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Collagen minipellet as a controlled release delivery system for tetanus and diphtheria toxoid

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Abstract

The use of biodegradable polymer matrices as a single-dose vaccine delivery system was investigated using tetanus toxoid (TT) and diphtheria toxoid (DT). BALB/c mice were immunized with TT or DT in different formulations including individual, in minipellet and aluminum hydroxide (alum), and the antibody responses were monitored for 48 weeks. Antigens entrapped in minipellet elicited higher antibody responses compared to those obtained with individual antigens and antigens adsorbed to alum and the antibody levels remained elevated over 48 weeks. In addition, minipellet formulations induced the same subclasses of antibodies induced by alum formulations. These results raise the possibility to obtain optimal and long-lasting immune responses by a single administration of minipellet formulations. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

Through the children's vaccine initiative, several areas in which the efficacy and cost-effectiveness of vaccines could be improved have been identified including those against diphtheria, tetanus and pertussis. To overcome the problems of compliance inherent to multi-dose vaccine schedules, systems for the delivery of a single dose of vaccine should be established [1]. At present aluminum hydroxide (alum) is the only widely used adjuvant available for humans. However, the use of alum-type adjuvant for immunization has several disadvantages, since it has been reported to induce inflammation and to stimulate local formation of granuloma [2]. Furthermore, conventional alum-type vaccines require multiple recall injections at approximately timed intervals to achieve long-lasting and optimal immune responses. But it is very difficult, especially in developing countries, to maintain a high re-immunization rate in the multiple-administration immunization program. Therefore, the development of more efficient and safe adjuvant/vaccine delivery systems to obtain high and long-lasting immune responses is of primary importance. Recently, biodegradable poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) microspheres were used to deliver vaccine preparations [3–6]. Unfortunately, the results obtained to date on the controlled release of antigens for a single-step immunization have not been satisfactory. No booster effect could be achieved, as the antigen in the microspheres was not stable. But, some improvements have been made to develop better vaccine preparations [7–10]. And the protective efficacy and the neutralizing capacity of the antibodies generated by PLA/PLGA microspheres have also been shown [11,12]. Alternatively, another delivery system using dextran without denaturing antigens has also been reported [13]. Meanwhile, the administration of interferon in minipellet has

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been attempted [14,15]. The advantages of the minipellet are as follows: the carrier material is a biodegradable natural protein, it is manufactured under mild processing conditions without any organic solvent or heating process, and it is easily administered in the same way as conventional injections. Therefore, it is applicable to various kinds of biologically active peptides and proteins and is expected to facilitate their potential therapeutic use [16].

Here we have examined minipellet formulations for the immunization of tetanus toxoid (TT) or diphtheria toxoid (DT) vaccine. The theoretical aim was to achieve similar or higher antibody titers and a booster effect to those seen with two or eventually three doses of alum-adsorbed TT or DT. It would probably be satisfactory to obtain sufficiently high, sustained, neutralizing antibody titers to protect the recipient for a desired period of time.

2. Materials and methods

2.1. Toxoid

The lyophilized TT (650 Lf/ml, 0.289 mg PN/ml, 2250 Lf/mg PN) and DT (2960 Lf/ml, 1361 mg PN/ml, 2170 Lf/mg PN) used for minipellet formulations were prepared at Kitazato Institute (Saitama, Japan). The purity of these samples was confirmed by SDS-PAGE.

2.2. Preparation of minipellet

TT/Collagen minipellet (TT-minipellet) and DT/Collagen minipellet (DT-minipellet) were prepared as described before [13,14]. In brief, an aqueous solution of toxoid was mixed with 2% (w/w) collagen solution, and the mixture was lyophilized. Then a small amount of distilled water was added to the spongy lyophilized product to allow it to swell. Distilled water was then added to the swollen product to obtain a 30% (w/w) collagen gel. The mixture was admixed thoroughly to obtain a homogenous mixture and was then passed through a nozzle having an inner diameter of 1.7 mm to make a rod, which was then air-dried at 4°C in a 75% relative humidity atmosphere for 24 h. The resulting dried rod, with a diameter of 1 mm collagen minipellet, contained 10 μg antigen per 1 cm.

2.3. Protein release study

TT- or DT-minipellets (30 μg/3 cm) were placed in tubes and then incubated in 3 ml of PBS, pH 7.4, under agitation at 37°C. At various determination points, 1 ml of the buffer was collected and replaced with 1 ml of fresh buffer to maintain sink conditions. The samples were centrifuged for 20 min at 6000 rev./min and the protein released into the buffer was determined by a micro BCA protein assay (Pierce, Rockford, IL). Release experiments were done independently in triplicate. The protein profile was determined by SDS-PAGE and quantified using Quality One version 3.0 (Toyobo, Osaka, Japan).

2.4. Animal experiment

Six-week-old BALB/c female mice were obtained from SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions. Each mouse received 20 μg of antigen using different formulations by subcutaneous injection.

In TT experiments, the following groups were provided: Group 1 received 2 cm of minipellet containing 20 μg of TT ($n = 7$); Group 2, 20 μg of alum-absorbed TT ($n = 7$); Group 3, 20 μg of alum-adsorbed TT twice (0 and 2 weeks later) ($n = 7$); Group 4, 20 μg of TT in PBS ($n = 7$).

In DT experiments, the following groups were provided: Group 1 received 2 cm of minipellet containing 20 μg of DT ($n = 7$); Group 2, 20 μg of alum-adsorbed DT ($n = 7$); Group 3, 20 μg of alum-adsorbed DT three times (0, 2 and 4 weeks) ($n = 7$); Group 4, 20 μg of DT in PBS ($n = 7$).

Blood samples were taken by retro-orbital puncture under ether anesthesia at 2- to 4-week intervals during 48 weeks. Sera were kept at -70°C until assayed. The present experiments were approved by the Institutional Animal Care and Use Committee of St. Marianna Medical University.

2.5. The gelatin-particle-agglutination (PA) test for TT antitoxin titers

PA test was carried out using a tetanus antibody assay kit (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) as described before [17]. Twofold serial dilutions of serum specimen ($n = 7$, each group at each time point) were made on a U-bottomed 96-well microplate in duplicate and each dilution was mixed with an equal volume of a TT-sensitized particle suspension. The mixtures were allowed to stand for 2 h at room temperature and read for agglutination with the naked eye. The highest dilution showing positive agglutination was determined and the titers were calculated from standard tetanus toxin obtained from the National Institute of Infectious Disease. Then TT antitoxin titers were expressed as international units (IU) per ml.

2.6. Cell culture method (CCM) for DT antitoxin titers

The micro cell culture color change method with VERO cells was carried out as described before [18].

Twenty-five microliters of twofold dilutions of serum specimen ($n = 7$, each group at each time point) were mixed with an equal volume of diphtheria toxin in duplicate and incubated for 30 min at 37°C after agitation. Then these samples were added to 50 μl of VERO cell suspensions (2×10^5 per ml) and 100 μl of Eagle's minimum essential medium containing 3% calf serum, 0.003% phenol red and 0.4% glucose, and the plates were sealed with pressure-sensitive film (Falcon) and incubated for 4 days at 37°C. The highest dilution in which the color changed from red to yellow was the end point for the titration and antitoxin titers were calculated from control antitoxin obtained from the National Institute of Infectious Diseases. Diphtheria antitoxin titers were expressed as IU per ml.

2.7. ELISA for TT and DT antibody subclass

ELISA for TT and DT antibody was performed as follows: 100 μl of TT or DT (5 μg/ml) in 0.05 M carbonate buffer (pH 9.5) was placed in individual wells of 96-well flat-bottom Immunoplates (Nunc, Roskilde, Denmark). After incubation for 24 h at 4°C, the plates were washed four times with PBS–Tween 80 (0.05%), then 200 μl of diluted blocking solution (PBS with 0.2% BSA) was added and incubated for 48 h. One hundred microliters of immunized sample ($n = 7$, each group at week 12), standard, or blank serum was added to each well in duplicate. After incubation for 24 h at 4°C, the plates were washed four times with PBS–Tween 80, then 100 μl of 1/2000 diluted using peroxidase-conjugated rat anti-mouse IgM, anti-mouse IgG3, anti-mouse IgG1, anti-mouse IgG2a, and anti-mouse IgG2b (Zymed, South San Francisco, CA) was added and incubated for 24 h at 4°C. After washing the plates four times, *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO) was added in citrate buffer (pH 5.0) containing H_2O_2. The enzymatic reaction was stopped by 2 M H_2SO_4, and the plates were read at 492 nm using a Bio-Rad Model 13550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA). The individual isotype antibody titer was expressed as the reciprocal of the highest positive serum.

2.8. Statistics

Statistical analysis was performed with the Student's *t*-test, and *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. In vitro release of TT and DT

The release of protein from minipellet is shown in Fig. 1. The major protein band by SDS-PAGE was toxoid and the released toxoid quantified by the densitometer paralleled the released total protein (data not shown); 27.5% of the TT toxoid and 32.6% of the DT toxoid were gradually and continuously released from the collagen minipellet within 4 days, followed by a very slow release. DT was released faster than TT and the cumulative release after 14 days was 32.5% of the TT-minipellet and 36.6% of the DT-minipellet.

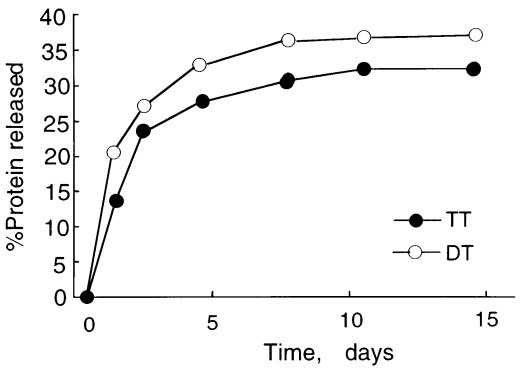

Fig. 1. Cumulative in vitro release of tetanus and diphtheria toxoid from minipellet in PBS at 37°C with agitation. Individual curves represent the mean of triplicates. S.D. is within 10%. ●, TT; ○, DT.

3.2. Antibody response

In a preliminary experiment, immunization with 20 μg of TT gave higher titers than 2 μg of TT regardless

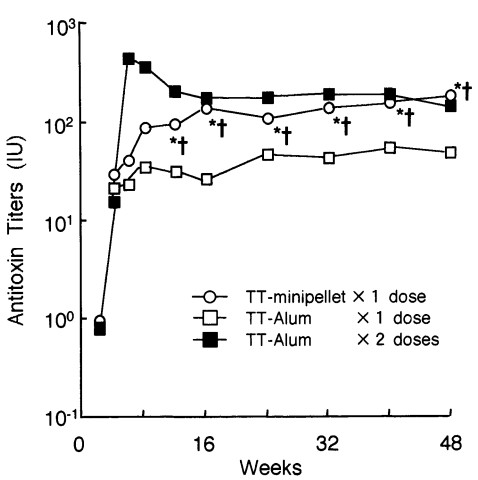

Fig. 2. Time course of TT antitoxin responses in BALB/c mice upon injection of TT in different formulations. Mice received 20 μg of TT under the conditions described in Section 2. Individual curves represent the mean antibody titer of each group ($n = 7$, each). The S.D. are less than 10% of the mean in all points. *$P < 0.01$ versus TT-Alum × 1 (Student's *t*-test). †Not significantly different versus TT-Alum × 2 (Student's *t*-test).

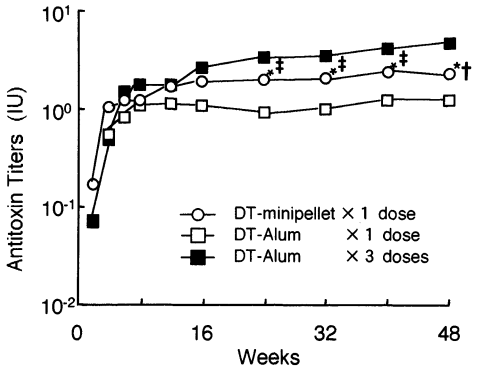

Fig. 3. Time course of DT antitoxin responses in BALB/c mice upon injection of DT in different formulations. Mice received 20 μg of DT under the conditions described in Section 2. Individual curves represent the mean antibody titer of each group ($n = 7$, each). The S.D. are less than 10% of the mean in all points. *$P < 0.05$ versus DT-Alum × 1 (Student's t-test). †$P < 0.01$, ‡$P < 0.05$ versus DT-Alum × 3, respectively (Student's t-test).

of the formulations employed. The highest titer with 20 μg of TT in PBS was 19 ($\pm$ 1) IU at week 6. The time course of antitoxin responses obtained with different preparations of 20 μg of TT is shown in Fig. 2. The S.D. were less than 10% of the mean in all points. The TT antitoxin titer in mice injected twice with alum-adsorbed TT increased the fastest and reached the highest levels (442 $\pm$ 30 IU) at week 6 remaining at those levels until week 32 (194 $\pm$ 18 IU), while the highest antitoxin titer (191 $\pm$ 18 IU) after 48 weeks was obtained by TT-minipellet. The titer with a single administration of alum-adsorbed TT (26 $\pm$ 7 IU) was as low as that obtained with the individual TT preparation. TT-minipellet gave a significantly higher antitoxin response than a single dose of alum-adsorbed TT ($P < 0.01$) and a similar response with alum-adsorbed TT twice (not significantly different) from week 12 to week 48.

Meanwhile, the DT antitoxin titer obtained with 20 and 2 μg of DT-minipellet did not differ significantly. The highest titer with 20 μg of DT in PBS was 0.7 $\pm$ 0.07 IU at week 12. The time course of antitoxin responses with different preparations of 20 μg of DT is shown in Fig. 3. The S.D. were less than 10% of the mean in all points. The titers obtained at week 48 were 3.6 ($\pm$ 0.1) IU with alum-adsorbed DT three times, 1.7 ($\pm$ 0.2) IU with DT-minipellet, and 1.0 ($\pm$ 0.1) IU with a single dose of alum-adsorbed DT. DT-minipellet gave a higher antitoxin response than a single dose of alum-adsorbed DT ($P < 0.05$) and a lower response than alum-adsorbed DT three times ($P < 0.05$ at weeks 24, 32, and 40 and $P < 0.01$ at week 48) from week 24 to week 48.

3.3. Isotype

Similar distributions of isotype/subclass antibodies were obtained in each group throughout the experiment period. The data obtained at week 12 are given in Table 1. IgG1 was the predominant antibody isotype in both alum and minipellet formulations and relatively high levels of specific IgG2a and IgG2b antibodies were also induced in minipellet formulations ($P < 0.01$ vs. fluid formulations). Only low amounts of IgM were detected in each group.

4. Discussion

In this study, we investigated whether a continuous or pulsatile release of antigen using collagen minipellet preparations of antigens could be obtained. The loading efficiency in the case of minipellet was about 90% (data not shown), whereas that by PLA/PLGA encapsulation has been reported to be 80% [19]. The release of toxoid from the minipellet was characterized by a rapid release phase, in which toxoid located on the surface of the minipellet might be released, followed by a slow release phase which resembled that observed with PLA/PLGA [19].

Table 1
Specific isotype/subclass antibodies induced in BALB/c mice using minipellet delivered and alum-adsorbed TT and DT[a]

Immunization group	Specific isotype/subclass antibody titer (U)				
	IgM	IgG3	IgG1	IgG2a	IgG2b
MP-20TT	$<10^2$	$<10^2$	3.0×10^4*	6.6×10^3*	3.3×10^3*
20TT	$<10^2$	$<10^2$	1.0×10^4	3.3×10^3	2.2×10^3
Alum-20TT × 2	$<10^2$	$<10^2$	7.0×10^4*	5.0×10^3	3.9×10^3*
MP-20DT	$<10^2$	$<10^2$	6.0×10^4*	4.6×10^3*	6.2×10^3*
20DT	$<10^2$	$<10^2$	8.0×10^3	1.0×10^3	3.3×10^3
Alum-20DT × 2	$<10^2$	$<10^2$	1.2×10^5*	1.2×10^3	2.9×10^3

[a] BALB/c mice (seven in each group) were immunized with TT or DT in the indicated formulations. The tested mouse sera were collected at week 12 after immunization. The figures are the mean subclass antibody titer obtained in each group ($n = 7$, each). The S.D. were less than 10% of the mean in all groups.

* $P < 0.01$ versus 20TT (Student's t-test).

Moreover, we found that the TT-minipellet preparation induced higher levels of TT antitoxin than fluid or aluminum-adsorbed TT, whereas the PLA/PLGA preparation was only superior to fluid toxoid [6]. But the DT-minipellet preparation was only superior to fluid or a single dose of the DT-alum preparation, but not to a triple dose of the alum formulation. Although the difference between DT-minipellet and a single dose of alum-adsorbed DT formulations was significant ($P < 0.5$) in this study, further studies are necessary to enhance the adjuvant effect of minipellet in the case of DT.

The minipellet preparations are able to induce a pattern of antibody isotype comparable to that obtained with alum preparations. As antibodies of some isotypes efficiently activate complement, bind to high-affinity receptors on monocytes, and act synergistically with antibody-dependent effector cells to produce cytotoxicity, a desirable adjuvant should preferentially elicit high-affinity antibodies of the IgG2a isotype in mice to confer protection against the infectious agents or toxin. Whereas cell-mediated immunity favors the production of IFN-γ as Th1 response, which augments production of antibodies of the IgG2a isotype in murine B cells, IL-4 augments the production of IgG1 and IgE through Th2 response. In this study we employed Th2-dominant BALB/c mice and IgG1 isotype was mainly produced by minipellet formulations as well as alum formulations. But the minipellet formulations could also induce higher IgG2 isotypes than fluid formulations. Moreover desirable adjuvant should elicit both cell-mediated and humoral immunity without unwanted side-effects. Although alum produces an appreciable granulomatous response at the injection site, minipellet formulations did not cause any local inflammation in a preliminary study.

It has been clearly demonstrated in this study that the minipellet preparation was associated with a significant adjuvant effect. This single-step immunization would be equivalent to a multi-dose schedule for tetanus and diphtheria immunization, which should lead to improved vaccination coverage as well as to a potential reduction in vaccination costs. This preparation also gave good results with respect to the duration of the response. For a vaccine to be effective, it is desirable to achieve high and long-lasting antibody titers for optimum protection in humans.

The use of this method as a single-shot vaccine delivery system has other potential applications, such as the possibility of incorporating several antigens in the same system, namely, diphtheria, tetanus, and pertussis (DPT), hepatitis B, and polio vaccine. Recently, the usefulness of collagen minipellet as a DNA vaccine carrier has also been proved [20].

More extensive in vivo studies are required to show the safety and to elucidate the ideal characteristics of collagen minipellet for a single-dose formulation of tetanus and diphtheria vaccine. In conclusion, we have demonstrated the viability of the development of a single-shot vaccine for an important pediatric vaccine.

References

[1] Shepard DS, Walsh JA, Kleinav E, Stansfield S, Bhalotra S. Setting priorities for the children's vaccine initiative: A cost effectiveness approach. Vaccine 1995;13:707–14.

[2] Allison AC, Byars NE. Immunological adjuvants: desirable properties and side effects. Mol Immunol 1992;28:279–86.

[3] Aguado MT, Lambert P-H. Controlled release vaccines — biodegradable polylactide/polyglycolide (PL/PG) microspheres as antigen vehicles. Immunobiology 1992;184:113–25.

[4] Singh M, Singh O, Singh A, Tawler GP. Immunogenicity studies on diphtheria toxoid loaded biodegradable microspheres. Int J Pharm 1992;85:R5–8.

[5] Alonson MJ, Gupta RK, Min C, Siber GR, Langer R. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccine 1994;12:229–306.

[6] Men Y, Thomasin C, Merkle MP, Grander B, Corradin G. A single administration of tetanus toxoid in a biodegradable microsphere elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. Vaccine 1995;13:683–9.

[7] Schwendeman SP, Constantino HR, Gupta RK, Siber GR, Klibanov AM, Langer R. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. Proc Natl Acad Sci USA 1995;92:11234–8.

[8] Chang A-C, Gupta RK. Stabilization of tetanus toxoid in poly (DL-lactic-co-glycolic acid) microspheres for the controlled release of antigen. J Pharm Sci 1996;85:129–32.

[9] Sanchez A, Gupta RK, Alonson MJ, Siber GR, Langer R. Pulsed controlled-release system for potential use in vaccine delivery. J Pharm Sci 1996;85:547–52.

[10] Tobio M, Schwendeman SP, Guo Y, McIver J, Langer R, Alonso MJ. Improved immunogenicity of a core-coated tetanus toxoid delivery system. Vaccine 2000;18:618–22.

[11] Singh M, Li X-M, Wang H, McGee JP, Zamb T, Koff W, Wang CY, O'Hagen DT. Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine. Infect Immun 1997;65:1716–21.

[12] Singh M, Li X-M, Wang H, McGee JP, Zamb T, Koff W, Wang CY, O'Hagen DT. Controlled release microparticles as a single dose diphtheria toxoid vaccine: immunogenicity in small animal models. Vaccine 1998;16:346–52.

[13] Diwan M, Misra A, Khar RK, Talwar GP. Long-term high immune response to diphtheria toxoid in rodents with diphtheria toxoid conjugated to dextran as a single contact point delivery system. Vaccine 1997;15:1867–71.

[14] Fujioka K, Takada Y, Sato S, Miyata T. Novel delivery system for protein using collagen as a carrier material: The minipellet. J Controlled Release 1995;33:307–15.

[15] Fujioka K, Takada Y, Sato S, Miyata T. Long-acting delivery system of interferon: IFN minipellet. J Controlled Release 1995;33:317–23.

[16] Fujiko K, Maeda M, Hojo T, Sano A. Protein release from collagen matrices. Adv Drug Deliv Rev 1998;31:247–66.

[17] Miyamura K, Sadahiro S, Kondo T, Takahashi M, Fujino R, Nishimura Y, Miyakoshi H, et al. Development and usefulness

of the gelatin-particle-agglutination test for titration of antibodies against diphtheria, pertussis, and tetanus toxins. Jpn J Med Sci Biol 1995;48:49–59.

[18] Miyamura K, Nishio S, Ito A, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titers using VERO cells. J Biol Stand 1974;2:189–201.

[19] Alonson MJ, Cohen S, Park TG, Gupta RK, Siber GR, Langer R. Determinants of release rate of tetanus vaccine from polyester microspheres. Pharm Res 1993;10:945–53.

[20] Ochiya T, Takahashi Y, Nagahara S, Sumita Y, Hisada A, Itoh H, Nagai Y, Terada M. New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet. Nat Med 1999;5:707–10.