Expression of a Functional Single-Chain Variable-Fragment Antibody against Complement Receptor 1 in *Streptococcus gordonii*

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Received 17 December 2007/Returned for modification 21 January 2008/Accepted 24 March 2008

*Streptococcus gordonii*, an oral commensal organism, is a candidate vector for oral-vaccine development. Previous studies have shown that recombinant *S. gordonii* expressing heterologous antigens was weakly immunogenic when delivered intranasally. In this study, antigen was specifically targeted to antigen-presenting cells (APC) in order to potentiate antigen-APC interactions and increase the humoral immune response to the antigen. To achieve this goal, a single-chain variable-fragment (scFv) antibody against complement receptor 1 (CR1) was constructed. Anti-CR1 scFv purified from *Escherichia coli* was able to bind to mixed lymphocytes and bone marrow-derived dendritic cells. The in vivo function of the anti-CR1 scFv protein was assessed by immunizing mice intranasally with soluble scFv and determining the immune response against the hemagglutinin (HA) peptide located on the carboxy terminus of the scFv. The serum anti-HA immunoglobulin G (IgG) immune response was dose dependent; as little as 100 ng of anti-CR1 scFv induced a significant IgG immune response, while such a response was minimal when the animals were given an unrelated scFv. The anti-CR1 scFv was expressed in *S. gordonii* as a secreted protein, which was functional, as it bound to dendritic cells. Mice orally colonized by the anti-CR1-secreting *S. gordonii* produced an anti-HA IgG immune response, indicating that such an approach can be used to increase the immune response to antigens produced by this bacterium.

*S. gordonii* is a commensal bacterium found in the oral cavities of humans. The organism is considered to be an attractive vector as a live-oral-vaccine vehicle. A number of heterologous antigens have been expressed in this organism as either secreted proteins or cell wall-anchored proteins. In the murine oral-colonization model, the recombinant *S. gordonii* was able to establish long-term colonization. However, there are difficulties in stimulating a strong protective immune response against recombinant antigens following oral colonization.

Antigen targeting to immune cells has the potential to manipulate the immune system and elicit an immune response more efficiently. Monoclonal immunoglobulin G (IgG) antibodies have long been used as specific targeting vehicles. A number of reports have indicated success in achieving enhanced immune responses using antibodies to complement receptor 1 (CR1) and CR2 (3, 8, 30), Fc receptors (1, 2), and enhanced immune responses using antibodies to complement receptor 1 (CR1) and CR2 (3, 8, 30), Fc receptors (1, 2), and dendritic cell DEC205 receptor (5, 6). However, there are limitations in using intact IgG as a targeting vehicle; these limitations include a weak extravascular transport ability for IgG and difficulties with expressing whole IgG by bacteria. Single-chain variable-fragment (scFv) antibodies, however, offer a number of advantages, e.g., they can be readily produced by bacteria and can be easily engineered genetically as fusion proteins carrying polypeptide antigens. In the context of antigen targeting, scFvs against CR1 and -2 (21, 24), DEC205 (9), CD3 (31), and natural killer NKG2D receptor (29) have been reported with some degree of success.

In this study, we have taken the approach of expressing an scFv antibody against CR1 in *S. gordonii* to target immune cells. CR1 is a phagocytic receptor expressed by a number of immune cells, including dendritic cells, macrophages, neutrophils, eosinophils, and B cells, as well as erythrocytes. The anti-CR1 scFv was tested for binding to target cells in vitro and used in intranasal immunization in mice.

### MATERIALS AND METHODS

**Bacteria and growth conditions.** *S. gordonii* was cultivated in Todd-Hewitt broth containing 0.5% yeast extract at 37°C aerobically without shaking. Kanamycin and tetracycline, when needed, were included in the medium at 250 μg/ml and 10 μg/ml, respectively. Recombinant *Escherichia coli* was grown aerobically with vigorous shaking at 37°C in Luria Bertani broth (1% tryptone, 0.5% yeast extract, and 1% NaCl [wt/vol]) or Super Broth (1% MOPS [morpholinepropanesulfonic acid], 3% tryptone, 2% yeast extract [wt/vol]) containing either 100 μg/ml of ampicillin or 50 μg/ml of kanamycin. All antibiotics were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada).

**Construction of the anti-CR1 scFv.** The anti-CR1 antibody gene was obtained from the anti-CR1 monoclonal antibody-producing hybridoma HB5S92 (American Type Culture Collection, Manassas, VA). The cells were grown in modified Dulbecco’s medium supplemented with 5 mM β-mercaptoethanol and 10% fetal calf serum (Sigma-Aldrich). Total RNA was isolated from 1 × 10⁶ hybridoma cells by extraction with the Trizol reagent (Invitrogen Life Technologies, Burlington, ON, Canada). The RNA obtained was dissolved in 40 μl of diethylpyrocarbonate-treated water. cDNA was synthesized from the RNA by reverse transcription using oligo(dT) as the primer and murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The variable light-chain (V_PASS) and heavy-chain (V_PASS) antibody fragments were amplified by PCR using mixed primers as described by Barbabs et al. (4). The resulting 0.4-kb V_PASS or V_PASS DNA fragments were gel purified and used in overlapping PCR to produce the scFv antibody DNA. The resulting 0.8-kb scFv fragment was

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\* Published ahead of print on 2 April 2008.
ligated into the SfiI sites of the phagemid pComb3X (4). The ligated DNA was transformed into *E. coli* XL1-Blue. The resulting construct (pCR1) was verified by restriction analysis and DNA sequencing (The John P. Robarts Research Institute DNA Sequencing Facility, London, ON, Canada).

**Cloning of the anti-CR1 scFv gene in *S. gordonii***.

To express the anti-CR1 scFv in *S. gordonii*, pSecCR1 was constructed. The construction of pSecCR1 is outlined in Fig. 1. Briefly, the DNA coding for the anti-CR1 scFv and the C-terminal histidine and hemagglutinin (HA) tags was obtained by PCR using the primer pair SL334 (CGGAATTCCGTTAAGAAGCGTAGTCCGGAACGTC; the EcoRI site is underlined) and SL335 (GAGGCCCAGGCGGCCGAGCTC). A 1-kb DNA fragment carrying the TetR repressor gene, the tetracycline-inducible *xyl/tetO* promoter, and the ribosomal binding site and signal sequence of *spaP* originating from *Streptococcus mutans*, was amplified from pPM14 (18) using primers P3 Universal (ACGCCAAGCTTGCATGCCTGC; the SphI site is underlined) and SL333 (GAGCTCGGCCGCCTGGGCCTCATCGGCAAAAACCTTTTG). The two fragments were joined together via overlapping PCR, and the resulting 1.8-kb PCR product was cloned into the SphI and EcoRI sites on the *E. coli*-Streptococcus shuttle vector pDL276, creating pSecCR1. pSecCR1 was transformed into *S. gordonii* (*hppG::Tetr*) via natural transformation (13).

**Isolation of scFvs from *E. coli***.

pCR1 was transformed into *E. coli* TB1 (New England Biolabs, Mississauga, ON, Canada) for the production of scFv. As a control, pK8 (pComb3X carrying scFv recognizing pertussis toxin [S. F. Lee, K. G. Chan, and S. A. Halperin, unpublished data]) was also introduced into *E. coli* TB1. The recombinant *E. coli* was grown in 500 ml of Super Broth for 16 h. Cells were harvested by centrifugation (10,000 × g) and resuspended in 5 ml NiCAM wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole) with 1 mM phenylmethylsulfonyl fluoride and sonicated (15 10-s bursts at amplitude 35 separated by 10-s cooling periods; Vibra cell; Sonics and Materials Inc., Danbury, CT). The sonicate was centrifuged at 20,000 × g for 20 min at 4°C. The clear supernatant was passed through a 1.5-ml NiCAM column (Sigma-Aldrich) essentially as described above. Briefly, the culture supernatant was passed through the NiCAM column twice, unwanted proteins were removed by a 20-ml wash, and scFv was eluted with four 500-μl elution buffers. The eluates were used in Western blotting and enzyme-linked immunosorbent assay (ELISA) as described below.

**FIG. 1. Construction of pSecCR1.** See Materials and Methods for details. SS, signal sequence; SD, ribosomal-binding site; HIS, hexahistidine tag.
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels using the buffer system of Laemmli (12). Proteins were stained with Coomassie blue. The scFv protein concentration was determined by comparison with bovine serum albumin (BSA) standards on the same SDS-PAGE gel using Image J software (National Institutes of Health, Bethesda, MD). For Western immunoblotting, proteins were transferred to nitrocellulose membranes (28) and antigens were detected using an anti-HA monoclonal anti-tibody (1/20,000; Sigma-Aldrich) and goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1/20,000; Sigma-Aldrich).

Isolation of mixed lymphocytes. Mixed lymphocytes were isolated from blood obtained from BALB/c mice using Ficoll Paque Plus (Amer sham Biosciences, Baie d’Urfe, PQ, Canada) according to the manufacturer’s instructions. The mixed lymphocytes were used to coat 96-well polystyrene microtiter plates.

Bone marrow-derived dendritic cells. Dendritic cells were isolated and cul tured from the femurs and tibias of BALB/c mice as described previously (7). The cells were harvested on day 6 and used in ELISA and immunofluorescence assays as described below.

Immunization. The anti-CR1 and -K8 scFvs isolated as described above were dialyzed against phosphate-buffered saline (PBS) and used to immunize BALB/c mice (female; 5 weeks old; n = 4; Charles River Laboratory, St. Constant, Quebec, Canada) using lipopolysaccharide (LPS) (10 μg) from E. coli 6127:B8 (Sigma-Aldrich) as an adjuvant. The mice were immunized intranasally with 0.1 g tetracycline in 100 μl PBS on days 1, 8, 16, and 22 (18). The animals were euthanized on day 29, at which time microbiological swabs were obtained from the nasal cavity, oral cavity, and pharynx (14, 16). The swabs were inoculated onto brain heart infusion agar plates and incubated at 37°C. The colonies obtained were observed to have colony morphology similar to that of S. gordonii and were gram-positive cocci in short chains. Western immunoblot analysis of culture supernatants of selected colonies confirmed the presence of the 33-kDa scFv protein. Saliva was collected 2 days prior to colonization and on day 28. Sera were obtained 1 day prior to colonization and at euthanasia.

ELISA. ELISAs were used to test the function of scFv in binding to mixed lymphocytes and dendritic cells and detection of HA-specific antibodies in saliva and sera. For the detection of scFv binding to splenic microtiter plates (Corning Inc., Corning, NY) were coated with mixed lymphocytes and dendritic cells (8,000 cells/well), and the cells were lightly fixed with 0.125% glutaraldehyde at 4°C overnight. After overnight incubation, the coated plates were used immediately or stored at −80°C until they were used. The plates were blocked with 1% wt/vol BSA in PBS containing 0.1% wt/vol Tween 20 (PBST) for 1 h at room temperature, and scFv (200 ng/well) was added. The plates were incubated at 4°C for 1 h and washed with PBST, and the bound scFv was detected with commercial anti-HA monoclonal antibody and goat anti-mouse alkaline phosphatase conjugates.

For the detection of HA-specific antibodies in sera and saliva, microtiter plates were coated with 100 ng/well recombinant cyclophilin 18 (rC18) in ELISA coating buffer at 4°C overnight. The C18 gene originating from T. gondii using methods described previously (11). The amplified 514-bp PCR fragment encoding the mature VKand VH domains. In addition, the glycine-serine linker and the histidine and HA tags introduced in the cloning were viewed with a Leica DM2500 microscope (495-nm excitation; 525-nm emission), and images were captured with a digital camera. The images were converted to black and white and pixel reversion to allow easier viewing using the PhotoShop program.

Nucleotide sequence accession number. The GenBank accession number for the anti-CR1 scFv nucleotide sequence is EF694984.
The results indicated that the anti-CR1 scFv was functional. Binding was also observed using immunofluorescence microscopy (Fig. 5). The anti-CR1 scFv clearly bound to bone marrow-derived dendritic cells (Fig. 5B), while the K8 scFv did not (Fig. 5C). A commercial anti-mouse CR1 antibody also bound to the dendritic cells (Fig. 5A). As expected, none of the antibodies bound to the control epithelial cells (Fig. 5D to F).

**Induction of immune response to HA.** The ability of the anti-CR1 scFv to induce an immune response was tested in a murine immunization experiment. The HA tag present on the carboxy terminus of anti-CR1 served as the antigen. The unrelated K8 scFv, which also contained the same HA tag, was used as a control. The animals were given scFv intranasally with LPS as the adjuvant. As shown in Fig. 6, all groups that received the anti-CR1 scFv showed a strong IgG response, with the strongest response in the group that received 5 μg of protein. The increase in immune response between animals immunized intranasally with 0.1 μg and 5 μg anti-CR1 was statistically significant (P = 0.03). Although an IgG response was also observed in the control group receiving the K8 scFv, the response was weaker. The responses from immunization
with 0.1 μg anti-CR1 and 0.1 μg K8 were significantly different ($P = 0.008$). The response by mice that received 5 μg of anti-CR1 was also higher than in mice that received 5 μg of K8 ($P = 0.048$). A response was not observed in the group that received only LPS. A salivary IgA response was not observed in any of the groups.

**Expression and function of anti-CR1 in S. gordonii.** The anti-CR1 scFv gene was subcloned into pDL276 as outlined in Fig. 1. The expression of scFv was under the control of a tetracycline-inducible xyl-tetO promoter. The promoter was shown to be functional in *E. coli* and *S. gordonii* (18). *E. coli* carrying pSecCR1 was shown to express a 33-kDa protein recognized by the commercial anti-HA antibody, indicating the gene had been successfully subcloned (data not shown). pSecCR1 was transformed into a tetracycline-resistant strain of *S. gordonii* DL-1 (hppG::tet). The transformant secreted a 33-kDa protein recognized by the commercial anti-HA antibody, while such a band was absent in the culture supernatant from the parent strain (Fig. 7A). The 33-kDa protein bound to the NiCAM column and could be eluted with imidazole. The eluted protein was able to bind to mouse dendritic cells in the ELISA (Fig. 7B), indicating that it was functional.

**Induction of an immune response following oral colonization with S. gordonii.** The ability of the anti-CR1-producing *S. gordonii* to induce an immune response was tested in a mouse oral-colonization study. The results showed that the two groups of mice were colonized to the same degree. In mice given *S. gordonii* SecCR1, the oral cavity, pharynx, and nasal cavity contained $1,013 \pm 200$ (mean ± standard error), $115 \pm 56$, and $20 \pm 10$ CFU of *S. gordonii*, respectively. In mice given *S. gordonii* SL3, there were $950 \pm 370$, $130 \pm 77$, and $25 \pm 7$ CFU of *S. gordonii* in these sites. Sera from mice colonized with the anti-CR1-producing *S. gordonii* showed an immune response, while that from mice colonized with the control *S. gordonii* SL3 did not (Fig. 8). No difference in IgA response was observed in saliva obtained from both groups.

**DISCUSSION**

In the present study, the single-chain recombinant antibody against CR1 was constructed from the cDNA obtained from a hybridoma. The construct was verified by DNA sequencing. The scFv was successfully expressed in *E. coli* and *S. gordonii*. The binding assay showed that the scFv was able to bind to dendritic cells and mixed lymphocytes, indicating that the scFv retained its function. The expression of a functional anti-CR1 scFv derived from a different hybridoma, 7G6, was previously reported by Prechl et al. (24), although that scFv remained in the insoluble fraction of *E. coli* while ours was mostly soluble. To the best of our knowledge, this is the first report of the
expression of anti-CR1 scFv in *S. gordonii*. Oggoni et al. (22) previously described the surface expression of an scFv against the *Streptococcus mutans* major surface protein antigen P1 in *S. gordonii* and demonstrated the ability of such an scFv to reduce dental caries in an animal model. These results collectively indicate that functional scFvs can be expressed in *E. coli* and *S. gordonii*.

The anti-CR1 scFv isolated from *E. coli* was able to elicit a very robust antibody response when given intranasally. The antibody response was significantly higher than that from mice immunized with the control K8 scFv, indicating that the anti-CR1 scFv works as postulated and efficiently targets antigen to phagocytic cells, resulting in an increased immune response. These data are in agreement with those observed for the anti-CR1/2 scFv derived from hybridoma 7G6, which failed to elicit a significant antibody response (24) even though the scFv was taken up and the influenza virus peptide carried on the scFv was efficiently presented by antigen-presenting cells in vitro (21). The difference may be due to the use of an LPS adjuvant in our experiment, whereas no adjuvant was used for the 7G6-derived scFv. LPS is known to up-regulate the expression of major histocompatibility complex molecules, CD40 ligand, and cytokines by antigen-presenting cells, leading to full activation of T cells and ultimately a strong immune response. Therefore, we believe that LPS played a role as an adjuvant in facilitating the robust immune response to the HA peptide in our experiment. The adjuvant effect of LPS is likely responsible for the observed immune response to the control K8 scFv, particularly at 5-µg doses.

It is noteworthy that the superior immune response observed in this study was obtained via intranasal immunization. All the studies reported in the literature were performed using the parenteral route. In view of the actual amount of HA administered (0.1 µg of anti-CR1 contains approximately 0.003 µg of HA peptide) and the fact that a large quantity of antigen is usually needed for mucosal immunization, the observed immune response is quite remarkable and further indicates the efficiency of the targeting ability of the anti-CR1 scFv.

The results of the oral-colonization experiment are in agreement with those observed using purified scFv delivered intranasally. We estimated that the amount of anti-CR1 scFv produced by *S. gordonii* was 17 ng/10^9 CFU (unpublished data), and in previous colonization studies, we reported that *S. gordonii* was present to the order of 10^6 CFU on the oral-nasal pharynx (14). Thus, the amount of scFv produced during colonization at a given time is small (but production is continuous), and yet an immune response was observed, further indicating the efficiency of the anti-CR1 scFv.

It is also interesting that in both scFv protein intranasal-immunization and bacterial-colonization experiments, only a systemic IgG response was observed. Typically, intranasal immunization elicits both a systemic and a mucosal antibody response, and this was certainly the case with other protein antigens (11, 14). On the other hand, our previous results from oral colonization with the recombinant *S. gordonii* elicited only a weak mucosal IgA response (16). The deviation from these past results suggests that the anti-CR1 scFv may have a unique feature in that it selectively promotes a systemic response, although the mechanism remains unclear.

In summary, an scFv against CR1 was constructed and expressed as a functional protein by both *E. coli* and *S. gordonii*. The scFv was able to elicit an enhanced immune response to the HA peptide linked to its C terminus, presumably due to its antigen-targeting ability. An immune response was also observed in a mouse *S. gordonii* oral-colonization experiment, indicating that the scFv may be used as an antigen-targeting tool in the bacterium.

**ACKNOWLEDGMENTS**

We thank Yi-Jing Li and Annette Morris for their valuable technical assistance. We also thank Carlos Barbas III for providing pComb3X and Keith Joiner for providing pkJ97.

J. B. Knight was an IWK graduate studentship recipient. This study was supported by the Canadian Institutes of Health Research (CIHR).

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