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Immunization by subcutaneous implants of polyester-polyurethane sponges coupled with antigen

S.L. Lima[1,4], C.B. Machado[1], M.A.C. Pereira[1], D.C. Cara[2], D.T. Velarde[4], S.P. Andrade[3] and C.M. Gontijo[1]

Departamentos de [1]Bioquímica e Imunologia, [2]Patologia and [3]Fisiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[4]Fundação Ezequiel Dias, Belo Horizonte, MG, Brasil

Abstract

A new protocol is described for immunization of outbred Swiss mice. The procedure is based on subcutaneous implantation of antigen-coupled polyester-polyurethane sponges cut into disks of 10 mm in diameter *vs* 2 mm in thickness. Antigen coupling was performed by overnight incubation of the sponge with a solution of ovalbumin (Ova) (2 mg/ml) diluted in sodium carbonate buffer, pH 9.6. The amount of ovalbumin that was taken up by the sponge was between 71.4 to 82.5 µg. This was estimated by comparing the Ova absorbance at 280 nm in coating buffer solutions before and after incubation. To compare the efficiency of the proposed method, experimental groups immunized with the antigen in the presence of adjuvants (10 µg in $Al(OH)_3$ or 100 µg in complete Freund's adjuvant (CFA)) were run in parallel. The data obtained after the 3rd week of immunization indicate that both cellular and humoral immune responses were achieved. These were assayed by antigen-induced footpad swelling and ELISA (specific antibodies), respectively. The levels of both immune responses elicited were similar to the responses observed in mice immunized with ovalbumin in the presence of $Al(OH)_3$. The method might represent an advantage when immunizing with pathogenic antigens. Preliminary experiments have suggested that the antigen remains immobilized or bound to the sponge for a long period of time, since there is an increment on the cell population inside the sponges after boosting the animals. If so, the undesirable effects of immunization would be reduced.

Correspondence
C.M. Gontijo
Departamento de Bioquímica e Imunologia, ICB, UFMG
Caixa Postal 486
30161-970 Belo Horizonte, MG
Brasil
Fax: +55-31-441-5963
E-mail: cgontijo@mono.icb.ufmg.br

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• Sponge
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• Vaccine

There is a great interest in the development of new methods for immunizing experimental animals with lethal antigens such as toxins and/or venoms in order to obtain specific immune sera for medical purposes. The use of adjuvants such aluminum hydroxide ($Al(OH)_3$) and complete Freund's adjuvant (CFA) in schemes of immunization has been extensively studied and defined in order to reach maximum antibody production as well as minimal damage to the animal and antigen loss. CFA is considered to be a potent adjuvant and has proved to be useful for the production of horse antisera, although it may cause undesirable side effects. Animals submitted to immunization with this adjuvant

for a long period of time often show skin lesions followed by a reduction in their ability to produce antibodies (1,2). $Al(OH)_3$, which does not induce these effects, is not as potent an adjuvant as CFA (3). Protein iodination (4) and encapsulation of the antigen into liposomes (5,6) are examples of methods that have been proposed to avoid these problems. These methods reduce toxicity but not immunogenicity. However, losses may occur and accidents have been reported (4-7).

Looking for an alternative way of immunizing, we became interested in an experimental model developed for studying angiogenesis in rats (8) and mice (9,10). In this model, subcutaneous implants of polyester-polyurethane sponges are performed in the dorsal region of the animals where they act as a matrix for the development of blood vessels and connective tissue. These implants stimulate an inflammatory infiltrate rich in mononuclear cells by the end of the first week. Antigens, on the other hand, can be associated with inorganic materials such as polystyrene plates (Nunc, Copenhagen, Denmark) that are used for ELISA reactions. Using a similar protocol, polyester-polyurethane sponges were treated with chicken egg albumin (ovalbumin - Ova) grade III (Sigma Chemical Co., St. Louis, MO, USA) in sodium carbonate buffer, pH 9.6 (coating buffer), and then implanted into experimental mice. Ova is not a pathogenic antigen and its immunogenicity is not considered to be high. Polyester-polyurethane sponges were cut into disks (10 mm in diameter *vs* 2 mm in thickness), treated with 70% ethanol for 1 h and then boiled in distilled water for 30 min. The sponges were then dried under sterile conditions and immersed in coating buffer (1 ml/sponge) containing Ova at different concentrations (0.2, 2 and 20 mg/ml), centrifuged to assure that all the internal surface was in contact with the solution and then incubated overnight at 4°C. The sponges were washed 3 times in sterile PBS before being subcutaneously implanted into 6-8-week-old outbred female Swiss mice. We estimated the incorporation of 71.4-82.5 µg of Ova/sponge by comparing the Ova absorbance at 280 nm in coating buffer solutions before and after incubation.

For positive control of immunization, mice were injected either intraperitoneally (*ip*) with 200 µl of saline containing 10 µg Ova mixed with 1 mg $Al(OH)_3$ or subcutaneously (*sc*) with 40 µl of saline containing 100 µg Ova mixed with CFA (Sigma). Negative control mice were implanted with a sponge that was not pretreated with Ova, or were injected as described above, but without the antigen. In some experiments, mice were boosted 3 weeks later with 10 µg Ova in 200 µl saline, *ip*. Blood samples were collected weekly from the tail of mice and diluted in PBS (1:3). After clotting and centrifugation, the supernatants were collected and stored at -20°C.

Anti-Ova antibodies were assayed by ELISA. Briefly, polystyrene plates (Nunc) were coated overnight at 4°C with 2 µg Ova diluted in 100 µl coating buffer per well, washed with saline containing 0.05% (w/v) Tween-20, saturated with 0.25% (w/v) casein in PBS, washed again and then coated with serial dilution of mouse antiserum starting at 1:100. After 1 h at 37°C, plates were washed, incubated for 1 h at 37°C with peroxidase-conjugated goat anti-mouse globulin antiserum (Southern Biotechnology, Birmingham, AL), washed and developed by the addition of H_2O_2 and ortho-phenylene-diamine (OPD; Sigma). The reaction was interrupted at 10 min by the addition of H_2SO_4 at 1/20 dilution and absorbance was read at 492 nm in EIA-reader (Biorad, Hercules, CA, USA). The absorbance values obtained in the assays are reported as a score (ELISA*) which represents the mean ± SEM (N = 5-7) of the sums of absorbance values of ELISA run with serum dilutions from 1/100 to 1/25600. The highest absorbance values obtained at 1/100 dilution were in the linear response region

and the values obtained at 1/25600 dilution always reached the blank level. This way of reporting the results is equivalent to reporting titration curves or selecting the absorbance at one particular serum dilution as representative. The significance of the difference between experimental and control groups was assessed by the Kruskal-Wallis test for nonparametric data. Positive and negative control samples were run on every plate. A mouse was considered to be immunized if its ELISA* score was higher than the mean of normal control mice plus 3 times the SD.

The kinetics of anti-Ova antibody production for a period of 8 weeks is shown in Figure 1A for mice immunized with implants of sponges containing Ova. Positive control groups were prepared by immunizing mice with the antigen in the presence of adjuvants (CFA and $Al(OH)_3$). Another group received 10 µg of Ova in saline, *ip*, without adjuvant. No antibody was produced by the animals of this last group, which actually exhibited the same profile as negative non-immunized control groups (data not shown). Although requiring a longer time, sponge-implanted mice produced specific antibodies at the same level as those immunized with $Al(OH)_3$. When the activity of CFA and $Al(OH)_3$ as adjuvants is compared, we observe that a higher level of antibody production was obtained with CFA. This may be explained by the difference in the amount of Ova injected. Figure 1B shows the profiles of the groups when animals received 10 µg of Ova in saline, *ip*, as a booster on day 21. One week after the booster, sponge-implanted mice reached the antibody level of the group immunized with $Al(OH)_3$. The levels reached by these 2 groups in Figure 1B were higher than those observed in Figure 1A. On the other hand, with the exception of the first week after the booster (week 4), no difference was observed between the profiles of mice immunized with Ova in CFA as adjuvant, when primary and secondary responses were compared.

We also tested whether the implant of Ova-coated sponges also elicited a cellular immune response. As shown in Figure 2, we have compared this procedure between groups that were immunized with Ova diluted in saline with or without the presence of CFA, incomplete Freund's adjuvant (IFA) or $Al(OH)_3$ as adjuvants. During the third week after implant or immunization, the groups were injected *sc* with 30 µl of a 2% aggregated Ova solution into the left footpad. The same volume of PBS was injected *sc* into the right footpad as control (11). The data in Figure 2 are reported as the difference in thickness between footpads observed 48 h after injection. With the exception of the group immunized with Ova in the absence of adjuvants, in all other groups a significant increase of footpad thickness was observed. Differences ($P<0.05$) were ob-

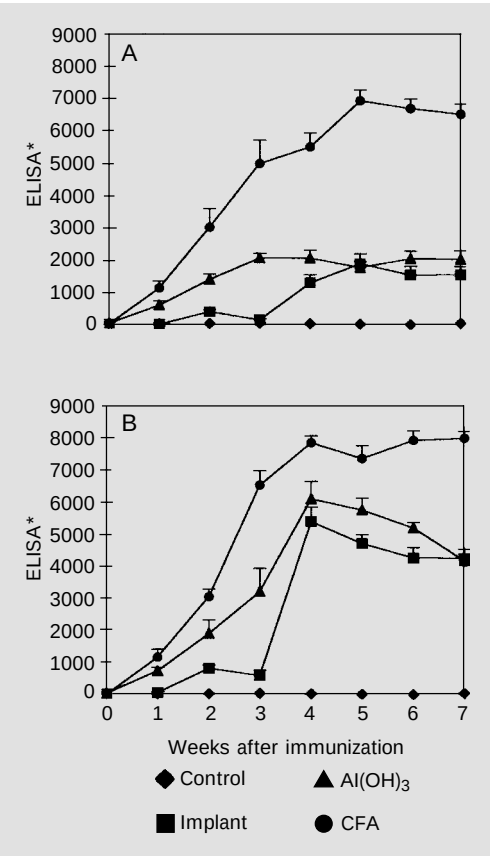

Figure 1 - Humoral immune responses elicited by Ova-conjugated implanted sponges. *A*, For the primary immune response, Swiss mice were immunized with Ova in $Al(OH)_3$ (10 µg, *ip*) or in complete Freund's adjuvant (CFA) (100 µg, *sc*), or were implanted subcutaneously with Ova-coupled sponges. Controls were either injected with 10 µg of Ova in saline, *ip*, or implanted with sponges treated without Ova. *B*, For the secondary immune response, the above protocol was repeated but mice received 10 µg of Ova diluted in saline, *ip*, during week 3 as a booster. Anti-Ova antibodies were detected by ELISA. Data are reported as ELISA* score, which represents the mean ± SEM (N = 5-7) of the sums of absorbance values from serum dilutions of 1/100-1/25600.

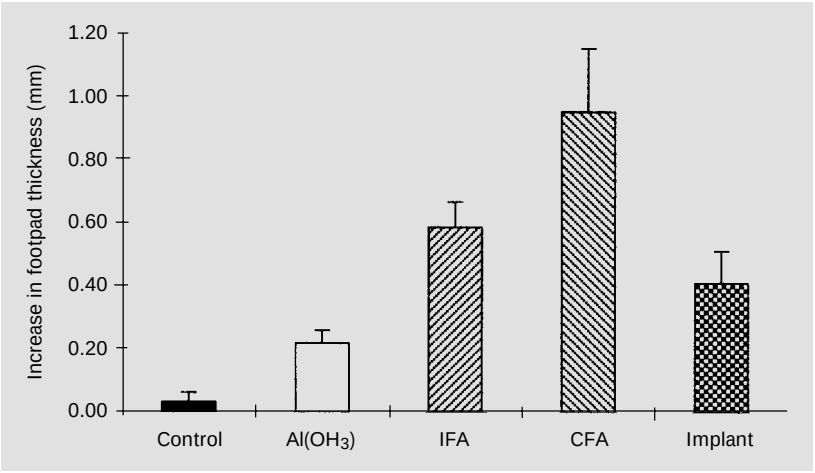

Figure 2 - Cellular immune response elicited by Ova-conjugated implanted sponges. Swiss mice were immunized with Ova in $Al(OH)_3$ (10 µg, *ip*), complete Freund's adjuvant (CFA) (100 µg, *sc*) or incomplete Freund's adjuvant (IFA) (100 µg, *sc*), or were implanted subcutaneously with Ova-coupled sponges. Controls were either injected with 10 µg of Ova in saline, *ip*, or implanted with sponges treated without Ova. During week 3, mice were injected subcutaneously with 30 µl of either 2% aggregated Ova (left footpad) or PBS (right footpad). Data are reported as the difference in thickness (mean ± SEM, N = 5-7) between both footpads observed 48 h after injection.

served between the three groups immunized with adjuvants (CFA, IFA and $Al(OH)_3$). No difference was observed between mice implanted with an Ova-treated sponge and those immunized with Ova in the presence of IFA or $Al(OH)_3$ as adjuvants.

The subcutaneous implantation of an antigen-coated sponge provided an alternative method for immunization. Both humoral and cellular immune responses were achieved at levels similar to those obtained when $Al(OH)_3$ was used as adjuvant. It is known that polyester-polyurethane sponge implants cause a granulomatous reaction characterized by an inflammatory infiltrate rich in polymorphonuclear cells, macrophages and giant cells around the trabeculae of the sponge matrix (9). A similar reaction is observed in granulomas formed when antigen is subcutaneously injected in the presence of $Al(OH)_3$ (12). The similarity of our proposed method and the use of $Al(OH)_3$ as adjuvant should be further analyzed. For instance, we are currently characterizing the isotypes that are formed when mice are implanted with antigen-coated sponges (13). It is known that, differently from CFA that usually elicits immune responses with a predominance of the T_H1 subset of T-lymphocytes, $Al(OH)_3$ is thought to elicit T_H2-type immune responses (14).

Antigen delivery from the sponge is another fact that deserves attention. Preliminary data from histological studies indicate that antigen delivery from the sponge should be slow. Histological changes occur in sponges when mice are boosted, indicating that antigens are probably still present at the site after at least 3 weeks. This possibility is very attractive since the method could represent an alternative for fixing pathogenic antigens at a site, with the consequent reduction of toxic effects during immunization procedures.

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Fellows will be selected on the basis of their promise as outstanding investigators, as well as the scientific merit of their research proposal, their record of training and how well their interests coincide with the laboratory of their sponsor in the United States. If potential applicants need assistance with the identification of an appropriate sponsoring laboratory in the United States, they may contact the Program Office before August 1, 1999. The Program will accept applicants from Mexico, Central and South America. Applications may be obtained from the Regional Committee contact listed here for your country or from our website at *http://futurehealth.ucsf.edu/pewlatin.html*

The application deadline is October 1, 1999. Winners will be notified in April 2000 and the fellowship should begin no later than August 2000.

Deadline for applications: October 1, 1999

Argentina

Israel Algranati
Instituto de Investigaciones Bioquímicas
Fundación Campomar
Av. Patricias Argentinas, 435
1405 Buenos Aires
Tel: 863-4018/Fax: 865-2246
e-mail: algra@iris.iib.uba.ar

Brasil

Carlos Eduardo Rocha-Miranda
Vice President, Academia Brasileira de Ciências
Rua Anfilófio de Carvalho, 29, 3º andar
20030-060 Rio de Janeiro, RJ, Brasil
Tel.: 220-4794/Fax: 240-4695
E-mail: cerm@abc.org.br

Mexico

Hugo Aréchiga
Facultad de Medicina UNAM
Ciudad Universitaria 04510
Mexico, D.F.
Tel: 622-0725/Fax: 550-8859
e-mail: arechiga@servidor.unam.mx

Chile

Manuel A. Kukuljan, Ph.D.
Universidad de Chile
Departamento de Fisiologia y Biofisica
Casilla 70005 Santiago 7, Chile
Tel: 2-678-6310/Fax: 2-777-6916
e-mail: kukuljan@bitmed.med.uchile.cl

All other countries

Silvia Montano de Jiménez
The Pew Latin American Fellows Program
3333 California Street, Suite 410
San Francisco, CA 94118
Tel: 415-476-5116/Fax: 415-476-4113
e-mail: montano@itsa.ucsf.edu