

Downregulation of IgE antibody and allergic responses in the lung by epidermal biolistic microparticle delivery

Mark Kendall, PhD,^a Thomas J. Mitchell, PhD,^a George Costigan, PhD,^a
 Mark Armitage, PhD,^a Jason C. Lenzo, PhD,^b Jennifer A. Thomas, BSc,^b
 Christophe von Garnier, MD,^b Graeme R. Zosky, PhD,^b Debra J. Turner, PhD,^b
 Phil A. Stumbles, PhD,^b Peter D. Sly, MD,^b Patrick G. Holt, PhD,^b and
 Wayne R. Thomas, PhD^b *Oxford, United Kingdom, and Perth, Australia*

Background: Biolistic injections provide a needle-free delivery of antigen-laden microparticles to the epithelium. The precision of the injection preferentially targets the Langerhans cell network, which, although ideal for vaccination, might not be suitable for the downregulation of immune responses in immunotherapy.

Objective: We sought to determine the ability of biolistic injection of antigen into the epithelium of sensitized mice to inhibit IgE antibody and lung inflammatory responses produced by further exposure to antigen.

Methods: Mice were sensitized by means of a needle injection of ovalbumin (OVA) in alum and given a series of biolistic injections of OVA or vehicle control, followed by a boost of OVA in alum. Serum IgE and IgG antibodies were measured before and after the boost. The mice were then challenged intranasally, and the infiltration of inflammatory cells was measured by means of bronchoalveolar lavage. Airway reactivity of the challenged mice was measured by examining responses to methacholine with forced oscillatory techniques.

Results: Biolistic injection of OVA into the dorsal skin of sensitized mice markedly inhibited IgE and IgG1 antibody

responses induced by boosting. IgG2a antibody responses were reduced rather than stimulated. The eosinophilic inflammation in the bronchoalveolar lavage fluid induced by intranasal challenge was also markedly inhibited. Lung hyperreactivity showed an initial increase and then a decrease of responsiveness to methacholine, with elastance returning to the level of unsensitized mice. Biolistic injection into the buccal epithelium was also inhibitory.

Conclusions: Biolistic injection of allergen inhibited the boosting of IgE antibody and eosinophilic lung inflammatory responses without inducing T_H1 immunity. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Desensitization, biolistic, Langerhans, IgE, eosinophilic, lung function

Biolistic delivery is a needle-free technique in which microparticles are accelerated in a high-speed gas jet to sufficient momentum to penetrate the epithelium of the skin or mucosal surfaces. It has its roots in gene-gun technology pioneered in the 1980s for genetic engineering¹ but has also been extensively applied to deliver medicaments,² as well as DNA³ and antigen-based⁴ vaccination. The advantages of biolistic delivery over needle or liquid jet injections are that it is pain free and does not require a cold chain for liquids.³ Desensitization for allergic disease is currently conducted by means of multiple subcutaneous injections of allergen. It can be efficacious, but the injections need to be continued for 3 years for consistent and lasting results.⁵ A major factor that compromises efficacy is that many patients do not finish the course of injections.^{6,7} Indeed, injection phobia can be a major impediment to the effective administration of many drugs and vaccines.⁸ The reduced trauma with biolistic delivery therefore offers the prospect of improved patient compliance and uptake of desensitization.

Biolistic injection delivers particles almost exclusively to the epithelium. They thus target Langerhans cells,³ which migrate and activate T cells in the lymph node.⁹ Langerhans cells have not been shown to present antigen for anergizing or inducing regulatory responses. Antigen presentation by dendritic cells is important for tolerance in autoimmunity,¹⁰ but this has been attributed to the

From ^aThe PowderJect Centre for Gene and Drug Delivery Research, University of Oxford, and ^bthe Telethon Institute for Child Health Research and Centre for Child Health Research, University of Western Australia, Perth.

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Reprint requests: Wayne Thomas, PhD, Telethon Institute for Child Health Research and the Centre for Child Health Research, The University of Western Australia, PO Box 855, West Perth, WA 6872 Australia. (E-mail: wayne@icmr.uwa.edu.au), and Mark Kendall, PhD, Department of Engineering Science, University of Oxford, 43 Banbury Rd, Oxford OX2 6PE, United Kingdom. (E-mail: mark.kendall@eng.ox.ac.uk).

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Abbreviations used

MCh: Methacholine

OVA: Ovalbumin

R_{aw}: Newtonian resistance

Zrs: Respiratory input impedance

functionally immature steady-state dendritic cells that have not been activated by inflammatory signals. Moreover, a study of sublingual tolerance to contact hypersensitivity found that during the tolerization, Langerhans cells only presented antigen with an immunogenic signal.¹¹ It was concluded that tolerance was induced by antigen that bypassed these cells. Additionally, epicutaneous application of protein antigen, which encounters the epithelial layer, induces strong T_H2 immunity.¹² There is therefore a need to ascertain the ability of biolistic delivery to inhibit allergic sensitization.

Biolistic DNA vaccination, with β -galactosidase as a model allergen,¹³ has been shown to vaccinate mice against allergic sensitization in a process that induced T_H1-type immunity. In contrast, the biolistic injection of antigen has been reported to be unable to decrease an ongoing allergic response of mice to ovalbumin (OVA).¹⁴ The study did not, however, examine IgE antibody or the ability of the biolistic injections to inhibit the boosting of sensitization, which is the typical outcome from immunotherapy,^{5,15} rather than inhibiting existing responses. The experiments reported herein examine the effect of biolistic injections on the boosting of IgE and eosinophilic inflammatory responses by using protocols optimized for the induction of IgE antibody responses of mice.¹⁶⁻¹⁸ Moreover, the biolistic devices were designed specifically for mice and were configured for the precise delivery of spray freeze-dried particles to the skin epidermis and the epithelium of the buccal mucosa.

METHODS

Biolistic devices for the skin epidermis and mucosal epithelium

Biolistic devices accelerate microparticles in a high-speed gas jet to impact the skin or mucosa with sufficient momentum to target the tissue layers of interest. The original concept from Oxford used a "cassette," storing the microparticles between 2 thin polycarbonate diaphragms, both instantly rupturing at a set pressure to initiate a high-speed gas flow. The biolistics system has been modified,^{19,20} leading to systems with better control of particle penetration.^{21,22} The biolistic devices used in this study are variants without diaphragms, entraining particles with the Venturi principle for delivery to murine epidermis (Fig 1, A) and buccal mucosa (Fig 1, B). These Venturi devices are quieter and release a larger fraction of the powder payload. An analysis of device operation of these systems is presented in the work of Mitchell.²³ Briefly, both use a "Venturi Gallery" to create low pressure, drawing the particles into a gas and accelerating to the target. The larger device for delivery to the skin (Fig 1, A) operates with a reservoir helium pressure of 6 MPa

and an exit diameter nozzle of 10 mm, which, unlike the diaphragm-based system, does not require a silencer. In contrast, the mucosal "Fanno Flow" device (Fig 1, B), with an initial helium reservoir pressure of 4 MPa, accelerates the particles to a lower velocity over a smaller diameter of 3.7 mm. The smaller diameter and quieter Venturi operation meets the requirement for the buccal delivery. Particles were formulated using a spray freeze-dried method at PowderJect technologies in Oxford, United Kingdom (PowderJect, Rudresha Prasad). The properties of the particles (approximately 45 μ m) are summarized in Table E1 in the Online Repository at www.jacionline.org.

Particle delivery assessment

Mice were injected in the shaved flank for dermal delivery or in excised buccal mucosa for mucosal delivery. Four administrations were given per operating condition, and the resulting footprints were fixed in formal saline and stained with hematoxylin and Masson's trichrome. Penetration depth analysis was carried out with Leica QWin, in which the particle penetration was measured to the leading (ie, deepest) edge of the particle from the viable epidermal or epithelial surface. Biolistic experiments were performed in ambient laboratory temperature and relative humidity conditions. For the penetration assessment studies, the particles were loaded with titanium dioxide to aid visualization.

Mice

Six- to 10-week-old specific pathogen-free female BALB/c mice were obtained from the Animal Resource Centre (Murdoch University, Murdoch, Western Australia). Procedures were approved by the Animal Ethics Committee of the Telethon Institute for Child Health Research.

Sensitization and desensitization

For sensitization, mice were injected intraperitoneally on day 0 with 1 μ g of chicken egg OVA (grade V; Sigma-Aldrich, St Louis, Mo) absorbed to 4 mg of alum (Amphogel; Wyeth Pharmaceuticals, Parramatta, Australia) in 100 μ L of PBS. The desensitization course was begun on day 7. Mice were biolistically administered 25 μ g of desensitizing antigen or control vehicle on the first and third weekday of each week for 4 weeks. They were boosted intraperitoneally with a further injection of 1 μ g of OVA in alum on day 34 (sensitization injection of 1 μ g in alum day 0; biolistic injections of 25 μ g on days 7, 9, 14, 16, 21, 23, 28, and 30; boost injection of 1 μ g in alum on day 34). Sensitization to papain (2 $\times$ crystallized, Sigma-Aldrich) was induced by using the same protocol to examine the antigenic specificity. Sera were collected before boosting and 17 (day 51) and 31 (day 65) days after boosting. For most experiments, the desensitization was performed biolistically to the dorsal skin. The first experiments also used the same protocol for epidermal delivery to the buccal mucosa of the cheek.

Aerosol challenge

Mice were exposed to 10 mL of an ultranebulized 1% OVA solution vented to a 30 $\times$ 30 $\times$ 20 cm box at a flow rate of 20 mL/min with a DeVilbiss Ultra-Neb 2000 (Sunrise Medical, Somerset, Pa) for 30 minutes.

Lung function

Lung function was measured 48 hours after 3 daily aerosol challenges with OVA. Mice were anaesthetized with 0.1 mL/10 g of a mixture of xylazine (2 mg/mL; Bayer, Pymble, Australia) and ketamine (40 mg/mL; Parnell Laboratories, Silverwater, Australia) and connected to a custom ventilator (Flexi Vent; Scireq, Montreal, Canada) by a polyethylene cannula (1.0 cm, id 0.0813 cm) inserted

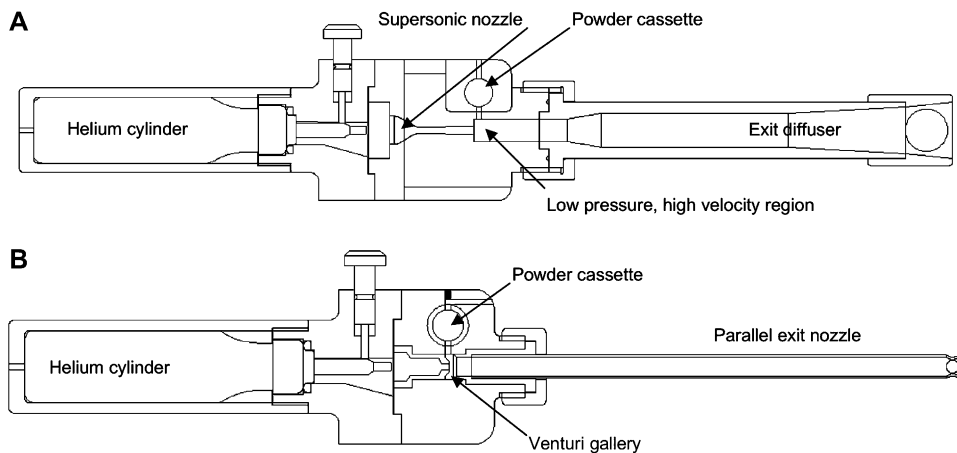


FIG 1. **A,** The biolistics system used for delivery to the mouse dorsal skin. **B,** The biolistics system used for buccal mucosal delivery.

into the trachea. Top-up anesthesia was given as necessary. Mice were ventilated at a tidal volume of 8 mL/kg and a rate of 450 breaths/min with a positive end-expiratory pressure of 0.2 kPa by using a computer-controlled piston. Respiratory input impedance (Z_{rs}) was measured at end expiration between 0.25 and 19.625 Hz by applying a composite signal (16 seconds long) containing 19 mutually prime sinusoidal waves during pauses in regular ventilation. The ventilator delivered both the regular ventilation and the oscillatory signals. After allowing the mice to acclimatize for 5 minutes, 5 discrete measurements of Z_{rs} were obtained, separated by 1 minute of regular ventilation. The constant-phase model ($Z_{rs} = R_{aw} + j\omega I + [G - jH]/\omega\alpha$)²⁴ was fitted to Z_{rs} spectrum, where R_{aw} is the Newtonian resistance (primarily located in the conducting airways but containing a component of the chest wall),²⁵ G is the coefficient of tissue damping, and H is the coefficient of tissue elastance (which reflects tissue stiffness). For hyperreactivity, mice were exposed for 90 seconds to aerosols from nebulized solutions of 0, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mg/mL methacholine (MCh) in normal saline generated from an online DeVilbiss Ultranebulizer. After each aerosol spray, Z_{rs} was measured each minute for 5 minutes. Concentration-response curves were constructed and the response in R_{aw} , G , and coefficient of tissue elastance to 30.0 mg/mL MCh was expressed as a percentage of the response to normal saline.

Antibody assays

IgE assays were performed by using passive cutaneous anaphylaxis. Doubling dilutions of sera (0.05 mL) were injected intradermally into the dorsal skin of male Sprague-Dawley rats. After 24 hours, the rats were injected intravenously with 2 mg of OVA or papain in 0.5 mL of PBS containing 0.05% Evans blue dye.²⁶ The titer is recorded as log₂ of the dilution of serum at the last site at which extravasation of dye was detected. IgG1 and IgG2a antibodies were measured by using immunoassays in microtiter wells (Nunc Maxisorb, Roskilde, Denmark) coated with 1 μ g/mL OVA in bicarbonate buffer.²⁶ Antibody binding was detected by using biotinylated anti-IgG1 and anti-IgG2a (Southern Biotechnology Associates, Birmingham, Ala), and the binding of these conjugates was detected by using europium-labeled streptavidin (Wallac, Turku, Finland). Dissociation-enhanced immunofluorescent DELFIA with proprietary reagents (Wallac) was used to detect the fluorescence of europium on a Wallac Victor2 multilabel counter.²⁷ The results are expressed as nanograms per milliliter interpolated from titration curves of immunoglobulin subclass standards captured and detected with mAbs.

Lung inflammation

Bronchoalveolar lavage was performed 48 hours after 3 daily exposures to aerosol performed according to the lung function protocol above. A cannula of 1-mm polyethylene tubing was inserted into the trachea and secured with thread. The tube was then connected to a syringe, and the lungs were then lavaged with 3 washes of 500 μ L of warm PBS containing 0.2% BSA (JRH Biosciences, Lenexa, Kan). Cells were counted with a hemocytometer, and differential counts were made from cytospin preparations stained with Diff Quik (Lab Aids, Narrabeen, Australia).

RESULTS

Particle delivery by means of biolistic injection of the skin and buccal mucosa

Fig 2 shows a histologic section demonstrating the lodgment of biolistically delivered microparticles into the skin. The titanium dioxide tracer reveals the particles as black dots. The sections were used to measure the penetration of the leading edge of the particles. The mean and SD of the penetration of 4 shots into the skin is shown in Fig 2 (means, 12–21 μ m). This shows that in each test particles breached the stratum corneum, which has a thickness of about 5 μ m.²⁸ Indeed, the particles targeted the viable epidermis of about 10 μ m, with the leading edge breaking through to the dermis. The back end of microparticles protruding from the tissue surface was not seen, as could be expected from their size, because they rapidly dissolved. Similar penetration depths were achieved to the thicker buccal epithelium (Fig 3). Mean penetration depths of between 12.8 and 20 μ m were achieved, and therefore few particles could breach into the underlying lamina propria, which has a mean thickness of about 50 μ m.²⁹

Antibody responses

The 10-mm device was used to deliver biolistic antigen to the dorsal epidermis, and the 4-mm device was used for the buccal cheek. Mice were sensitized with OVA in alum

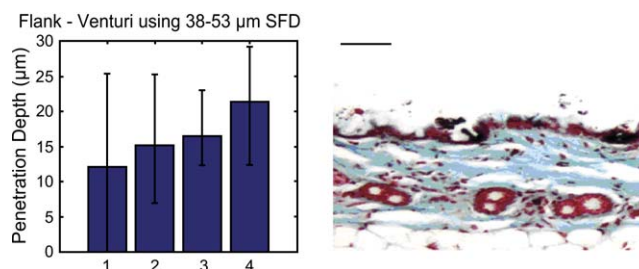


FIG 2. A histologic section of skin after biolistic injection of particles loaded with titanium dioxide tracers with the 10-mm Venturi injector. The scale bar is 100 μ m. Four sites were injected. The mean penetration for each site was calculated by measuring the depth of the leading edge of 20 particles. Results are shown as mean values (SD). *SFD*, Spray freeze-dried.

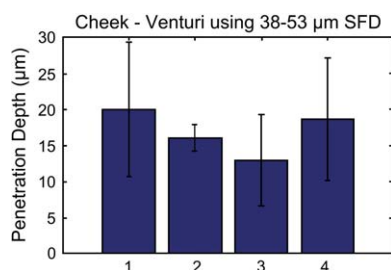


FIG 3. Mean penetration depths of biolistically injected particles loaded with titanium dioxide tracers from a 4-mm Venturi injector. Results are shown as mean values (SD) of 20 particles counted in shots at 4 sites in the buccal mucosa. *SFD*, Spray freeze-dried.

and after 1 week were given a course of biolistic or needle injections (see the Methods section). The IgE titers before boosting (day 31, Fig 4) showed no difference between the control groups and the desensitized groups. After boosting with OVA in alum on day 34, the IgE titers of control mice increased about 8-fold. The titers of the mice desensitized by means of biolistic injections to the skin or the buccal cheek mucosa, however, showed little increase. Qualitatively and statistically, highly significant differences from the corresponding vehicle-treated and untreated control animals were found on days 51 and 63. Mice desensitized by the subcutaneous injections of OVA also had lower responses than the control animals, but these were more variable. When analyzed by using ANOVA, the biolistically desensitized groups were different from their control animals ($P < .001$), as was the subcutaneous injection group from the untreated control group ($P < .01$). There were no differences between the different desensitized groups. The effect of the biolistic delivery to the skin has been examined 3 times with similar results. The specificity of the inhibition induced by biolistic delivery of OVA was examined by measuring the effect on IgE responses to the unrelated allergen papain. Mice were sensitized with papain and given the course of biolistic injections of OVA or the vehicle control. They were then boosted with papain, and the IgE titers to papain were determined. Mice injected biolistically with OVA had the same antipapain titers as the mice injected with the vehicle

(Fig 5). The anti-OVA titers of mice sensitized and boosted with OVA, set up for comparison, showed the expected inhibition induced by biolistic injections.

In addition to measuring IgE titers, the IgG subclass antibodies were measured on days 51 and 65 in 2 experiments (Table I). Mice immunized with OVA and alum had high IgG1 titers, and these were markedly inhibited by the biolistic injections to the skin (Table I). The IgG2a titers were expectedly low. The titers of the biolistically injected mice were, however, lower than those of the control mice, although this did not reach statistical significance ($P > .05$, Table I).

Lung inflammation

Mice were sensitized, given biolistic injections of OVA or vehicle, and then boosted with OVA by using the protocol used for the antibody responses. Starting on day 56 (25 days after the boost), the mice were challenged with 3 daily aerosol exposures of OVA. Cells infiltrating into the lumen of the airways were examined by means of lavage. Mice that had not been desensitized by the biolistic injections had a highly eosinophilic infiltrate at 48 hours. This was reduced approximately 3-fold in the mice given the biolistic injections (Fig 6).

Lung function

Mice were sensitized, given biolistic injections of OVA or vehicle, and then boosted with OVA by using the protocol used for the antibody responses. Lung function and MCh responsiveness were measured 48 hours after 3 daily aerosol challenges of OVA beginning on day 56 (25 days after boosting) or on day 77 (46 days after boosting). There were no differences in baseline function between any groups (see this article's Table E2 in the Online Repository at www.jacionline.org). MCh hyperresponsiveness of the sensitized groups was readily detected (Table II). At the early time point (day 56), mice treated biolistically with OVA had an increased MCh responsiveness (R_{aw} , G, and H) compared with that seen in control animals receiving the biolistic vehicle. When measured after a further 21 days, however, the values of R_{aw} , G, and coefficient of tissue elastance from mice treated with biolistic injections of OVA had decreased markedly. The elastance (H) dropped below the corresponding vehicle

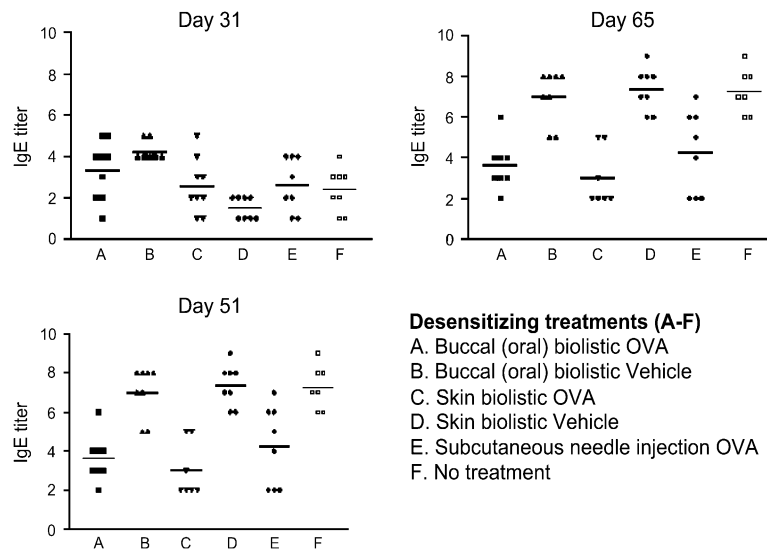


FIG 4. Inhibition of IgE by biolistic injections. OVA-sensitized mice were given desensitizing or control injections (as shown for groups A through F) and boosted on day 34. The titers (shown for individual mice) showed no difference between groups before boosting (day 31). Mice receiving desensitizing injections (A, C, and E) had lower titers than control animals (B, D, and F) after boosting but not compared with each other (see text for analysis).

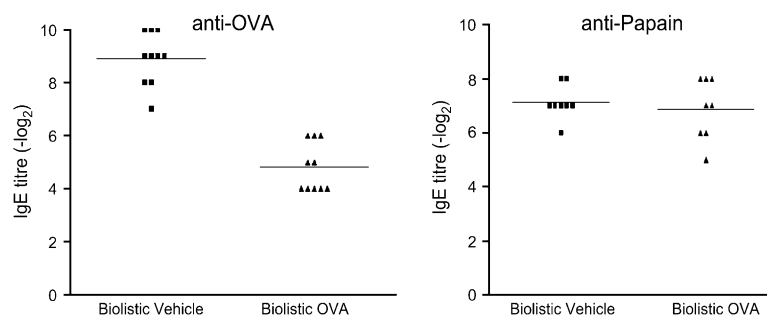


FIG 5. Specificity of IgE inhibition. The effect of biolistic injections of the vehicle and OVA were examined in mice sensitized and boosted with papain or OVA. The results show titers of sera from groups of 10 mice for OVA and 8 mice for the papain taken after boosting (day 51). There was a marked inhibition of anti-OVA but not antipapain responses.

control value and to levels similar to those seen in nonsensitized mice.

DISCUSSION

Injection phobia from a needle can be a major impediment to the delivery of vaccines and allergens.^{8,30} Needle-free, pain-free delivery could therefore be expected to increase the compliance of allergen desensitization and its scope of application. Immunotherapy for children is an example. Children respond at least as well as adults,³¹ and specific immunotherapy to one source of allergen reduces the development of sensitivity to other sources of allergen.³² The potential benefit of early immunotherapy is, however, offset by a reluctance to subject children to multiple injections.¹⁵ Liquid jet injection systems are needle-

free alternatives, but they cause pain and tissue damage and deposit the antigen in the dermal and deep subcutaneous regions.³³ Indeed, the potential for viruses to spread from the bleeding caused by liquid jets has limited their use for mass vaccination.³³ In contrast, biolistic injections atraumatically deliver microparticles to the epithelium. Although this has a clear advantage for comfort and compliance, epithelial delivery for desensitization could potentially be flawed. Antigen delivery to the network of Langerhans cells might induce an immune response instead of a regulatory or anergic response. The experiments here, however, show that biolistic injections do downregulate allergic responses.

The biolistic injection of OVA almost completely inhibited the IgE and IgG1 antibody responses induced by boosting sensitized mice. IgG2a antibody responses were typically low because alum adjuvant induces a T_H2

TABLE I. IgG subclass titers

		Day 51		Day 65	
		IgG1	IgG2a	IgG1	IgG2a
Experiment 1	Vehicle	310 (127)	2.0 (3.2)	253 (128)	4.9 (8.9)
	OVA	97 (91) [†]	0.4 (0.3)	61 (78) [†]	0.4 (0.8)
Experiment 2	Vehicle	252 (143)	0.4 (0.1)	159 (68)	0.5 (0.5)
	OVA	25 (42) [†]	0.1 (0.1)	133 (88)*	1.8 (2.2)

Results are shown as mean values (SD) of groups of 10 mice for 2 separate experiments. The significance was determined by using the Student *t* test:

**P* < .01, [†]*P* < .001. The values for the comparison of the IgG2a titers were as follows: *P* < .1 and .2 for days 51 and 65 of experiment 1 and *P* < .1 and .3 for experiment 2, respectively.

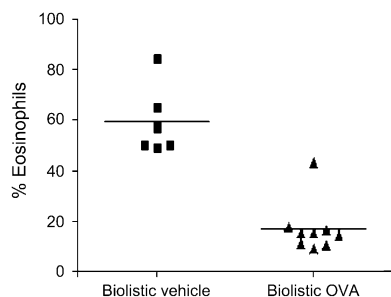


FIG 6. Effect of biolistic injection on eosinophilia. Sensitized mice given biolistic injections and boosted with OVA were challenged with 3 daily aerosol exposures on day 56. Results show eosinophilia as a percentage of eosinophils in the bronchoalveolar lavage fluid of individual mice (6 and 9 for biolistic vehicle control and OVA, respectively). Mice given biolistic injections of OVA had decreased eosinophil counts in the lavage fluid (*P* < .01, Student *t* test).

response, but importantly, the titers of this isotype tended to decrease and not increase. This demonstrates an overall downregulation and not a shift in the T_H1/T_H2 balance. This is clearly different to the gene-gun DNA vaccination of Ludwig-Portugall et al,¹³ which induced T_H1 immunity. It has been shown that a tolerizing procedure that inadvertently induced delayed hypersensitivity severely exacerbated lung inflammatory responses to allergen, and therefore downregulation instead of vaccination can be an advantage.³⁴ The biolistic injections also markedly affected the lung response induced when the sensitized and boosted mice were challenged with aerosols of OVA. The eosinophilia in lung lavage was clearly reduced. The biolistic injections did not affect the IgE responses to an unrelated antigen, papain. Therefore the response to repeated injection of antigen did not create a nonspecific inhibition. Biolistic delivery to the buccal mucosa was also efficacious. Although mucosal delivery was not investigated further, it is possible that investigations could reveal some advantage over the skin, such as longevity. The reason for not pursuing buccal delivery was that because skin desensitization was effective, the resources available were focused on the route currently used for injection therapy. Buccal delivery, however, warrants further investigation.

In contrast to the striking effect on the boosting, IgE titers measured before the boost were not inhibited, which is consistent with the results of Burger et al.¹⁴ The biolistic

injections thus produced a response similar to that of current immunotherapy, which can even increase existing IgE levels while blocking the response to further allergen exposure.^{5,15} The tissue hyperreactivity to MCh revealed a similar phenomenon. It was transiently increased in biolistically injected mice but then decreased toward normal levels and was reduced to less than that seen in animals receiving the vehicle control for elastance. The subcutaneous injection of allergen during conventional immunotherapy can similarly induce transient respiratory reactions.³⁵ The 25- μ g doses of OVA are greater than the 5- to 15- μ g doses used for human immunotherapy,³⁶ and therefore some systemic reactivity could be expected. Increased airway hyperreactivity can be mediated by both nonspecific inflammation and specific immune responses. Paradoxically, increases in the regulatory cytokine IL-10,³⁷ as well T_H1 cytokines,³⁸⁻⁴⁰ have been shown to enhance bronchial hyperreactivity while counterregulating T_H2 responses, perhaps by affecting cell trafficking.

The penetration experiments showed that the biolistically injected microparticles were lodged in the epidermis or the buccal epithelium. They were thus delivered to the proximity of the Langerhans cells, which are only found in these regions.⁹ The reported density of Langerhans cells in the abdominal epidermis is 800 cells per square millimeter.⁴¹ The 10-mm biolistic device would access 60,000 Langerhans cells. The Langerhans cell density is similar in the buccal mucosa,⁴² but the smaller 4-mm diameter of the mucosal device would mean that the same payload was delivered to the vicinity of one sixth of the number of Langerhans cells. The effect of the biolistic delivery on IgE was similar for the skin and buccal mucosa, but dose-response titrations would be required for a comparison. There are dendritic cells in both the dermis and epidermis, but the Langerhans cells are confined to the epidermis. A variety of data show that the immune responses induced by the Langerhans cells and dermal cells differ. It is likely that the precision of the predominant epidermal biolistic delivery will generate a unique balance of presentation by different antigen-presenting cell populations and distinctive immunologic sequelae.⁴³⁻⁴⁵ The results here show a downregulation of IgE and eosinophilia in allergic sensitization. It is speculated that further analysis of the delivery system might yield information of direct relevance to the development of improved strategies for desensitization.

TABLE II. Responses to MCh challenge (30 mg · mL⁻¹) as a percentage of response to saline

	Naive	Day 56		Day 77	
		Vehicle	OVA	Vehicle	OVA
R _{aw} (%)	235.5 (56.9)	393.2 (150.8)	518.0 (198.9)	428.0 (111.9)	458.1 (193.1)
G(%)	138.8 (16.1)	310.4 (104.5)	473.4 (152.4)	301.5 (70.6)	242.3 (130.7)
H(%)	145.4 (13.5)	258.8 (54.5)	379.2 (97.6)	265.4 (60.6)	190.8 (52.1).
<i>P</i> < .02					

Mean values (SD) of groups of 8 mice show that all values except H of OVA/biologic mice on day 77 were different from those of naive mice (*P* < .05, ANOVA), and this was different from values seen in animals receiving the vehicle control (*P* < .02, Student *t* test). R_{aw} and G of the OVA/biologic mice were significantly higher than those of the animals receiving the vehicle control on day 56 (*P* < .05 and .01, Student *t* test).

G, Coefficient of tissue damping; H, coefficient of tissue elastance.

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