Large-scale screening of human B-lymphoblastoid cell lines reveals super sensitive and completely resistant lines to *E. coli* Shiga toxin 2

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ABSTRACT

Diarrheal disease R&D is a top priority of the US Military. These diseases can severely hamper and disrupt mission operations, especially in deployment areas where the outbreaks are common. Among the causative agents of diarrheal disease are certain toxigenic strains of such bacteria as *Escherichia coli*, *Shigella dysenteriae*, and *Campylobacter jejuni*. The protein exotoxins produced by them are the central players in the virulence and disease pathogenesis of their respective strains. Among these toxins are the shigatoxins. This toxin family comprises the prototypic shigatoxin produced by *S. dysenteriae* and shigatoxin 1 (STx1) and shigatoxin 2 (STx2) produced by shigatoxigenic strains of *E. coli*. These strains include the highly virulent enterohemorrhagic *E. coli* strains such as 0157:H7. STx1 and STx2 are crucial for the infectious pathogenesis, including hemorrhagic colitis and hemolytic uremic syndrome, which can be fatal. However, STx2 has a notable significance because it more frequently associates with the diarrheal disease severity, including hemorrhagic colitis and hemolytic uremic syndrome. Human susceptibility to the shigatoxigenic virulent strains and the ensuing pathogenesis is quite variable. Susceptibility of various cells and tissues to shigatoxins is also variable. The ultimate causal factors that account for this variability are genetic.

The overall objective of the work reported here was to ascertain differences in cellular sensitivity or resistance to STx2. Our long-term goal is to elucidate subcellular, molecular, and genetic determinants that account for differences in sensitivity or resistance to STx2. For the initial work, we screened 220 individual-specific, immortalized B-lymphoblastoid cell lines. To determine sensitivity to STx2, 72-h cytotoxicity assays were performed. The toxin IC_{50} values were used as an index of sensitivity or resistance of the tested cells to STx2. We found that these cells exhibit a broad spectrum of sensitivity to STx2. Many cell lines were totally resistant to the toxin (IC_{50} > 500 ng/ml; > 7 nM), while many others proved highly sensitive to it (IC_{50} < 0.07 ng/ml; < 1 pM). The toxin IC_{50} differences between some cells were about five to six orders of magnitude. Further work is now in progress to determine the mechanisms that underlie these vast differences in cellular sensitivity or resistance to STx2.

INTRODUCTION

Diarrheal diseases pose persistent risks to the US Military service members both in garrison and during deployment in various parts of the world, especially the areas where hygienic conditions are insufficient, even absent, to control localized or widespread outbreaks. The outbreaks can severely hamper and disrupt mission operations. These diseases are typically food-borne and their common causative agents are bacteria, protozoans, and viruses. The diarrheal disease bacteria include certain toxigenic strains of *Escherichia coli*, *Shigella dysenteriae*, and *Campylobacter jejuni*. Although these bacteria have a number of virulence factors that make them pathogenic, the protein exotoxins they produce are the central players in the disease pathogenesis. Among these toxins are the shigatoxins, a toxin family that comprises the prototypic shigatoxin produced by *S. dysenteriae* and shigatoxin 1 (STx1) and shigatoxin 2 (STx2) produced by shigatoxigenic strains of *E. coli*. These strains include the highly virulent enterohemorrhagic *E. coli* strains such as 0157:H7, which has been associated with numerous outbreaks. *E. coli* 0157:H7 and other like strains also cause hemorrhagic colitis and hemolytic uremic syndrome, which can be fatal.

Both STx1 and STx2 are crucial virulence factors in the infectious pathogenesis of shigatoxin-producing *E. coli*. However, STx2 has a notable significance; it more frequently associates with diarrheal disease severity, including hemorrhagic colitis and hemolytic uremic syndrome. STx1 sequence is essentially identical to that of STx produced by *S. dysenteriae*. But STx2 sequence differs from both considerably. Shigatoxins belong to the general class of protein toxins termed A-B toxins, which comprise an enzymatically active, cytotoxic A-chain (A-moiety) and a receptor-binding B-chain (B-moiety). Stoichiometrically, the A-B toxins are commonly composed of one A-chain and one B-chain (A_1B_1), or one A-chain and five B-chains assembled as a donut-shaped homoheptamer (A_1B_5). Examples of A_1B_1 toxins are the plant toxins ricin and abrin. Examples of A_1B_5 toxins are cholera toxin and shigatoxins.

Like other A-B toxins, shigatoxins enter cells by receptor-mediated endocytosis, and for this entry all three shigatoxins use the same receptor, globotriaosylceramide (Gb3; CD77), a glycosphingolipid. The entry process spans several major stages, which culminate in delivery of the enzymatically active portion of the toxin to cytosol, where the toxin's target is located. **First**, the toxin binds its receptor. **Second**, the toxin is internalized and routed to endosomes. **Third**, the toxin is retrogradely transported to the lumen of endoplasmic reticulum. **Fourth**, the enzymic portion of the toxin enters cytosol. **Fifth**, the toxin catalytically inactivates its target, which results in cell death if the toxin is cytocidal, or severe disruption of cellular functions if the toxin is cytotoxic but not necessarily cytocidal. For shigatoxins, like the A-B type plant toxins, the target is 28S rRNA. As enzymes, the A-chains are N-glycosidases, and they remove a particular adenine in a functionally essential short stem-loop in the 28S rRNA. This catalytic depurination inactivates the essential role of 28S rRNA in ribosome function, which ultimately arrests protein synthesis, killing the cell. Thus, shigatoxins, like other toxins that target the 28S rRNA (e.g., plant toxins ricin and abrin), are potent cytocidal proteins.

Human susceptibility to the shigatoxigenic strains and the ensuing pathogenesis is quite variable. Susceptibility of various cells and tissues to shigatoxins is also variable. The ultimate causal factors that account for this variability are genetic. The **objective** of the work reported here was to ascertain differences in cellular sensitivity or resistance to STx2. The **long-term goal** of the larger study is to elucidate the underlying mechanisms by which the genetic determinants influence susceptibility and resistance to STx. Precise knowledge of genetic and cellular factors that confer toxin sensitivity or resistance to cells would help develop better disease prevention and treatment approaches.

METHODS

Human B-lymphoblastoid cells were from the Coriell Institute (Camden, NJ) and all other cells from ATCC (Manassas, VA). STx2 was purchased from List Biological Labs (Campbell, CA). The cells were propagated as directed by the suppliers. Before screening of B-lymphoblast lines for sensitivity or resistance to STx2, the toxin was tested on 4 different cell lines to confirm its functionality (Fig. 1). These were 48-hour assays. The screening assays with B-lymphoblasts were 72 hours. To do cytotoxicity assays, cells were seeded in 96-well plates and incubated with STx2 in 10-fold serial dilutions. The cell viability was assessed with CellTiter-Glo (Promega, WI). Viability of cells that received no toxin was considered 100%, and all other values were then normalized to it. STx2 concentrations that resulted in 50% viability (IC_{50}) were used to compare sensitivity or resistance of various cells.

RESULTS

Fig. 1. Control assays performed to evaluate the functionality of STx2.
As expected, STx2 proved highly toxic to Vero E6, an African Green Monkey kidney line. These cells are known to be very sensitive to both STx1 and STx2. HeLa cells are less sensitive than Vero E6, but HEK 293T/17 and the mouse fibroblast line LMTK$^-$ are totally resistant to STx2.

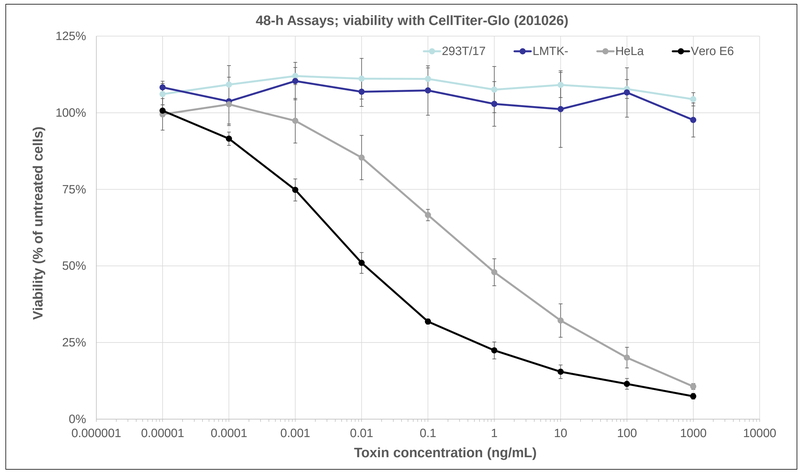
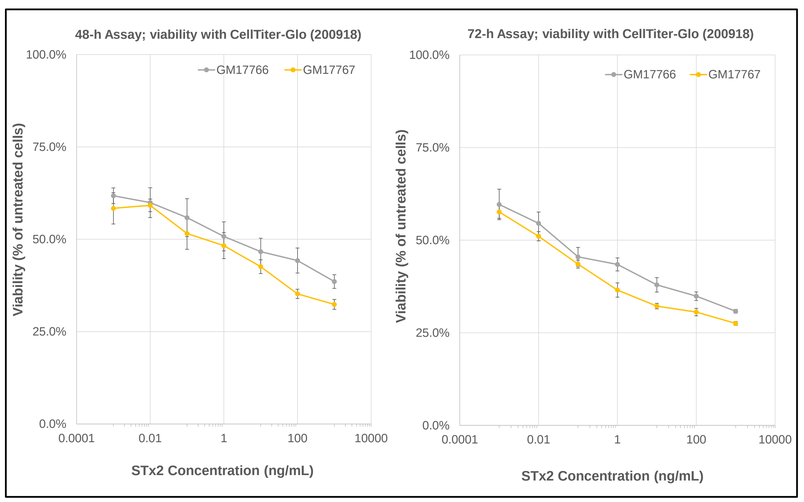
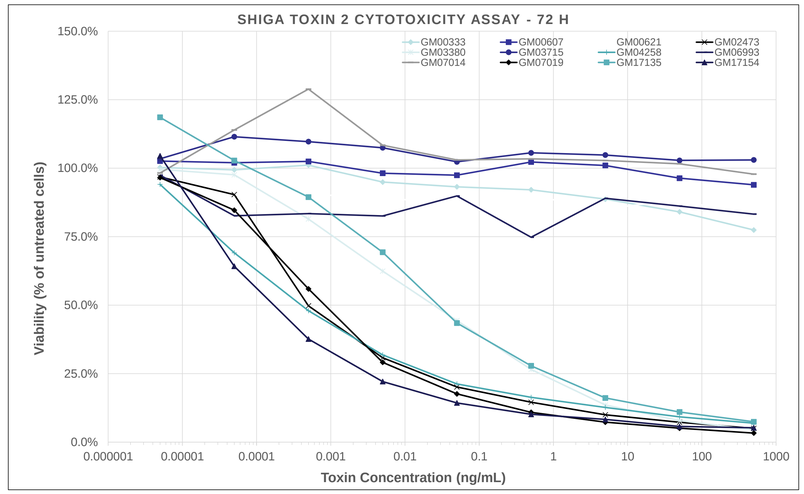


Fig. 2. Suitability of 48-h vs. 72-h STx2 toxicity assay for screening of B-lymphoblasts.
It is important that the length of toxicity assays be sufficient to reveal IC_{50} values for quantitative comparisons. Short assays mask sensitivity of some cells, leading to the false determination that such cells are resistant to the toxin. We performed 24-h, 48-h, and 72-h assays to determine the appropriate length of incubation with STx2. These assays proved progressively better at revealing STx2 toxicity differences, with 72-h assays being the most suitable. The 24-h and 48-h assays did not reveal sensitivity of many cells (not shown).



RESULTS

Fig. 3. Confirmatory assays performed following initial screening of B-lymphoblasts with STx2.
These 72-h assays were performed to confirm sensitivity or resistance of certain B-lymphoblasts after initial screening. We considered it important to confirm the STx2 sensitivity or resistance profile of these cells before further work, as screening was done only in duplicate or triplicate. The toxin sensitivity or resistance phenotypes of these cells held in confirmatory assays as in initial screening.



DISCUSSION

1. The work reported here represents a portion of a larger study whose overall goal is to identify genetic and cellular determinants of susceptibility to diarrheal disease bacterial protein toxins.
2. Our approach was to use individual-specific human B-lymphoblastoid cells to ascertain differences in cellular sensitivity to STx2. The rationale is that these cells represent different individuals, are like in histologic and differentiation lineage, likely retain the native genetic variations present in the donor individuals, and can be obtained in large numbers needed for such studies.
3. Thus, the fundamental factors that account for differences in toxin sensitivity or resistance are already present in these cells. This is obviously a great advantage as the goal is to identify naturally occurring genetic variations that confer sensitivity or resistance.
4. Next we plan to: **a)** Screen more cells to have large enough numbers for statistical analyses to determine genetic variation associations with sensitivity or resistance. **b)** Perform biochemical and cell biological studies to determine steps where intoxication is disrupted in resistant cells (e.g., STx2 binding to receptors, internalization). **c)** Do transcriptome sequencing (RNA Seq) to ascertain differences that may explain STx2 toxicity differences among the sensitive and resistant cells. **d)** If we find, for example, that a particular gene is expressed at low levels or not at all in totally resistant cells and its absence associates with resistance, we will express that gene in relevant cells to determine whether it complements the genetic lesion and restores the resistance phenotype to sensitivity.

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ACKNOWLEDGMENTS

We are greatly thankful to the US Air Force for funding this study. We are also indebted to the able support provided by the program officials in Science and Technology, 59 MDW, JBSA-Lackland.