk intervals. Sera were collected from individual mice 1 day before the next immunization or the final challenge. Vm, Win, Vd, Wd, P, and NMS refer to sera from i.m. V3-, i.m. W-, i.d. V3-, i.d. W-, and GST-PyC2 protein-immunized mice, and normal mice, respectively. SEs are indicated in Figure 2, both A and B.

As shown in Figure 2A, while there were no PyC2-specific Abs induced by the W DNA control, both i.m. and i.d. V3-immunized mice produced specific Abs against PyC2. The titers of anti-PyC2 Abs from the DNA i.m. group were similar to those of the DNA i.d. group after the fifth immunization (p > 0.05), but they were significantly lower than those of protein-immunized mice after the third inoculation (p < 0.05) (Fig. 2A). In addition, the relative concentrations of anti-PyC2 Abs after each immunization from both DNA- and protein-immunized mice were measured at a dilution of 1:400. As shown in Figure 2B, the anti-PyC2 Abs in both DNA groups became detectable after the third immunization, then increased after the fourth inoculation. There was no further increase in relative concentration of anti-PyC2 Abs after the fourth immunization in both DNA groups (p > 0.05). This indicates that the DNA vaccination takes longer to generate detectable specific Abs in mice than protein vaccination, in which the anti-PyC2 Abs became detectable after the first immunization (Fig. 2B).

**FIGURE 3.** Isotype profiles of anti-PyC2 Abs from DNA- and protein-immunized mice. Vm-5, Vd-5, P-3, and NMS refer to prechallenge sera from i.m. V3-, i.d. V3-, and GST-PyC2 protein-immunized mice, and normal mouse sera, respectively. SEs are demonstrated in each group.

**DNA immunization elicits anti-PyC2 Abs with different isotype profiles from those of protein immunization**

The specificity of the anti-PyC2 Abs induced by DNA immunization was assessed by immunoprecipitation of metabolically labeled total parasite proteins with sera from immunized mice taken before challenge infection. As shown in Figure 4, the pooled sera from i.d. V3- and i.m. V3-immunized mice, as well as the protein-immunized sera, immunoprecipitated radiolabeled native PyMSP-1 (apparent molecular mass 220 kDa), indicating that the Abs induced by V3 plasmid immunization recognize native parasite proteins. However, the sera from V3-immunized mice precipitated much less native PyMSP-1 than those from protein-immunized mice at the same dilution (1:50) (Fig. 4). To investigate whether this differential ability of Abs to immunoprecipitate native protein was due to the different levels of anti-PyC2 Abs in sera, pooled sera from protein-immunized mice were further diluted to 1:1400, at which they showed the same OD value with a 1:50 dilution of sera from either i.m. V3 DNA- or i.d. V3 DNA-immunized mice. When the final dilution of 1:1400 protein-immunized sera was used, the bands immunoprecipitated by pooled protein-immunized

**FIGURE 4.** Immunoprecipitation of radiolabeled native proteins of *P. yoelii* with prechallenge sera from DNA- and protein-immunized mice. *P. yoelii* proteins were labeled with [35S]methionine, and 3 × 10⁶ TCA-precipitable counts of soluble plasmodial Ags were added to each reaction. The final dilution of pooled sera in each reaction is indicated. Anti-Vd, anti-Vm, anti-P, NMS, and mAb302 refer to pooled prechallenge sera from i.d. V3-, i.m. V3-, and GST-PyC2 protein-immunized mice, normal mouse sera, and mAb302 tissue culture fluid, respectively.
sera (lane 4, Figure 4) showed similar intensity to those obtained with 1:50 diluted, pooled DNA-immunized sera (lane 2 and lane 3, Figure 4). These results suggest that anti-PyC2 Abs from both DNA- and protein-immunized mice have similar ability to react with native parasite proteins after normalizing titers against PyC2.

DNA immunization induces anti-PyC2 Abs with the same mAb302 specificity as protein immunization

Since both the GST-PyC2 fusion protein and the polypeptides expressed in V3-transfected Cos 7 cells contain the conformational mAb302 epitope, the fine specificity of the elicited Abs was measured using an mAb302 competition ELISA assay. The diluted antisera were incubated in immunoplates coated with PyC2 for 45 min, and radiolabeled mAb302 was subsequently added to each well and incubated for another 45 min. After extensive washing, bound mAb302 was dissolved from each well, and the radioactivity was measured. NMS and unlabeled ascites fluid from mice carrying mAb302 hybridoma cells were used as negative and positive controls, respectively. As illustrated in Figure 5, while NMS did not compete with \[^{35}S\]mAb302, mAb302 did compete completely with \[^{35}S\]mAb302. This assay was used to assess the relative level of the subpopulation of anti-PyC2 Abs in immune sera, which can compete with \[^{35}S\]mAb302 for binding its epitope.

To rule out the effect of different anti-PyC2 Abs concentrations, the sera from individual mice in both DNA- and protein-immunized groups were normalized against PyC2 according to OD values in ELISA. The average dilutions and their corresponding OD value in ELISA to PyC2 in different groups are shown in Table I. After normalizing the level of anti-PyC2 Abs, the different dilutions of individual sera from each group were assayed for their ability to compete with \[^{35}S\]mAb302 for its epitope. As shown in Table I, there were no significant differences in bound \[^{35}S\]mAb302 from sera from i.m. DNA-, i.d. DNA-, and protein-immunized mice (p > 0.05). These data indicate that anti-PyC2 Abs induced by DNA (both i.m. and i.d.) and protein immunization have the same fine specificity for the mAb302 epitope in the range of concentrations tested.

DNA immunization induces anti-PyC2 Abs with lower avidity than protein immunization

The avidity of anti-PyC2 Abs from both DNA- and protein-immunized mice was measured by an ELISA-based assay using thiocyanate elution (47). This method was based on the susceptibility of Ag-Ab complexes to dissociation by the chaotropic thiocyanate ion. Sera that had been diluted 1:400 were added to immunoplates coated with PyC2, and then increasing concentrations of NH\(_4\)SCN were added to each well to disrupt the bound immune complexes. After extensive washing, the specific Abs bound to PyC2 were measured by ELISA. The absorbance readings in the absence of thiocyanate were assumed to represent effective total binding of specific Abs, and subsequent absorbance readings in the presence of increasing concentrations of thiocyanate were converted to the appropriate percentage of the total bound Abs. The avidity index represents the molar concentration of thiocyanate required to reduce the initial OD value by 50% (47). The avidity index has been reported to be independent of the level of specific Abs in sera (47), and we also determined that the avidity index changed minimally from 2.6 to 2.1 when a tertiary serum from protein-immunized mice was diluted from 1:250 to 1:10,000 (data not shown). These data suggest that the level of anti-PyC2 Abs has little effect on the avidity index over a relatively wide range.

We then compared the avidity indices of anti-PyC2 Abs from DNA- and protein-immunized mice. As shown in Figure 6A, the avidity index of anti-PyC2 tertiary sera from protein-immunized mice was significantly greater than that of anti-PyC2 sera after the fifth immunization with V3 plasmid (both i.m. and i.d.). There was no significant difference in avidity indices of anti-PyC2 Abs between V3 i.m. and V3 i.d. groups. The same results were obtained even after normalizing levels of anti-PyC2 Abs from different groups (Table I). As shown in Table I, the avidity indices of anti-PyC2 Abs from protein-immunized mice were still significantly higher than those from both DNA-immunized groups (p = 0.016 for i.m. group and p = 0.001 for i.d. group), and there was no difference in avidity indices of anti-PyC2 Abs between DNA i.m. and DNA i.d. groups (p > 0.05, Table I). These results indicate that the avidity of anti-PyC2 Abs from protein-immunized mice is significantly higher than that from DNA-immunized mice (both groups).

We then compared the avidity indices of anti-PyC2 Abs after each immunization from DNA- and protein-immunized mice. Surprisingly, while there were remarkable increases in avidity indices of anti-PyC2 Abs from protein-immunized mice during the course of immunization (p < 0.05), avidity indices of anti-PyC2 Abs from both V3 i.m. group and V3 i.d. group did not increase during the course of immunization (Fig. 6B). These data suggest that there

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<thead>
<tr>
<th>Table I</th>
<th>mAb302 specificity and avidity of anti-PyC2 Abs after normalizing Ab titers</th>
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<tbody>
<tr>
<td>Groups</td>
<td>Vm-5*</td>
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<tr>
<td>Serum dilutions</td>
<td>175 ± 93</td>
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<tr>
<td>OD(_{max}) in ELISA</td>
<td>1.34 ± 0.09</td>
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<tr>
<td>Bound [^{35}S]mAb302 in the mAb302 competition assay</td>
<td>8342 ± 550</td>
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<tr>
<td>Avidity index in the avidity assay</td>
<td>1.79 ± 0.36</td>
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* Vm-5, Vd-5, and P-3 refer to prechallenge sera from i.m. V3-, i.d. V3-, and GST-PyC2 protein-immunized mice, respectively. Values are means ± SD.

* p < 0.05.
incubated in immunoplates coated with PyC2. The Ag-Ab complexes (Vd-5), and protein-(P-3) immunized mice were diluted 1:400, then immunized mice. Prechallenge sera from both i.m. V3- (Vm-5), i.d. V3-, and GST-PyC2 protein-immunized mice. The sera collected after each immunization are designated by the number of immunizations with DNA or protein (p = 1.0). SEs are demonstrated.

Discussion

Our previous data showed that the fusion protein GST-PyC2 expressed in recombinant E. coli could immunize BALB/c mice against an otherwise lethal erythrocytic infection with P. yoelii 17XL (38). Humoral immune responses played a predominant role in this protection (39). In this study, we compared humoral responses to PyC2 in BALB/c mice induced by vaccination either with a plasmid that contained the DNA sequence encoding GST-PyC2 or with GST-PyC2 protein. Specifically, we examined the titer, Ig isotype profile, specificity, and avidity of the anti-PyC2 Abs, as well as the protective capacities of these Abs.

We and others have shown that the configuration of the immunizing Ag is critical for the induction of protective humoral immune responses in this model (45, 48). Therefore, it was important to determine initially whether the DNA vaccine could direct synthesis of the encoded polypeptide correctly in mammalian cells. The conformation of the expressed polypeptides in a V3-transfected Cos 7 cell line was examined in immunoprecipitation experiments with mAb302 and anti-GST-PyC2. Our results demonstrated that, although approximately half of the protein secreted from V3-transfected Cos 7 cells had undergone N-glycosylation, the mAb302 epitope is still available on both N-glycosylated and nonglycosylated polypeptides expressed from transfected Cos 7 cells. This lends support to the native configuration of the C-terminal region of PyMSP-1. This observation was also important since there is evidence that plasmadia do not N-glycosylate their proteins (49) and that the GST-PyC2 produced by recombinant E. coli would also not be N-glycosylated.

Our results demonstrated that V3 DNA vaccination successfully induced production of anti-PyC2 Abs in BALB/c mice, while the
negative W plasmid control elicited no specific Abs. However, the induction of anti-PyC2 Abs by DNA immunization took 6 wk longer, and the titer was considerably lower than that elicited by the protein immunization. This result is similar to preliminary data from Gardner et al., who reported lower anti-PyC2 Ab titers after immunization with a DNA vaccine encoding the C-terminus of PyMSP-1 (25). Moreover, we also established that switching to IgG isotypes does occur during the course of V3 DNA immunization.

Two approaches to examination of the specificity of these Abs were taken. First, pooled sera from DNA- (both i.d. and i.m.) and protein-immunized mice were shown to immunoprecipitate native parasite protein at similar levels after normalizing their titers to PyC2. Second, the fine specificity of anti-PyC2 Abs to mAb302 epitope was evaluated by the mAb302 competition assay. We found that both DNA- and protein-immunized sera partially prevented mAb302 from binding its epitope, but protein-immunized sera competed with mAb302 better than DNA-immunized sera. However, after adjustment for relative concentration, the anti-PyC2 Abs induced by DNA and protein immunizations have the same ability to compete with mAb302 for binding to its epitope. These data demonstrated that, although the titer of anti-PyC2 Abs induced by the DNA inoculation was lower, the anti-PyC2 Abs resulting from DNA immunization showed the same ability to recognize native parasite protein and the mAb302 epitope.

Another important parameter for measuring humoral immune responses is Ab affinity. Because of the difficulties involved in measurement of true Ab affinity, Ab avidity was determined using an ELISA-based thiocyanate elution assay (47). The avidity index, representing the molar concentration of thiocyanate required to reduce the initial OD by 50%, was quantified. Importantly, the avidity index obtained from this assay was reported to be relatively independent of the level of specific Abs (47). Our data also showed that the avidity index of anti-PyC2 Abs in prechallenge serum from protein-immunized mice varied by just 0.5 over a 40-fold concentration (data not shown). As assessed by this assay, the avidity index of anti-PyC2 Abs from DNA-immunized mice (both groups) is considerably lower than that from protein-immunized mice. This is also true even after normalizing the titers of anti-PyC2 Abs from DNA- and protein-immunized mice (Table I). Surprisingly, while a clear increase in the avidity index of anti-PyC2 Abs was observed during the course of GST-PyC2 protein immunization, there was no significant increase in the avidity indices of either the anti-PyC2 population or the anti-GST Abs during the course of DNA immunization. Since avidity of Abs is primarily determined by their dissociation rates, this observation suggests that there is little or no avidity maturation of Abs induced by DNA immunization. To our knowledge, this is the first report determining the avidities of Abs induced by DNA vaccines and demonstrating a lack of maturation of avidity of Abs during DNA vaccinations in mice.

Avidity maturation of Abs elicited by immunization with haptens and protein Ags has been well documented in animal models. In addition, infection of humans with pathogens such as rubella (50), cytomegalovirus (51), and Toxoplasma gondii (52) also elicits Abs whose avidities increase with time. For example, the avidity of anti-Toxoplasma IgG is considered as a criterion in the first half of pregnancy to discriminate between primary infection acquired in early pregnancy (low avidity) and infection that occurred before pregnancy (high avidity) (52). However, a few exceptions have been noted. Neutralizing Abs against vesicular stomatitis virus (VSV) seem not to undergo avidity maturation during acute infection in mice (53). In addition, severe measles occurs in immunized and nonimmunized HIV-infected individuals, and the immunized and nonimmunized HIV-infected individuals, and the im- paired immune responses in these patients seem to be associated with defective avidity maturation of anti-measles Abs (54).

Avidity maturation is the consequence of somatic hypermutation of the Ig genes followed by selection of B cells with high affinity surface Ig in germinal centers (GCs), and depends upon the interaction of activated B cells with follicular dendritic cells (55). We do not know whether the apparent lack of affinity maturation of specific Abs during DNA immunization is the result of deficiencies in somatic hypermutation of Ig genes or in the selection of higher affinity B cells in germinal centers. It is also unclear whether the lack of avidity maturation during DNA immunization occurs only with particular Ags or in certain strains of mice.

We have considered several possibilities that might explain our observations. First, the glycosylation of the Ag in vivo may alter its localization in germinal centers. Using the same expression vector, DNA-based immunizations for HA in an influenza model were more effective than DNA immunizations for Env in a human immunodeficiency model (56). Further study showed that the Env protein had been more heavily glycosylated than the HA (56). In our case, about half of the secreted protein had been glycosylated in V3-transfected monkey cells. Although the N-glycosylation of the Ag did not change the mAb302 epitope, the difference in physical structure between glycosylated and non-glycosylated Ag expressed in vivo may result in differences in the efficiency with which the Ag-specific B cells enter into GCs for hypermutation and affinity maturation of specific Abs.

Localization of Ag within GCs not only is important for affinity maturation but also may affect specific B cell memory. The role of follicular dendritic cells in driving the maturation of the humoral immune responses depends chiefly on their ability to retain Ags on their surface for long periods of time. Only B cells with higher affinity surface Ig survive and differentiate into either plasma cells or memory B cells. Therefore, deficiency in affinity maturation not only results in generation of Abs with lower affinity but also may affect specific B cell memory.

Another possible explanation for the lack of avidity maturation is the schedule chosen for DNA vaccination. Injection with DNA vaccines leads to lower but longer in vivo expression of the encoded Ags, and, since DNA vaccination takes longer to induce Ab immune responses, it may take longer for avidity increases to become apparent. In support of this possibility, Lyon and his colleagues recently reported that increasing the interval between DNA immunization of mice increased the levels of Abs produced (57). To address the issue of longer intervals between DNA injections, we have done an experiment with the V3 DNA vaccine, immunizing three times at weeks 0, 12, and 32. Despite this very extended schedule, the avidities of the Abs produced were similar to those reported here with more frequent immunization (data not shown).

Additionally, it is of interest that the production of these low avidity Abs after DNA vaccination is similar to the phenomenon observed in mice for a protein Ag injected without adjuvant (58). While the protein Ag administered in CFA induced specific Abs with higher affinity, the same Ag in PBS elicited lower affinity Abs (58). In our case, the V3 DNA vaccine was given in PBS, and thus it is possible that the absence of appropriate adjuvant may contribute to the lack of maturation of Abs during DNA immunization. We are therefore currently investigating the effect of adjuvants on DNA vaccination in this model.

Finally, when DNA- and protein-immunized mice were tested for protection against lethal challenge of P. yoelii infection, the results showed that all the protein-immunized mice resolved the parasitemias, whereas the DNA-immunized mice died of infection.
HUMORAL RESPONSES TO PyMSP-1 DNA AND PROTEIN VACCINES

The data from our laboratory as well as others indicates that iso-type is not a critical parameter for protection in this P. yoelii model (46, 59). Thus, we believe that the significantly lower titer and lower avidity of anti-PyC2 Abs induced by DNA immunizations are responsible for the lack of protection against lethal challenge in DNA-immunized mice. We have also seen a similar correlation of Ab avidity and titer with protection in mice immunized with GST-PyC2 protein in CFA (P. A. Calvo and C. A. Long, unpublished observations). C57BL/10 mice produced higher titer Abs with an avidity index comparable to the protein-immunized BALB/c mice reported here (2.35) and were protected. In contrast, B10.BR mice produced Abs of both lower titer and avidity (1.35) comparable to those reported here for DNA immunization and were not protected.

In summary, V3 DNA vaccine encoding GST-PyC2 successfully elicited anti-PyC2 Abs in BALB/c mice, which recognized native MSP-1 protein and had similar specificity for the epitope recognized by mAb302 as Abs from protein-immunized mice. However, such Abs had significantly lower titer and lower avidity, different isotype profiles, and different protective capacity against lethal challenge. More importantly, our results suggest that there may be little or no affinity maturation of specific Abs during the course of V3 DNA immunization. Further understanding of the process by which DNA vaccines elicit humoral immune responses in vivo may allow us to obtain protective immune responses through this strategy.

Note added in proof. Lack of avidity maturation of anti-PyC2 antibodies was also observed in other strains of mice, including C57BL/10 and Swiss Webster mice, which were immunized with V3 DNA vaccine.

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References


