Meningococcal disease is one of the most dreadful conditions because of its propensity to affect children and adolescents, its often fulminant course, and its tendency to cause epidemics (1, 2). The incidence varies from 1 to 50 cases per 100,000 but can reach much higher numbers during epidemics. Mortality ranges from 1 to 10% in meningitis and from 20 to 40% in sepsis, with up to 25% of the survivors developing permanent neurological sequelae (2). The causative agent, \textit{Neisseria meningitidis}, is an encapsulated bacterium classified into different serogroups based on the chemical composition and immunologic features of the capsular polysaccharide (CP) (3). Human isolates are almost totally accounted for by five serogroups (A, B, C, Y, and W135), with A, B, and C accounting for >90% of all infections. Group A strains cause large epidemics in developing countries, whereas group B, C, or Y strains are prevalent in Europe and the United States (4). The main virulence factor of these organisms is the CP, which protects against complement-mediated bacteriolysis and phagocytosis. Polysaccharide vaccines against serogroups A, C, Y, and W135 have been available for decades but are not effective in the age groups that are most susceptible to the disease (i.e., infants and young children). Recently developed polysaccharide–protein conjugate vaccines, however, are likely to overcome this limitation (5, 6). No vaccine is currently available for the prevention of infections caused by serotype B strains, which often account for more than half of meningococcal disease cases in developed countries (4). Major obstacles to the development of capsule-based vaccines are the poor immunogenicity of the group B polysaccharide (even after protein conjugation) and concerns over the induction of autoantibodies (7). These features are probably related to the...
structural identity between serogroup B *N. meningitidis* (MenB) CP and human polysialic acid (PSA), both consisting of α(2→8)N-acetyl neuraminic acid. To circumvent this problem, the immunogenicity of various derivatives of the MenB CP was tested (8). A protein-conjugated polysaccharide in which the N-acetyl groups of the sialic acid residues were replaced with N-propionyl groups induced bactericidal IgGs, which protected mice against experimental infection (8, 9). Further studies using mAbs defined two different classes of capsular epitopes naturally present on the meningococcal surface (10–12). One class is cross-reactive with human PSA, whereas the other is non–cross-reactive and protective. Therefore, a reasonable approach may involve immunization with mimics of the protective epitope. Using the bactericidal non–cross-reactive mAb Seam 3 as a template (11), we developed antiidiotypic antibody single chain variable fragments (scFvs), which could induce, after immunization, human non–cross-reactive anticapsular IgGs (13). These antibodies, however, could not consistently produce serum bactericidal activity, likely because of their insufficient avidity and/or concentration. Although bactericidal activity is only one aspect of host defense and may underestimate the efficacy of vaccines directed against MenB (14, 15), it remains the hallmark of protection in humans and is desirable in vaccine development. In this paper, we sought to increase the immunogenicity of our scFv constructs by exploiting their adaptability for DNA vaccination. This is a logical extension of the use of recombinant mimics and offers many practical advantages, including ease of manipulation and production of the immunogen (16–18). After exploring different strategies, we were able to induce satisfactory bactericidal activity and protection against experimental MenB infection by immunization with scFv gene–containing plasmids devoid of a secretory signal sequence. These data may be useful in the development of effective immunogens for the prevention of diseases caused by encapsulated bacteria.

**RESULTS**

**In vitro scFv expression**

In initial experiments, COS-7 cells were transiently transfected with a plasmid containing the G1 scFv gene fused to an adenoviral secretory signal peptide sequence (pS.scFvG1; Table I). Protein expression was analyzed by the ability of permeabilized cells to bind the Seam 3 mAb (e.g., the antigen against which the G1 scFv was raised) using immunofluorescence flow cytometry. After treatment with Seam 3 followed by FITC-conjugated anti–mouse IgG, pS.scFvG1-transfected cells showed increased fluorescence relative to cells transfected with the empty plasmid (Fig. 1). Increased fluorescence was not detected when cells were treated with an irrelevant, isotype-matched mAb in place of Seam 3 (not depicted). These data indicated that pS.scFvG1 transfection resulted in the expression of the G1 scFv in a functional form, as defined by its ability to bind to the Seam 3 idiotope.

### Table I. Plasmids used

<table>
<thead>
<tr>
<th>Description</th>
<th>Leader sequencea</th>
<th>T helper sequenceb</th>
<th>Antigenic sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCI-neo</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pS.scFvG1</td>
<td>MRYMLGILALAAVCSAEF</td>
<td>–</td>
<td>G1 scFv</td>
</tr>
<tr>
<td>pT.scFvG1</td>
<td>–</td>
<td>MKLOYKANSSKFITELEF</td>
<td>G1 scFv</td>
</tr>
<tr>
<td>pST.scFvG1</td>
<td>MRYMLGILALAAVCSAA</td>
<td>QYIKANSSKFITELEF</td>
<td>G1 scFv</td>
</tr>
<tr>
<td>pscFvG1</td>
<td>–</td>
<td>–</td>
<td>G1 scFv</td>
</tr>
</tbody>
</table>

a Underlined is the leader sequence from a 19-kD glycosylated membrane protein of human adenovirus C, serotype 2 (available from the National Center for Biotechnology Information [NCBI] under accession no. CAC67720, aa 1–18), which was previously used in the design of vaccination plasmids (reference 30).

b Underlined is the T helper sequence from tetanus toxin (available from NCBI under accession no. AAK72964, aa 831–845), which was previously used in the design of vaccination plasmids (reference 19).

c Available from NCBI under accession no. AAQ83756, aa 1–248.

**Figure 1. Flow cytometry analysis of in vitro expression of the scFv.** Binding of Seam 3 mAb to permeabilized COS-7 cells transfected with an expression plasmid containing the scFv G1 gene (bottom, pS.scFvG1) or the empty vector pCI-neo (top). After being transiently transfected, cells were permeabilized and exposed to the Seam 3 mAb. FITC-conjugated anti–mouse IgG was used to detect Seam 3 binding. Horizontal lines indicate the gates that were defined to exclude background binding.
Vaccination with the scFv gene induces serum bactericidal activity

Next, mice were injected i.m. with different doses (25, 75, and 150 μg) of an expression plasmid containing the scFv G1 gene fused to a signal peptide sequence (pS.scFvG1) or with 150 μg of the G1 protein (G1-scFv-KLH). Sera from mice administered with the empty vector (pCI-neo) were used as controls. Arrows indicate immunization times. Data represent geometric means + SD of eight determinations, with each conducted in duplicate on a different animal. *, P < 0.05 compared with empty vector sera as determined by ANOVA and Student-Newman-Keuls test.

Figure 2. Bactericidal activity in sera from scFv-immunized mice. (A) Serum bactericidal activity in mice immunized with different doses (25, 75, and 150 μg) of an expression plasmid containing the scFv G1 gene fused to a signal peptide sequence (pS.scFvG1) or with 150 μg of the G1 protein (G1-scFv-KLH). Sera from mice administered with the empty vector (pCI-neo) were used as controls. Arrows indicate immunization times. Data represent geometric means + SD of eight determinations, with each conducted in duplicate on a different animal. *, P < 0.05 compared with empty vector sera as determined by ANOVA and Student-Newman-Keuls test. (B) Bactericidal titers in sera from mice immunized with various types of G1 scFv gene-containing plasmids (for a description see Table I). 10 animals per group were immunized on days 0, 21, and 42 with 150 μg DNA in PBS i.m. or, for comparison, with N-Pr MenB–TT (4 μg sialic acid) s.c. in CFA (day 0) and IFA (days 21 and 42). Sera were collected at day 56. Each marker indicates data from an individual mouse with mean geometric titers represented by a horizontal bar. P < 0.05, as determined by ANOVA and Student-Newman-Keuls test.

Speciﬁcity of the antibodies induced by DNA vaccination

Next, we veriﬁed that the antibodies induced by scFv gene immunization were directed against their intended target, that is the MenB CP. We used this conjugate because it contains the epitope mimicked by the G1 scFv and can elicit high levels of anti–MenB CP antibodies when used in Freund’s adjuvant (8, 13). Vaccination with plasmids devoid of a signal peptide sequence resulted in bactericidal titers that were markedly higher than those induced by the corresponding plasmids bearing such a sequence and that were similar to those of animals vaccinated with the N-Pr MenB–TT in Freund’s adjuvant (Fig. 2 B). The presence of a T cell epitope sequence in the immunizing plasmid was associated with a slight increase in antibody titers, although this effect did not reach statistical signiﬁcance. These data indicated that immunization with the G1 scFv gene devoid of a secretory signal sequence was effective in inducing high-level serum bactericidal activity.

Figure 3. Speciﬁcity of antibodies induced by scFv gene immunization. (A) Sera from pT.scFvG1-immunized animals were incubated with puriﬁed N-Pr MenB CP or the G1 scFv protein but not with a control, irrelevant scFv (designated H6; Fig. 3 A). Moreover, bactericidal activity was observed only with encapsulated MenB strains, but not with serogroups MenA or MenC (Fig. 3 B). These data indicated that vaccination with the G1 scFv gene induced anti-scFv antibodies that speciﬁcally cross reacted against the MenB capsule, thereby producing bacterial killing. Although we have previously shown that the
antibodies induced by our antiidiotypic protein did not cross react with human PSA (13), it was of interest to verify that this also occurred after DNA immunization, especially in consideration that the latter can broaden the repertoire of recognized epitopes (20). Therefore, sera from pT.scFvG1-immunized animals were tested against neuraminidase-treated or untreated cells from the CHP 212 human cell line expressing high levels of PSA. The Seam 26 mAb, which is known to react with both human and MenB PSA, was used as a positive control. As expected, this mAb showed strong reactivity against untreated, but not neuraminidase-treated, cells (Fig. 3 C). Immune sera were totally devoid of reactivity against either untreated or neuraminidase-treated CHP 212 cells, indicating that human PSA cross-reactive antibodies were not induced by scFv gene immunization.

Because the antibodies cross reacting with the MenB surface should be a fraction of anti–G1 scFv antibodies, in further experiments we determined whether there was a correlation between serum bactericidal activity and total anti-scFv titers. To this end, serum samples from animals immunized with either the scFv-KLH protein (from the experiments reported in Fig. 2 A) or the pT.scFvG1 plasmid (from the experiments reported in Fig. 2 B) were tested for antibody binding to plates sensitized with the G1 scFv. These groups of sera were chosen because they differed widely in bactericidal activity. Surprisingly, however, they did not differ in reactivity against the scFv (Fig. 4, A and B), suggesting that DNA immunization selectively increased the fraction of anti–G1 scFv antibodies that cross reacted with MenB and caused bactericidal activity.

To determine the isotype distribution of anti-MenB antibodies, we used an ELISA assay in which plates were sensitized with whole meningococcal cells. Preimmune sera showed some background reactivity that was mostly accounted for by IgM (Fig. 4 C). Antibody binding was significantly higher in immune sera, with a predominance of IgG2a (Fig. 4 D). These data suggested that immunization with the G1-containing plasmid induced a Th1-type response and that IgG2a, which can mediate complement-dependent bacterial killing (11), likely accounted for the observed serum bactericidal responses.

Protective effects of passively administered antibodies
To completely assess the functional properties of the antibody response induced by scFv gene immunization, we ascertained the ability of immune sera to passively protect infant rats against meningococcal bacteremia. In these experiments we measured the number of blood CFU in pups inoculated with pools of sera obtained before or after pT.scFvG1 immunization and challenged i.p. with MenB strain 2996. As positive controls, groups of pups were treated with a pool of sera from N-Pr MenB–TT–immunized animals or with the Seam 3 mAb. Pretreatment with the pT.scFvG1 immune serum pool (diluted up to 1:8) (P < 0.05) significantly protected pups from bacteremia (Table II). These effects were similar to those observed with the serum pool from N-Pr MenB–TT–immunized animals. In contrast, animals inoculated with a preimmune serum pool or with a pool obtained after immunization with the empty plasmid were not protected (Table II). These data indicated that immunization
with pT.scFvG1 induced serum antibodies capable of affording passive protection against systemic spreading of MenB.

**DISCUSSION**

Capsule–based vaccines are not available for the prevention of MenB infection because the MenB CP is cross-reactive with human tissue and is not immunogenic, even after protein conjugation. This is a serious problem, because MenB strains can account for up to 80% of devastating meningococcal disease in developed countries (2, 11). Because distinct human cross-reactive and non–cross-reactive epitopes exist on the MenB surface (11, 12), it was possible to induce anti-capsular antibodies and at the same time avoid the risk of autoimmune by mimicking a single non–cross-reactive epitope of the MenB CP (13). However, the antibodies induced by our surrogate antigen were of insufficient avidity and/or concentration to consistently induce bacterial killing, which is an accepted marker of vaccine-induced protection in humans. This is not surprising, because the induction of weak antibody responses has been a problem with many antigenic mimics, including peptide mimotopes or antiidiotypic antibodies (21).

After exploring different strategies in this paper, it was possible to consistently induce bactericidal and protective activity in the sera of animals immunized with the gene encoding for our scFv mimic. Serum bactericidal activity was totally accounted for by scFv–specific antibodies that cross reacted with the MenB capsule, as shown by complete abrogation of killing by competing G1 scFv or MenB CP. Moreover, immune sera could not kill meningococci with MenA or MenC capsules. The antibodies induced by scFv gene immunization were predominantly of the IgG2a isotype, which is typical of a Th1 response. Finally, the induced antibodies were non–cross-reactive with human PSA.

The importance, in terms of protection in humans, of the bactericidal responses observed in our experiments after scFv gene vaccination remains, of course, to be established. It should be noted, in this respect, that bactericidal activity was tested using rabbit complement, which produces considerably higher bactericidal titers than human complement (22). Nevertheless, it is encouraging that the antibodies induced by scFv gene immunization were protective in a well characterized in vivo model, such as the infant rat model. This paper illustrates the potential of DNA vaccination–based strategies to augment antibody responses against protein mimetics. The versatility and ease of manipulation of...
gene vaccination allowed us to quickly and efficiently screen several approaches, including the addition of T helper epitopes (23, 24). Unexpectedly, the most successful strategy that resulted in markedly augmented bactericidal titers involved deletion of the secretory signal peptide sequence initially placed before the scFv (Fig. 2 B). In DNA vaccination, homologous or heterologous signal sequences are generally used to direct the antigen into the endoplasmic reticulum of the transfected cell, thus leading to secretion, with the rationale of augmenting availability of the immunogen for uptake by antigen-presenting cells. However, there are few data on the relationships between the cellular localization of the antigen and the type and extent of the immune response. In a comparative study, two plasmids either containing or lacking a signal sequence produced similar antibody levels despite differential intracellular targeting of the encoded antigen (25). In another study, cytoplasmic ovalbumin induced lower IgG1 but higher IgG2a than secreted ovalbumin (26). Interestingly, mouse immunization with a plasmid encoding a modified version of carcinoembryonic antigen (CEA), devoid of its signal peptide and fused to a T helper epitope, resulted in higher anti-CEA antibody levels relative to those observed using the wild-type CEA plasmid (27). Further studies will be necessary to clarify whether the mechanisms underlying the effects reported in these papers, as well as in the present one, involve differential antigen uptake and/or processing by antigen-presenting cells. Irrespective of the mechanisms involved, our data strongly indicate that manipulation of secretory signals deserves further exploration in the challenging task of optimizing antinfectious DNA vaccines. In our hands, removal of secretory signals recently proved challenging task of optimizing antiinfectious DNA vaccines. DNA vaccination was recently used to redirect the immune system from a Th2 to a more effective Th1 response (30). Moreover, a similar approach was successful in inducing serum bactericidal activity against MenC CP (31) and antibodies directed against the type 4 pneumococcal CP (32).

Collectively, these data indicate that DNA immunization offers new ways of stimulating the immune system and suggest that these features can be exploited in the prevention of diseases caused by encapsulated bacteria. This is especially relevant for infections, such as those caused by MenB, for which no vaccine is available because of the failure of conventional approaches. Moreover, despite considerable success, existing conjugate vaccines are not without problems, including complexities in polysaccharide production and conjugation. These difficulties can become particularly challenging with vaccines consisting of multiple conjugates. In contrast, it seems relatively easy to clone different mimics in a single vector for vaccinating against pathogens with multiple serotypes. Thus, the global use of vaccines directed against encapsulated bacteria would be facilitated by the development of effective DNA vaccines, especially in consideration of some additional advantages such as their low cost and independence from the cold chain.

MATERIALS AND METHODS

Bacterial strains and reagents. Meningococcal strains 2996, 8047, and MC58 (MenB), F8238 and A1 (MenA), and C11 (MenC) were provided by M.M. Giulani (Chiron Corp., Siena, Italy). Purified N-Pr MenB CP and the Seam 3 and Seam 26 mAbs were provided, respectively, by A. Bartoloni and M. Mariani (Chiron Corp., Siena, Italy). The G1 and the irrelevant H6 scFvs were expressed recombinantly in Escherichia coli and purified as previously described (13, 18, 33). For immunization experiments, the G1 scFv–KLH and the N-Pr MenB–TT conjugates were prepared as previously described (13).

DNA constructs and expression analysis. To generate plasmids for DNA vaccination (Table I), we used pCI-neo, a mammalian expression vector (Promega). The G1 scFv–encoding sequence was PCR-amplified as previously described (13–18) and ligated into the multiple cloning site of pCI-neo, generating pScFvG1. In the design of pScFvG1, the leader sequence was inserted at the beginning (i.e., at the S' NheI/ EcoRI flanking site) of the G1 scFv gene. To produce the pT.xScFvG1, a tetanus toxin universal T helper cell epitope (19) was inserted at the beginning of the scFv. Finally, a plasmid (pST.scFvG1) was generated containing both the secretory sequence and the T helper cell epitope before the scFv gene.
Plasmid preparation. Plasmids for in vitro transfection or mouse immunization were grown in E. coli DH5α and purified using EndoFree Plasmid Maxi or Giga kit (QIAGEN). Each lot of plasmid DNA had a A260/A280 ratio ≥1.8 (as determined by UV spectrophotometry), endotoxin content ≤0.1 EU/μg DNA (as determined by Limulus Amoebocyte Lysate assay kit, Associates of Cape Cod Inc.), and a predominantly supercoiled form.

FACS analysis. The ability of engineered DNA constructs to express functional G1 scFv was analyzed by flow cytometry. A subconfluent monolayer of COS-7 cells (CCL-70, a monkey kidney fibroblast cell line; American Type Culture Collection) was transiently transfected with 2.5 μg of vector DNA per 10^5 cells in a synthetic cationic lipid solution (TransFast; Promega). The pCI-neo mammalian vector (empty vector) was used as a control. After 48 h, the transfected cells were washed in PBS (0.01 M phosphate, 0.15 M NaCl, pH 7.2), trypsin treated, and fixed overnight with 1 ml paraformaldehyde (2.5 mg/ml). The cells were then permeabilized with 1 ml PBS containing 0.2% (vol/vol) Tween 20 (PBS-Tween) and incubated with the anti-G1 scFv (4 μg/ml in PBS) for 2 h at 37°C. 10 μg/ml FITC-labeled rabbit anti-mouse IgG (Abcam Ltd.) was used to detect bound ScFv 3.

Immunizations. For DNA immunization, 6–8-wk-old BALB/c mice (Charles River Laboratories) were injected in the quadriceps muscle with purified DNA at the doses indicated in the figures in equal volumes with serially diluted (ranging from 1:3 to 1:768) heat-inactivated serum and undiluted baby rabbit complement (Cederlane). The reciprocal of the highest final serum dilution causing ≥50% killing of the inoculum was recorded as the bactericidal titer. Because the lowest final serum dilution tested was 1:9, the lower limit of detection was an arbitrary titer of 4.5 (i.e., half the lower detection limit). Differences in the frequency of protected animals were analyzed using Fisher’s exact test.

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