![CVI Journals.ASM.org]

A Nonadjuvanted Transcutaneous Tetanus Patch Is Effective in Boosting Anti-Tetanus Toxoid Immune Responses

Robert C. Seid, Jr., Christoph Reinisch, Robert Schlegl, Michael Moehlen, Andreas Meinke, Urban Lundberg

Valneva Austria GmbH, Vienna, Austria

Dry tetanus toxoid (TTx) patches were formulated without any adjuvant, with excipients to impart antigen stabilization and to enhance skin delivery. The booster effects of the TTx patches were assessed using a guinea pig model. The study revealed significant rises in TTx IgG titers induced by the TTx patches after a low-dose subcutaneous (s.c.) prime with TTx adsorbed to aluminum hydroxide. The TTx patch can therefore be considered an effective alternative to a subcutaneous booster.

Transcutaneous immunization (TCI), using a topically applied needle-free patch, allows the delivery of an antigen and/or adjuvant into the skin, providing a powerful and effective alternative to traditional immunization (1). By taking advantage of antigen-presenting cells in the skin, TCI has been shown to induce robust immune responses to a wide variety of protein antigens, including those derived from bacterial and viral pathogens (2).

In this communication, we describe a research study to develop a nonadjuvanted, monovalent tetanus toxoid (TTx) booster vaccine patch to deliver TTx antigen into the skin and induce adequate serum anti-TTx IgG responses. We envision that a transcutaneous TTx patch may be conveniently used to reduce maternal and neonatal tetanus cases, observed mostly in developing countries, and as a booster vaccination for the adult/at-risk population, which includes those who have not received a tetanus booster in the preceding 10 years, as well as those persons who are exposed to hot, damp climates with soil rich in organic matter.

The main goal of our study was to compare the booster effect (i.e., immunogenicity) offered by the TTx patch versus that of a subcutaneous (s.c.) booster injection of aluminum hydroxide-adsorbed TTx in primed guinea pigs. Our study model was based on the European Pharmacopoeia (Ph. Eur.) section 2.7.8, in which standard methods (i.e., methods A to C) are described and used to determine the potency of a tetanus vaccine (3). For our study, we used a modified version to measure serum (enzyme-linked immunosorbent assay [ELISA]) anti-TTx IgG antibodies induced in s.c. immunized guinea pigs; instead of determining IU/ml for the serum samples, we calculated the half-maximum titer (50% effective concentration [EC_{50}]). It is also worth mentioning that the guinea pig is the preclinical model of choice to evaluate TCI, since the skin properties (e.g., stratum corneum thickness, presence and concentration of skin immune cells, etc.) of this animal are similar to those of humans (4).

We utilized our patch technology (5) to prepare dry 3-cm^2 nonwoven patches containing TTx antigen (Statens Serum Institut, Denmark) at two doses, i.e., 18 (45 μg) and 100 (250 μg) Lf (limit of flocculation) units. The dry patches were formulated with a proprietary mixture of GRAS (generally regarded as safe) excipients (a mixture of carbohydrates and surface active substances) for antigen stabilization (maintaining a noncrystalline, amorphous phase and minimizing denaturation/aggregation) and to facilitate a uniform coating of the patch. The carbohydrates replace water during drying and therefore increase the concentration gradient generated on the skin during rehydration of the patch by transepidermal water (TEW). In addition, the surface-active ingredients enhance the delivery of TTx following *in situ* patch hydration on the skin (5).

In contrast to earlier TCI studies (6), the transcutaneous TTx patches, as used in this study, did not contain any coadministered adjuvant. Because transcutaneous delivery via a skin patch is dependent on passive diffusion, the TTx patches were purposely prepared at higher dosages than those used for the s.c. boost injections (see below). The 18-Lf-unit TTx patch dose was based on the concentration of a commercial available TTx bulk solution and on the maximum volume of the final patch formulation blend that could be loaded onto the patch matrix. The 100-Lf-unit TTx patch dose was prepared with a 5- to 6-fold-concentrated, diafiltered bulk TTx solution.

All animal experiments were conducted in accordance with Austrian law (BGB1 no. 501/1989) and approved by Magistratsabteilung 58. The design of the guinea pig immunogenicity study is outlined in Table 1. Essentially, eight groups of female Hartley guinea pigs (5 animals per group, 4 weeks old; Charles River, France) were primed on day 1, boosted on day 37, and bled on day 51. On day 1, guinea pigs were primed with a s.c. injection of a 200-μl mixture containing aluminum hydroxide-adsorbed TTx vaccine at either a low dose (0.5 Lf units [1.25 μg]) or a high dose (5.0 Lf units [12.5 μg]). For the s.c. primary and secondary (boost) immunizations, the low and high TTx doses were adsorbed onto 25 or 250 μg of aluminum hydroxide (Brenntag, Denmark), respectively. On day 36, the first bleeds were collected. On day 37, groups 1 and 5 and groups 2 and 6 were boosted, respectively, by s.c. route with the low- and high-dose aluminum hydroxide-adsorbed TTx. For the TCI patch boost, groups 3 and 7 and groups 4 and 8, respectively, received TTx patches of 18 and 100 Lf units. Prior to patch applications, the abdominal skins of the guinea pigs were shaved and gently treated with a sandpaper device (5) to partially disrupt the stratum corneum. As a negative control,

TABLE 1 Design of the TTx boosting study in guinea pigs[a]

Group no.	Primary immunization, s.c.	Adjuvant dosage (µg)	Secondary immunization (boost)	Application route	Adjuvant dosage (µg)
1	0.5 Lf units TTx (low dose)	25	0.5 Lf units TTx, low dose	s.c.	25
2	0.5 Lf units TTx (low dose)	25	5.0 Lf units TTx, high dose	s.c.	250
3	0.5 Lf units TTx (low dose)	25	18 Lf units TTx, patch	TCI	NA
4	0.5 Lf units TTx (low dose)	25	100 Lf units TTx, patch	TCI	NA
5	5.0 Lf units TTx (high dose)	250	0.5 Lf units TTx, low dose	s.c.	25
6	5.0 Lf units TTx (high dose)	250	5.0 Lf units TTx, high dose	s.c.	250
7	5.0 Lf units TTx (high dose)	250	18 Lf units TTx, patch	TCI	NA
8	5.0 Lf units TTx (high dose)	250	100 Lf units TTx, patch	TCI	NA
9	Adjuvant alone (negative control)	250	Adjuvant alone, negative control	s.c.	25

[a] The primary immunizations were given on day 1 via the subcutaneous (s.c.) route. The first bleeds were collected on day 36. The booster immunizations were performed on day 37, by either s.c. injections or TCI patches. The second bleeds were collected on day 51. See the text for further description of the preclinical study. NA, not applicable.

group 9 animals received s.c. injections of 250 µg aluminum hydroxide for the primary and secondary (boost) immunizations. On day 51, the final bleeds were collected for all groups.

The serum anti-TTx titers for the primary s.c. immunizations (i.e., day 36 bleeds) obtained for the combined group 1 to 4 animals (primed with 0.5 Lf units TTx) were lower and statistically different from those combined for groups 5 to 8 (primed with 5.0 Lf units TTx) (Fig. 1A). As expected, the negative-control group (adjuvant only) did not produce any significant anti-TTx titers following the primary immunization.

The effects of the booster immunization were significantly stronger for the animals primed with the low-dose than for those primed with the high-dose aluminum hydroxide-adjuvanted TTx (Fig. 1B and C). Upon low-dose priming (Fig. 1B), a significant rise (~25-fold) in anti-TTx IgG titers was observed in the animals of groups 1 to 4. Although the low-dose s.c. boost (group 1) induced a noticeable rise in the titer of anti-TTx antibody, the titer was significantly ($P < 0.05$) lower (one-way analysis of variance [ANOVA], multiple-comparison Tukey *post hoc* test) than the anti-TTx titer induced by the high-dose patch boost (group 4) (Fig. 1B).

For the high-dose-primed animals (Fig. 1C), weaker (<5-fold rise) or no booster effects were evident for both the s.c. and transcutaneous boost immunizations. A significant induction (paired *t* test, two-tailed analysis) of anti-TTx antibody levels was observed only in group 6 (5.0 Lf units; P value = 0.0005) and group 8 (100 Lf units; P value = 0.0215). The anti-TTx antibody titer after the booster in group 6 was significantly higher (one-way ANOVA, multiple-comparison Tukey *post hoc* test) than in groups 5 ($P < 0.01$) and 7 ($P < 0.05$) (Fig. 1C). These results can likely be explained by the already high titers of anti-TTx antibodies induced by the high-dose primary immunizations, i.e., the high level of antibodies might allow binding and sequestering of the majority of antigen administered by the boost immunization, thus limiting the potential activation of memory B cells.

These data suggest that the TTx patch can induce a significant booster effect in animals with a low level of preexisting anti-TTx antibody titers. This was evidenced by an ~25-fold rise in anti-TTx IgG titers for the low-dose-TTx-primed animals (Fig. 1B, groups 1 to 4), which had lower preexisting anti-TTx antibody titers than the high-dose-TTx-primed animals at day 36 bleeds (Fig. 1A). Of particular importance and relevance, Fig. 1B shows that the boosting effect obtained with TTx patches (groups 3 and

4) was equally effective and at least comparable to the high-dose s.c. injected aluminum hydroxide-adsorbed TTx. The strong boosting effect seen with the 18-Lf-unit TTx patch (Fig. 1B, group 3) suggests that a significant boosting effect could possibly already be achieved with a lower patch dose. However, the currently used higher patch dose becomes of secondary importance when a TTx vaccine can be administered without adjuvant and needle injection and with the reduction in logistics due to lack of cold chain storage and transportation requirements.

Our preclinical data confirm that transcutaneous delivery of a complex high-molecular-weight formalin-treated antigen, such as TTx, is feasible and that high levels of anti-TTx IgG antibodies can be induced in guinea pigs having low preexisting anti-TTx antibody titers in the absence of an adjuvant. Moreover, the TTx patches without adjuvant demonstrated an acceptable safety profile, since local side reactions were minimal and transient at the site of the patch booster immunization (data not shown). Other research laboratories have reported on the use of TCI as part of a heterologous prime-boost regimen (7, 8). Glynn et al. (7) studied the effect of homologous and heterologous prime-boost regimens on the immune response of mice to a *Yersinia pestis* recombinant fusion protein. Tarique et al. (8) used TCI with a *Vibrio cholerae* O1 Ogawa synthetic hexasaccharide glycoconjugate after an oral immunization with a whole-cell cholera vaccine. For both preclinical studies, immune adjuvants, such as cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) or mutants thereof, were coadministered during the TCI applications. In our study, we have intentionally avoided the use of an adjuvant(s) for TCI, thereby producing a less complex patch formulation and reducing the occurrence of local skin reactions. In addition to TTx, we have also formulated the recombinant protective antigen from *Bacillus anthracis* without an adjuvant on a dry patch which was stable and generated an immune response similar to that with s.c. immunization (data not shown). Thus, it seems that some proteins when correctly formulated on a dry patch are capable of inducing a strong immune response without adjuvant.

The Grand Challenges in Global Health, a partnership between the Bill & Melinda Gates Foundation, Canadian Institute of Health, National Institutes of Health, and Wellcome Trust initiated in 2003, has as one of its goals the development of needle-free delivery systems for vaccines (9). In this sense, our preclinical results bode well for the prospect that a dry patch, containing TTx antigen, can be developed as a needle-free and painless alternative
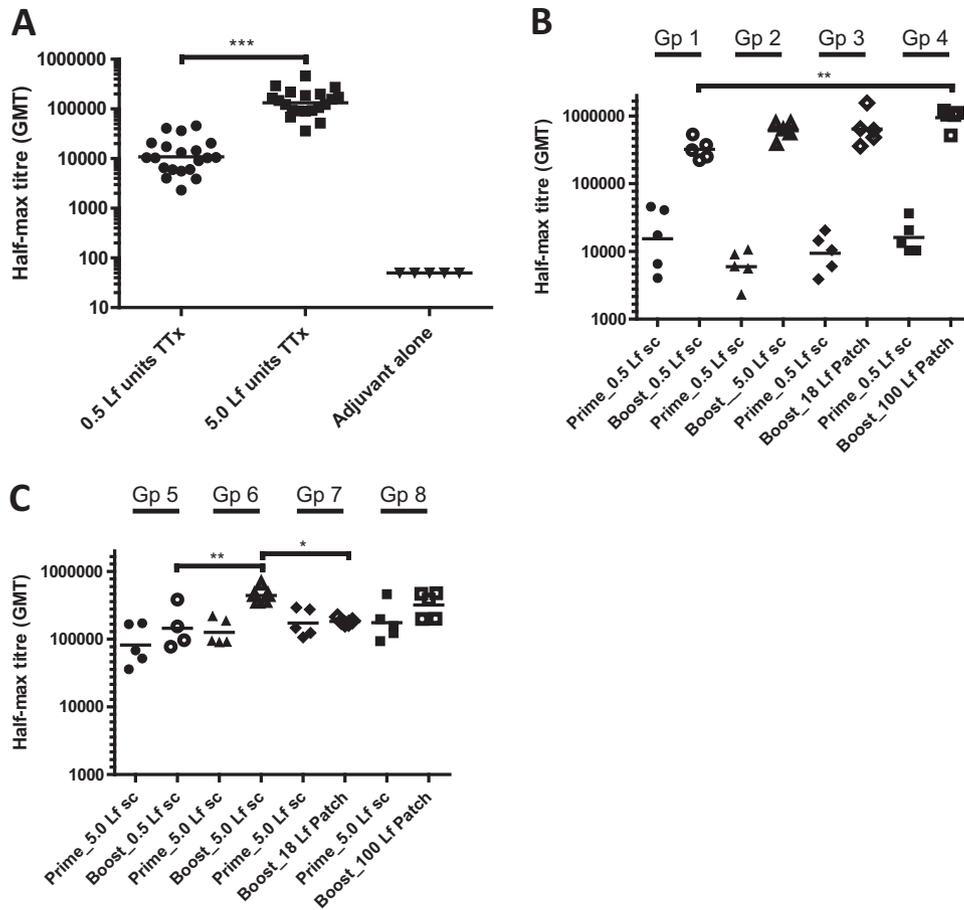
FIG 1 (A) Total anti-TTx IgG titers after primary s.c. immunization. The circles ($n = 20$) represent individual animals immunized with the low-dose, aluminum hydroxide-adsorbed 0.5-Lf-unit TTx vaccine; the squares ($n = 20$) are individual animals immunized with the high-dose, aluminum hydroxide-adsorbed 5.0-Lf-unit TTx vaccine; the triangles ($n = 5$) are animals immunized with 250 μg of aluminum hydroxide. Asterisks represent the statistical difference between the low-dose and high-dose aluminum hydroxide-adsorbed s.c. vaccine groups (see below). See the text for further experimental conditions. (B and C) The booster effect of transcutaneous patch or s.c. injection following primary s.c. immunization. Panel B shows strong booster effects in anti-TTx IgG titers for animal groups (see Table 1) primed with the low-dose, aluminum hydroxide-adsorbed 0.5-Lf-unit TTx vaccine. Panel C shows weak or no booster effects in anti-TTx Ig titers for animal groups primed with the high-dose, aluminum hydroxide-adsorbed 5.0-Lf-unit TTx vaccine. See the text for further details on experimental conditions. Only significant differences as determined by either an unpaired t test (A) or two-tailed analysis or one-way ANOVA (multiple-comparison Tukey *post hoc* test) (B and C) are shown in the graphs (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

route to strongly boost anti-TTx IgG levels and yield strong immune responses.

REFERENCES

1. **Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR.** 2000. Transcutaneous immunization: a human vaccine delivery strategy using a patch. Nat. Med. **6:**1403–1406. http://dx.doi.org/10.1038/82225.
2. **Seid RC, Jr, Glenn GM.** 2012. Transcutaneous immunization via vaccine patch delivery system, p 303–323. *In* Morrow WJW, Sheikh NA, Schmidt CS, Davies DH (ed), Vaccinology: principles and practices. Wiley-Blackwell, West Sussex, United Kingdom.
3. **European Directorate for the Quality of Medicines & HealthCare.** 2013. Assay of tetanus vaccine (adsorbed), p 2791–2795. *In* European pharmacopoeia, 7th ed, 5.1 monograph, section 2.7.8. European Directorate for the Quality of Medicines & HealthCare, Council of Europe, Strasbourg, France.
4. **Frolov VG, Seid RC, Jr, Odutayo O, Al-Khalili M, Yu J, Frolova OY, Vu H, Butler BA, Look JL, Ellingsworth LR, Glenn GM.** 2008. Transcutaneous delivery and thermostability of a dry trivalent inactivated influenza vaccine patch. Influenza Other Respir. Viruses **2:**53–60. http://dx.doi.org /10.1111/j.1750-2659.2008.00040.x.
5. **Seid RC, Jr, Look JL, Ruiz C, Frolov V, Flyer D, Schafer J, Ellingsworth L.**
6. 2012. Transcutaneous immunization with Intercell's vaccine delivery system. Vaccine **30:**4349–4354. http://dx.doi.org/10.1016/j.vaccine.2011.09.113.
6. **Scharton-Kersten T, Yu J, Vassell R, O'Hagan D, Alving CR, Glenn GM.** 2000. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. Infect. Immun. **68:**5306–5313. http://dx.doi.org/10.1128/IAI.68.9.5306-5313.2000.
7. **Glynn A, Freytag LC, Clements JD.** 2005. Effect of homologous and heterologous prime-boost on the immune response to recombinant plague antigens. Vaccine **23:**1957–1965. http://dx.doi.org/10.1016/j.vaccine.2004 .10.025.
8. **Tarique AA, Kalsy A, Arifuzzaman M, Rollins SM, Charles RC, Leung DT, Harris JB, Larocque RC, Sheikh A, Bhuiyan MS, Saksena R, Clements JD, Calderwood SB, Qadri F, Kovác P, Ryan ET.** 2012. Transcutaneous immunization with a Vibrio cholerae O1 Ogawa synthetic hexasaccharide conjugate following oral whole-cell cholera vaccination boosts vibriocidal responses and induces protective immunity in mice. Clin. Vaccine Immunol. **19:**594–602. http://dx.doi.org/10 .1128/CVI.05689-11.
9. **Varmus H, Klausner R, Zerhouni E, Acharya T, Daar AS, Singer PA.** 2003. Public health. Grand challenges in global health. Science **302:**398– 399. http://dx.doi.org/10.1126/science.1091769.