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# Neutralization of Enteric Coronaviruses with *Escherichia coli* Cells Expressing Single-Chain Fv-Autotransporter Fusions

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**We report here that fusions of single-chain antibodies (scFvs) to the autotransporter β domain of the IgA protease of *Neisseria gonorrhoeae* are instrumental in locating virus-neutralizing activity on the cell surface of *Escherichia coli*. *E. coli* cells displaying scFvs against the transmissible gastroenteritis coronavirus on their surface blocked in vivo the access of the infectious agent to cultured epithelial cells. This result raises prospects for antiviral strategies aimed at hindering the entry into target cells by bacteria that naturally colonize the same intestinal niches.**

Secretory immunoglobulins present on mucosal surfaces (e.g., immunoglobulins A [IgAs]) provide an early barrier against most virulent agents that invade their hosts through that port of entry (14, 16). Such Igs are as efficient when provided by the host organism itself as when acquired from an external source, typically, the Igs present in the maternal milk (19). The barriers built by secretory Igs in the mucosa are particularly important in naturally immunocompromised hosts, such as newborn animals and infants, which are more susceptible to a fatal outcome after an initial infection (6). Typically, Igs neutralize viruses in the mucosa by preventing their adherence to epithelial cells (14). Unfortunately, this mechanism is not efficacious in all cases and disease does occur.

The transmissible gastroenteritis coronavirus (TGEV), which infects respiratory and enteric tissues, is an important porcine disease that causes nearly 100% mortality in infected newborn animals (7). Previous studies have identified a mouse monoclonal antibody (MAb), named 6A.C3, which fully neutralizes TGEV and TGEV-related coronaviruses infecting pigs, cats, and dogs (3). The outstanding neutralizing ability of 6A.C3 is maintained in various contexts in vivo. Transgenic mice engineered to secrete 6A.C3 in milk (2, 18) produced an antibody which maintained in full its intrinsic neutralizing activity. This finding suggested a plausible approach for developing a sort of passive immunity against TGEV in young animals who feed on such a milk.

In this work, we explored a different approach for creating scenarios of passive immunity, e.g., the use of live bacteria as the vehicle to deliver the TGEV-neutralizing activity at the required sites. The rationale is that the locations of entry of the infectious agent (the mucosal epithelia) are also the natural niches of enteric bacteria (i.e., *Escherichia coli*) that can be programmed genetically to provide neutralizing antibodies. Construction of such bacteria requires the expression and secretion of active antibodies in *E. coli*. This process needs (i) the expression of the heavy ($V_H$) and light ($V_L$) variable domains that assemble the antigen-binding site of the antibody, (ii) the formation of disulfide bonds in the V domains for correct folding, and (iii) the selection of suitable vector systems to target active antibodies to the cell surface and the external medium. As shown below, we have successfully met these needs by exploiting some key features of the mechanism of secretion of the IgA protease (IgAP) from *Neisseria gonorrhoeae*.

**Expression of the anti-TGEV antibody 6A.C3 in *E. coli*.** The antigen-binding site of the original 6A.C3 MAb was recreated as a single-chain Fv protein (scFv) by employing the corresponding $V_H$ and $V_L$ domains from the 6A.C3 hybridoma (8). Although the apparent affinity of the resulting scFv was reduced 50-fold (8), probably due to the conversion of the bivalent MAb into a monovalent scFv molecule (5, 11, 17), the new scFv (i.e., 6AC3-scFv) retained the TGEV-neutralizing activity of the full-size 6A.C3 MAb. The 6AC3-scFv protein was, therefore, still helpful in validating the in situ neutralization concept. To this end, we next attempted to fuse the 6AC3-scFv to a carrier protein able to translocate the antibody moiety to the *E. coli* surface. The vehicle of choice was the transporter domain of the IgAP from *N. gonorrhoeae*. IgAP belongs to the autotransporter family of secreted proteins (10). All members of this family, which are described as present in an increasing number of pathogenic bacteria (9), share the same modular structure, with an N-passenger domain that is exposed to the external medium and a C-terminal transporter β domain driving the translocation of the passenger across the outer membrane (OM). The N-passenger module can be replaced by heterologous domains, which may become exposed to the medium provided that the hydrodynamic radius of the folded passenger protein is not longer than ~2 nm (4, 13, 15, 20; unpublished results). Such a secretion system tolerates the folding and the passage of disulfide-bond-containing proteins (1, 21; unpublished results). Furthermore, when fused to the β domain of an autotransporter, active scFvs can be targeted to the external medium of *E. coli* (21). On this basis, we set out to produce a hybrid protein that fused in frame the sequence of the 6AC3-scFv protein to the C-terminal transporter module of the IgAP (Fig. 1A).

For generating the constructs expressing the desired scFv-β domain hybrid (hereafter named 6AC3β protein), the sequence encoding 6AC3 was excised from plasmid p6AC3g3 (8)

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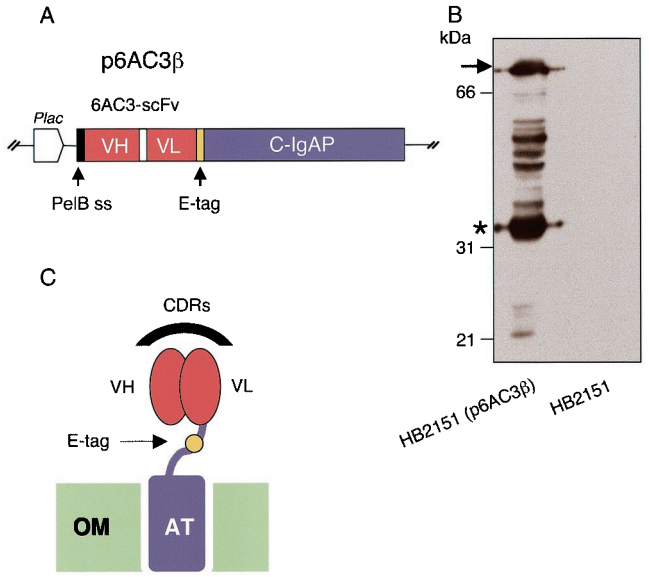


FIG. 1. (A) Organization of the relevant insert of plasmid p6AC3β, encoding the 6AC3β fusion. The sequences corresponding to the *pelB* leader (ss), the scFv, and the C-IgAP segments are indicated along with the *lac* promoter (*Plac*) and the fragment encoding the E-tag epitope. (B) Expression of the 6AC3β hybrid in *E. coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of crude extracts of induced *E. coli* HB2151(p6AC3β) cells expressing 6AC3β were probed by immunoblotting with an anti E-tag MAb. The location of the full-size 80-kDa 6AC3β protein is indicated with an arrow. The 35-kDa major proteolytic band corresponding to 6AC3-scFv is indicated with an asterisk. (C) Simplified sketch of the localization and predicted domain structure of the 6AC3β protein. Although the autotransporters (ATs) are structured as oligomeric complexes (22), only a monomer is shown. The illustration includes the AT domain of the IgA protease of *Neisseria* inserted into the bacterial OM and bound to the $V_H$ and $V_L$ modules of the scFv by a linker which incorporates the E-tag. This assembly allows the presentation of the complementarity determinant regions (CDRs) of the scFv to the cell exterior.

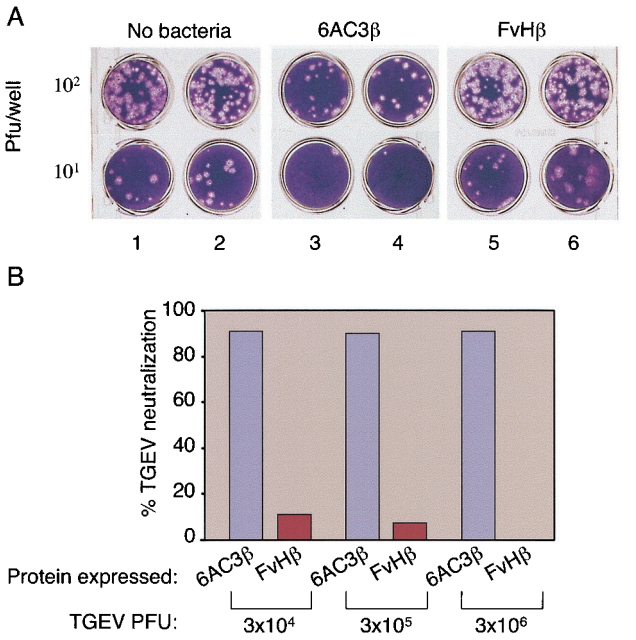


FIG. 2. Neutralization of TGEV infection by the *E. coli* cells expressing the 6AC3β hybrid. (A) TGEV ($3 \times 10^6$ PFU) was incubated for 30 min at 37°C with $10^8$ *E. coli* HB2151 cells expressing the proteins indicated (6AC3β and FvHβ) in each case. After centrifugation, serial dilutions of the supernatant were added to monolayers of ST cells grown in vitro. The plaques caused by TGEV replication were visualized after 48 h by fixing and staining the ST cells. (B) Quantification of TGEV neutralization. The numbers of plaques produced by TGEV infection in samples incubated with *E. coli* cells expressing the proteins indicated or without this incubation were compared. Neutralization rates are shown as percentage ratios of samples with various amounts of PFU treated with bacteria to those lacking any treatment. The data are the average of results for at least three independent experiments.

as a 0.7-kb *Sfi*I-*Not*I fragment, which was then cloned in vector pF11β (21). The resulting plasmid (named p6AC3β; Fig. 1A) fused the 6AC3-scFv protein to the transporter C domain of the IgAP (C-IgAP). To ensure secretion, the hybrid sequence was placed in frame to the N-terminal signal peptide of the *pelB* gene (Fig. 1A). Furthermore, expression of the hybrid protein 6AC3β is controlled by the *lac* promoter and can be induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). Production of protein 6AC3β is easily detected with a MAb that recognizes the E-tag epitope engineered in the region between the scFv module and the C-IgAP transporter domain (21, 22).

To test expression of the hybrid protein, *E. coli* HB2151 cells (8) were transformed with plasmid p6AC3β and induced with 0.1 mM IPTG for 3 h at 30°C in Luria-Bertani medium. As shown in Fig. 1B, a major band of the expected size (~80 kDa) along with a series of smaller extra products corresponding to proteolysis of the hybrid protein was observed. The presence of such degradation products has been noticed before in other scFv–C-IgAP fusions targeted to the OM (21, 22). The major ~35-kDa band was proven to correspond to the scFv that remained trapped in the hydrophilic pore formed by the autotransporter complex (21, 22). This proteolysis can be prevented if other scFv types that are less prone to aggregation are fused with C-IgAP (23; unpublished results). Figure 1C depicts the putative topology on the bacterial surface of the scFv passenger of a 6AC3β monomer.

**TGEV-neutralizing activity of *E. coli* cells expressing 6AC3β.** In order to test whether the *E. coli* cells expressing the 6AC3β hybrid showed TGEV-neutralizing activity, we used a viral infection assay (8). To this end, $3 \times 10^6$ PFU of the TGEV strain PUR46-MAD (3) were incubated in 200 μl of PBS buffer with $10^8$ *E. coli* HB2151 cells expressing the 6AC3β hybrid (Fig. 2A, lanes 3 and 4). Controls (Fig. 2A, lanes 5 and 6 and lanes 1 and 2) included *E. coli* HB2151 cells expressing the control scFv–C-IgAP fusion FvHβ (bearing an antibody raised against C-terminal His tags [21]) as well as buffer without bacteria. After 30 min, samples were centrifuged to remove bacteria and adsorbed TGEV particles. Supernatants containing the free viruses were added in 10-fold serial dilutions to duplicate monolayers of swine testis (ST) cells grown in tissue culture plates. After a further 48 h of incubation, the ST cell monolayers were stained with crystal violet to visualize the plaques formed by TGEV replication. As shown in Fig. 2A, a distinct and specific neutralization of TGEV became evident in samples in which the virus had been preincubated with the bacteria expressing 6AC3β. No neutralization was seen in the samples treated with bacteria expressing the control FvHβ protein (Fig. 2A). TGEV-neutralizing activity was also not

detected in the culture supernatants of induced *E. coli* HB2151 (p6AC3β) cells (data not shown), thus indicating that the neutralizing scFv remained attached to the *E. coli* cell surface.

Next, the neutralization brought about by *E. coli* populations expressing 6AC3β preset at $10^8$ bacteria/200 μl by using various titers of TGEV ($3 \times 10^4$ to $3 \times 10^6$) was measured (Fig. 2A). It is noteworthy that the percentage of virus neutralization by *E. coli* producing 6AC3β was always around 93% ± 2% (mean ± standard deviation), regardless of the number of TGEV PFU employed in the assay. This result was not due to the escape of viral mutants lacking the target antigen. Instead, the percentage of nonneutralized viruses may reflect the lower affinity of monomeric 6AC3-scFv relative to that of the bivalent 6AC3 MAb, which provides >99.99% neutralization (8). Consistent with this notion, an increase of purified 6AC3-scFv from 0.5 to 5 μg/ml in TGEV neutralization assays elicited a neutralization of ~99% (8). A minor decrease in the titer of active viruses (<10%) was caused by *E. coli* cells expressing the unrelated FvHβ on viral populations of $<10^6$ PFU. This was probably due to some residual nonspecific adhesion of viroids to the charged surface of bacterial cells. This effect was not observed when higher TGEV titers (e.g., $10^6$ PFU) were used.

Together, the data presented above indicate that bacteria expressing fusions of autotransporter domains to antiviral scFvs are capable of placing a neutralizing activity on the bacterial cell surface. These observations expand and generalize our previous efforts to design *E. coli* strains that are able to produce functional antibodies in various cellular compartments as well as our efforts to exploit in situ their target-binding abilities (8, 12, 21).

In conclusion, our data show that the anti-TGEV activity of 6AC3-scFv is significantly preserved when it is fused to C-IgAP and that this hybrid protein is displayed on the surface of *E. coli* cells in a form able to reach out and neutralize the infectious agent prior to their binding to epithelial cells. These results put forward the enticing possibility of engineering antibody-producing *E. coli* strains that are able to colonize the very same intestinal niches engaged by TGEV and other pathogens and so create a protective barrier to infection in vivo.

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