

Block Copolymers Have Differing Adjuvant Effects on the Primary Immune Response Elicited by Genetic Immunization and on Further Induced Allergy[▽]

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Block copolymers were recently used to promote gene delivery in various tissues. Using a plasmid encoding a food allergen, bovine β -lactoglobulin (BLG), we studied the effects of block copolymers on gene expression levels and primary immune response and on further induced allergy. Block copolymers (i.e., Tetronic 304, 908, and 1107) and various quantities of DNA were injected into the tibialis muscles of BALB/c mice. The BLG levels in injected muscle and the BLG-specific induced immune response were analyzed after injection. DNA-immunized mice were further experimentally sensitized with BLG, and the effects of block copolymer and DNA doses on allergic sensitization and elicitation were compared. Tetronic 304 induced a 12-fold increase in BLG production, while Tetronic 1107 increased the duration of BLG expression. Different Th1 primary specific immune responses were observed, either strong humoral and cellular (304), only cellular (1107), or weak cellular and humoral (908) responses. After BLG sensitization, increased BLG-specific IgG2a production was observed in all groups of mice independently of the presence and nature of the block copolymer. Increased BLG-specific IgG1 production was also detected after sensitization, except with Tetronic 1107. Compared with naked DNA, Tetronic 304 was the only block polymer that decreased BLG-specific IgE concentrations. However, after allergen challenge, Tetronic 1107 was the only block copolymer to reduce eosinophils and Th2 cytokines in bronchoalveolar lavage (BAL) fluid. Tetronic 304 amplified local inflammation. Each block copolymer elicited a different immune response, although always Th1 specific, in BALB/c mice.

We previously demonstrated that DNA immunization using the β -lactoglobulin (BLG) gene, one of the major cow's milk allergens, elicited a Th1-specific immune response that inhibited Th2 cell induction and prevented further allergic sensitization (2). This approach has also been successfully applied to allergens from pollen, latex, and peanut (for a review, see reference 23). However, patients with cow's milk allergy are predominantly multisensitized, i.e., they produce IgE directed against more than one cow's milk protein (22). Moreover, many allergic patients are polyallergic, i.e., they are allergic to various allergens (food, pollen, and dust). Putative use of DNA delivery in allergy would therefore imply multigene immunization, i.e., the administration of a pool of plasmids containing the cDNAs of different allergens. This suggests that decreasing quantities of DNA should be used to determine the minimal quantity of plasmid necessary to decrease the IgE level after sensitization. In this context, adjuvants such as block copolymers, which promote the expression of various reporter genes, e.g., luciferase and β -galactosidase genes (11, 12, 16), are of interest.

Block copolymers are synthesized using propylene oxide

(PO) and ethylene oxide (EO), which are organized as "blocks" of polyoxyethylene (POE) and polyoxypropylene (POP). These copolymers can be designed and synthesized using various amounts of PO and EO and with differential arrangements of POP and POE blocks. Block copolymers are used for their adjuvant capacities (for a review, see reference 21) and were recently found to promote gene delivery in tissues, such as skeletal and cardiac muscle (11, 15–18), lung (8), and eyes (12). Copolymers can be used to increase the intensity and/or duration of the expression of reporter genes, such as those encoding green fluorescent protein (GFP) or luciferase, but also of therapeutic genes, such as those encoding erythropoietin or dystrophin. We used poloxamine block copolymers, which have a tetrafunctional structure consisting of four PEO/POP blocks centered on an ethylenediamine moiety (15). The DNA delivery efficiencies of 3 nonionic block copolymers, i.e., Tetronic 304, 908, and 1107, with molecular masses ranging from 1,650 to 25,000 Da, were studied by monitoring *in situ* protein production at different time points after intramuscular (i.m.) injection of BLG-encoding plasmid DNA. Different doses of injected DNA were used (0.02, 1, and 50 μ g). The adjuvant capacities of copolymers were evaluated using various criteria, such as primary specific antibody response and cytokine secretion after splenocyte reactivation. Lastly, we evaluated the effects of DNA immunization with the different copolymers and with the different plasmid doses on further allergic sensitization and challenge with BLG protein.

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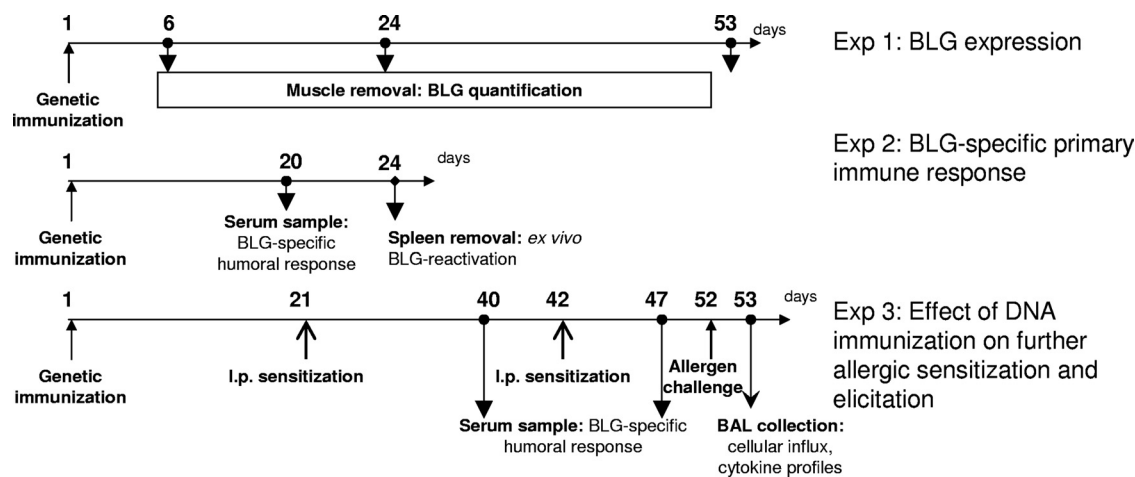


FIG. 1. Experimental protocols. Exp, experiment.

MATERIALS AND METHODS

Apparatus and reagents. All enzymatic immunoassays were performed in 96-well microtiter plates (Immunoplate Maxisorb; Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland). Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, MO). BLG was purified from cow's milk as previously described (13). The block copolymer poloxamines Tetronic 304, 1107, and 908 were provided by the BASF Corporation and prepared as 20% stock solutions in sterile water (culture tested; Gibco). The approximate molecular masses of the block copolymers were, respectively, 1,650, 15,000, and 25,000 Da. The percentages of POE in block copolymers 304, 1107, and 908 were, respectively, 40, 70, and 80%.

Mice. Female BALB/c mice from CERJ (Centre d'Élevage René Janvier, Le Genest Saint-Isle, France) were housed under normal husbandry conditions. The mice were acclimated for 2 weeks before experiments and were used when they were 6 weeks old. All experiments were performed according to European Community rules of animal care and with authorization 91-122 of the French Veterinary Services.

Genetic immunization. The plasmid pcDNA3BLG carrying the cDNA of BLG under the control of the human cytomegalovirus promoter (7) was purified from recombinant *Escherichia coli* using the Endofree Gigaprep kit (Qiagen). Complexes of block copolymers and DNA were prepared as previously described (16) by mixing equal volumes of block copolymer stock solution in water and plasmid DNA solution at the desired concentration in 2× Tyrode's solution. Injection of 50, 1, or 0.02 µg of DNA in 50 µl was performed in the tibialis muscle as previously described (2, 7) but without the use of sucrose and using mice anesthetized by intraperitoneal (i.p.) injection of 200 µl/mouse of ketamine (15 mg/ml)-xylazine (2 mg/ml) cocktail (Imalgène 500 [Merial, Lyon, France] and Rompum 2% [Bayer Pharma, Puteaux, France]). Control mice received Tyrode buffer.

BLG production in muscle after immunization with block polymer-DNA complexes (Fig. 1, experiment 1). Six, 24, and 53 days after genetic immunization, mice were humanely killed and the injected muscles were removed ($n = 5$ mice/group). The muscles were immediately put in cold phosphate-buffered saline (PBS) (Gibco) containing an antiprotease cocktail (50 µg/ml bacitracin, 300 µg/ml benzamidine, 80 µg/ml leupeptin, 20 µg/ml chymostatin, 25 µg/ml pepstatin, 200 µM phenylmethylsulfonyl fluoride). Muscle tissue suspensions were prepared in ice using an Ultrathurax grinder (Janke & Kunkel, IKA Labortechnik, Germany) and sonicator (Sonics & Materials Inc.; VibraCell VC 40). After centrifugation (13,000 × g ; 20 min; +4°C), the supernatants were collected. The protein content was determined using a bicinchoninic acid (BCA) kit from Pierce. Two-site enzyme immunometric assays (EIA) for native BLG (BLGn) were performed as previously described (13). Briefly, the assays were performed in 96-well microtiter plates coated with a monoclonal antibody (MAb) specific for BLGn. Fifty microliters of standard or 50 µl of the samples was added; then, 50 µl of tracer was added, consisting of a second MAb labeled with AChE. After an 18-h reaction at 4°C, the plates were washed and solid-phase-bound AChE activity was measured using Ellman's method (9). A detection limit of 30 µg/ml was obtained.

Primary immune response induced by DNA immunization (Fig. 1, experiment 2). (i) **Quantification of BLG-specific IgE, IgG1, and IgG2a.** Blood samples were obtained from the retro-orbital venous plexus 20 days after genetic immunization and centrifuged, 0.1% sodium azide was added as a preservative, and the sera were stored at -20°C until further assays were performed. Naïve mice were bled on the same days to assess nonspecific binding. Each immunization group was composed of 5 mice. BLG-specific IgE, IgG1, and IgG2a were measured using immunoassays, as previously described, allowing quantification of antibodies recognizing both native and denatured BLG (1).

(ii) **Cytokine production.** On day 24, mice were humanely killed and spleens were harvested under sterile conditions and pooled per immunization group. After lysis of red blood cells (180 mM NH₄Cl, 17 mM Na₂-EDTA) and several washes, the splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, and 100 mg/ml streptomycin). The cells were incubated for 60 h at 37°C (5% CO₂) in 96-well culture plates (10⁶ cells/well) in the presence of BLG (20 µg/ml) or concanavalin A (1 µg/ml; positive control). Incubations with PBS or ovalbumin (20 µg/ml) were done as negative controls. The supernatants were then removed and stored at -80°C until further assays were performed. Gamma interferon (IFN-γ) and interleukin 4 (IL-4) were assayed using CytoSets kits (BioSource International Europe, Nivelles, Belgium). IL-5 was assayed using an immunometric assay with TRFK4 monoclonal antibodies for capture and AChE-labeled TRFK5 monoclonal antibodies for development.

Effect of block copolymer-DNA complex administration on further allergic sensitization and elicitation (Fig. 1, experiment 3). (i) **Allergic sensitization.** On day 1, BALB/c mice received block copolymer-DNA complexes as described above (naked DNA or block copolymer 304, 908, or 1107 complexed with 50, 1, or 0.02 µg of DNA; $n = 7$ per group). One group of 10 mice received saline solution instead of DNA (positive control of sensitization). On days 21 and 42, all mice were sensitized by i.p. injection of 5 µg BLG adsorbed on alum (1 mg/mouse; Alhydrogel 3%; Superfos Biosector als, Denmark) (3). The injected volume was 0.2 ml per mouse. On days 40 and 47, serum was collected to assess BLG-specific IgE, IgG1, and IgG2a concentrations as described above. Naïve mice ($n = 7$) were bled the same day to assess nonspecific binding.

(ii) **Elicitation of allergic reaction.** On day 52, all mice were challenged by intranasal administration of 10 µg of BLG in 50 µl of PBS under light anesthesia (Isoflurane; Baxter) (Fig. 1, experiment 3). This protocol induces a well-characterized allergic reaction, as demonstrated previously (3). Twenty-four hours after challenge, the mice were deeply anesthetized by i.p. injection of 200 µl/mouse of a cocktail of ketamine (15 mg/ml) and xylazine (2 mg/ml) (Imalgène 500 [Merial, Lyon, France] and Rompum 2% [Bayer Pharma, Puteaux, France]). The trachea was cannulated, and bronchoalveolar lavage (BAL) samples were collected in sterile saline and kept on ice. Markers of the elicitation of a local allergic reaction were local Th1/Th2 cytokine production and cellular influx, particularly eosinophilia. The total cells in BAL fluid were counted on Malassez slides after trypan blue exclusion, and differential cell counts were performed after cytocentrifugation and staining with May-Grunwald and Giemsa stains (LaboModerne, France). Morphological characteristics were used for cell differentiation of at least 300 cells/sample and allowed the quantification of eosinophils, lymphocytes,

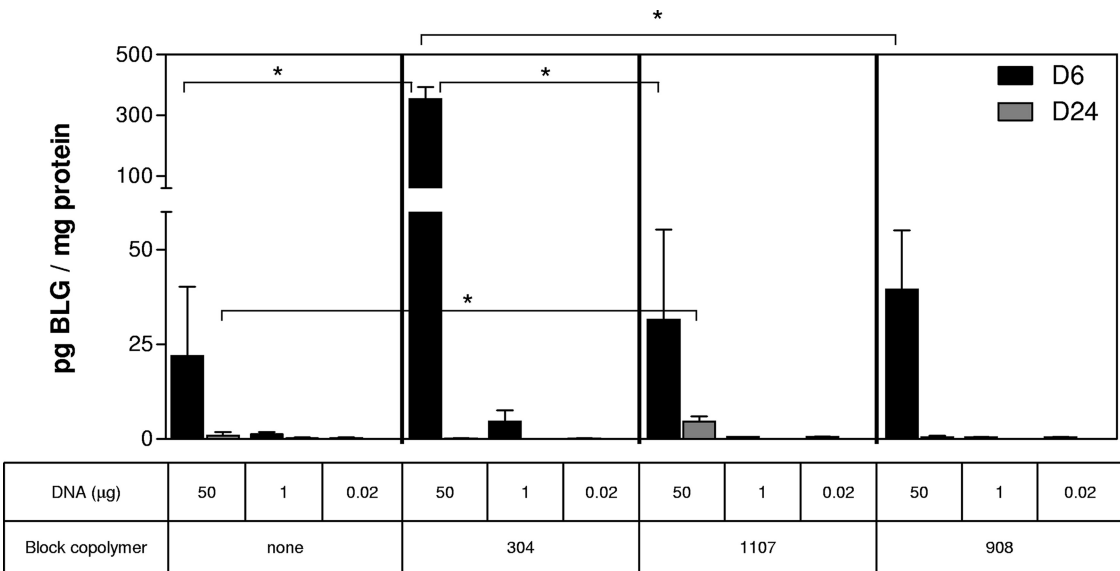


FIG. 2. BLG expression in tibialis muscle after DNA immunization. Fifty, 1, or 0.02 µg of plasmid pcDNA3BLG was injected either alone or with block copolymer 304, 908, or 1107 into the tibialis muscles of BALB/c mice. Tyrode buffer was administered to control mice. Six or 24 days later, the tibialis muscles were retrieved. Proteins were extracted, and BLG expression was assayed using specific enzyme immunoassays. Each group was composed of 5 mice. The asterisks indicate a significant statistical difference between corresponding groups ($P < 0.05$; ANOVA and Tukey's multiple-comparison test). The error bars indicate standard deviations.

neutrophils, and macrophages. Aliquots of the remaining BAL fluid were centrifuged and stored at -80°C until a cytokine assay was performed. Th1 and Th2 cytokines (i.e., IL-2, IL-4, IL-5, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor [GM-CSF], IFN- γ , and tumor necrosis factor alpha [TNF- α]) were assayed using BioPlex technology and the mouse Th1/Th2 cytokine kit from Bio-Rad, following the provider's recommendations. All BAL samples were analyzed individually for cytokine assays but pooled for differential cell counts.

Statistical analyses. All statistical calculations were done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data were analyzed using analysis of variance (ANOVA) and Tukey's multiple-comparison test or the Mann-Whitney nonparametric test when only two groups of mice were compared. A P value of less than 0.05 was considered significant.

RESULTS

Effect of DNA immunization with block copolymer on BLG expression. A plasmid encoding BLG under a eukaryotic promoter (pcDNA3BLG [2, 7]) was administered intramuscularly at different doses with or without the block copolymers Tetronic 304, 1107, and 908. Six, 24, and 53 days after injection, the injected muscle was retrieved and BLG was assayed in muscle protein extract (the protocol is shown in Fig. 1). At day 6, BLG was detectable in the muscles of all mice receiving 50 µg of naked DNA, but not at lower doses or at other time points (Fig. 2). The highest expression was obtained on day 6 in muscles from mice receiving 50 µg of plasmid with block copolymer 304 (Fig. 2). In this group of mice, BLG expression was homogeneous, as demonstrated by a low standard error of the mean (SEM). With this block copolymer, BLG expression was even detected after injection of 1 µg of DNA. BLG expression on day 6 using the other block copolymers was not significantly different from that without block copolymer. However, BLG was detected on day 24 only in mice receiving 50 µg of DNA with block copolymer 1107 (Fig. 2). No BLG was detectable on day 53, regardless of the presence or absence of

block copolymer, the type of block polymer, or the dose of plasmid (not shown).

Effect of DNA immunization with block copolymer on the primary immune response. (i) Humoral response. BLG-specific IgG1 and IgG2a were detected in sera 20 days after intramuscular injection of 50 µg of pcDNA3BLG with or without block copolymer (the protocol is shown in Fig. 1). Administration of naked DNA or of DNA with block copolymer 908 induced similar significant BLG-specific IgG1 and IgG2a production levels (Fig. 3). The highest concentrations of BLG-specific IgG1 and IgG2a were induced after injection of plasmid added to block copolymer 304 (Fig. 3). Administration of pcDNA3BLG plus block copolymer 1107 elicited barely detectable BLG-specific IgG1 and IgG2a responses. Whether or not block copolymer was present, and regardless of its nature, the IgG1/IgG2a ratios were comparable and indicated a Th1 immune response (the individual IgG1/IgG2a ratios were around 10). No specific primary immune response was obtained with other doses of plasmids in any group (data not shown).

(ii) Cellular response. Splenocytes from treated mice were reactivated *ex vivo* with BLG, and cytokines in the supernatant were assayed. Splenocytes from naked-DNA-immunized mice secreted the Th1 cytokine IFN- γ after reactivation with BLG (Fig. 4). Sevenfold-higher IFN- γ concentrations were assayed after BLG reactivation of splenocytes from mice receiving 50 µg of plasmid with block copolymers 304 and 1107. The use of block copolymer 908 gave an IFN- γ concentration comparable to that seen with naked DNA. With lower doses of plasmid, IFN- γ secretion was detectable only in mice given 1 µg of plasmid added to block copolymer 1107 (Fig. 4).

The Th2 cytokines IL-4 and IL-5 were not detected in the supernatant (data not shown).

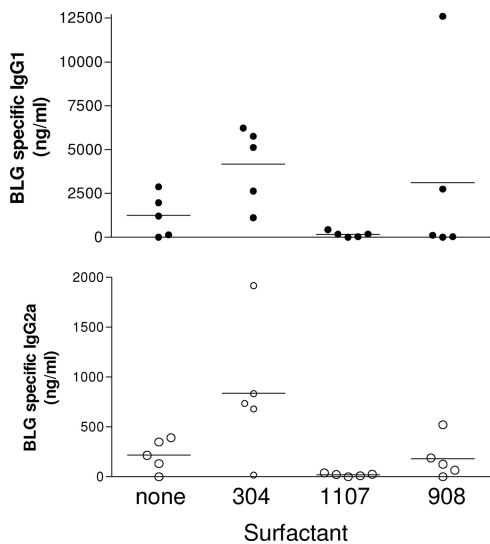


FIG. 3. BLG-specific IgG1 and IgG2a responses elicited by DNA immunization. Fifty micrograms of pcDNA3BLG was injected with or without (none) block copolymer into the tibialis muscles of mice. Twenty-one days later, serum samples were collected and BLG-specific IgG1 (top) and IgG2a (bottom) were assayed in individual sera. Mean values (horizontal lines) are given for each group ($n = 5$ mice). No specific antibody was detected in mice receiving Tyrode's solution (not shown).

Effect of DNA immunization with block copolymer on further allergic sensitization. On days 21 and 42, mice pretreated or not with pcDNA3BLG with or without block copolymers were sensitized by two i.p. injections of BLG adsorbed on alum in order to induce a high BLG-specific IgE response (3) (the protocol is shown in Fig. 1). On day 40, saline-pretreated mice (0 μ g DNA) showed a typical Th2 immune response characterized by the presence of high concentrations of BLG-specific IgG1 (Fig. 5A) and IgE (Fig. 5B), whereas almost no BLG-specific IgG2a was induced (Fig. 5C). Pretreatment with pcDNA3BLG with or without block copolymer 304 greatly

increased the concentration of specific IgG1 at the highest quantity of DNA used (Fig. 5A). At the same dose, DNA treatment dramatically increased the concentration of BLG-specific IgG2a, whatever the block copolymer. The influence of DNA administration on the IgG2a concentration disappeared totally for plasmid doses of 1 and 0.02 μ g (Fig. 5C). On day 49, i.e., after the second i.p. injection, levels of BLG-specific IgG1 and IgG2a were enhanced (data not shown). On day 40, untreated mice showed a BLG-specific IgE concentration of 2 μ g/ml. DNA administration without block copolymer had no effect on the BLG-specific IgE level (Fig. 5B) for the highest dose of plasmid. Surprisingly, the lower doses tended to increase the BLG-specific IgE concentration, although not significantly ($P = 0.08$). In contrast, the administration of 50 μ g of pcDNA3BLG added to block copolymer decreased the IgE level by 75 to 95%. Due to the heterogeneity of responses between mice, the only statistically significant decrease was observed with block copolymer 304 ($P = 0.02$ compared with the control group). On day 49, when the Th2 immune response was fully developed, the lowering of IgE concentrations was detectable in the same proportions (data not shown).

Effect of DNA immunization with block copolymer on elicitation of the allergic reaction. Ten days after the booster injection of BLG adsorbed on alum, mice were challenged intranasally with BLG, and BAL samples were collected 24 h later. The elicitation of the allergic reaction in mice pretreated with saline and then sensitized by BLG is demonstrated by eosinophil influx and local production of Th2 cytokines in BAL, as previously demonstrated (3). Genetic immunization with naked DNA or block polymer 304 elicited a very high cellular influx of both eosinophils and neutrophils (Fig. 6A). Conversely, block copolymer 1107 was the only one to reduce the influx of eosinophils. The greatest decrease was observed with 0.02 μ g of DNA. Naked DNA or block copolymer 304 increased the concentrations of both Th1 and Th2 cytokines compared with untreated mice, as demonstrated by the levels of IL-4, IL-5, IFN- γ , and TNF- α detected in BAL fluid (Fig. 6B), indicative of marked local inflammation after allergen

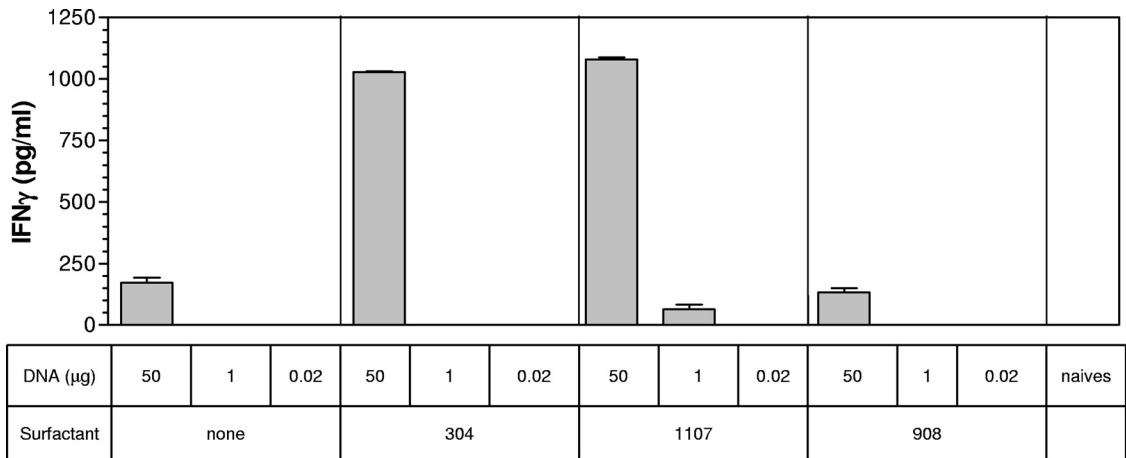


FIG. 4. Cytokine secretion by reactivated splenocytes. Fifty, 1, or 0.02 μ g of plasmid pcDNA3BLG was injected with or without (none) block copolymer into the tibialis muscles of mice. On day 24, splenocytes from treated mice were reactivated with irrelevant protein or BLG, and IFN- γ was assayed in the supernatants collected 60 h later. BLG-specific secretion was obtained after subtraction of nonspecific secretion. The error bars indicate standard deviations.

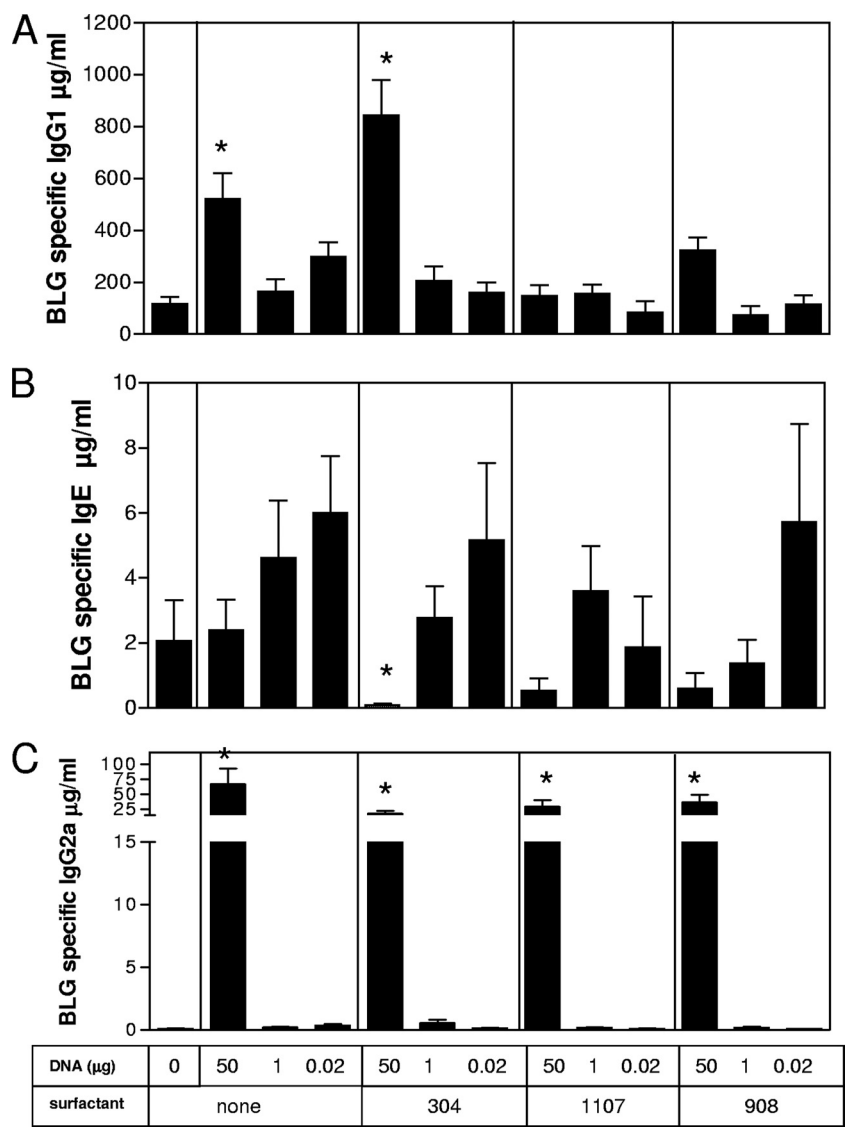


FIG. 5. BLG-specific IgG1 and IgG2a after sensitization. Fifty, 1, or 0.02 µg of plasmid pcDNA3BLG was injected with or without (none) block copolymer into the tibialis muscles of mice ($n = 7$ /group). Ten mice received saline solution instead of DNA (0 µg). The mice were then sensitized by two i.p. injections of BLG adsorbed on alum on days 21 and 42. BLG-specific IgG1 (A), IgE (B) and IgG2a (C) were assayed in sera collected on days 40 and 49 (not shown). Naive mice did not produce BLG-specific antibodies ($n = 7$) (not shown). The asterisks indicate a significant statistical difference between the corresponding DNA-pretreated group and the group pretreated with saline ($P < 0.05$; Mann-Whitney nonparametric test). The error bars indicate standard deviations.

challenge. Interestingly, and according to the eosinophil influx, administration of 0.02 µg of DNA with block copolymer 1107 decreased both Th1 and Th2 cytokines in BAL fluid compared with control mice (Fig. 6B). No inflammatory response was detected after allergen challenge.

DISCUSSION

We compared the effects of various block copolymers (i.e., Tetronic 304, 908, and 1107) and various quantities of BLG-encoding plasmid DNA (from 50 to 0.02 µg) on genetic immunization efficiency. BLG levels in injected muscle and BLG-specific induced immune responses were analyzed at different time points after injection. DNA-immunized mice were further

experimentally sensitized with BLG, and the effects of the block copolymer and DNA dose on allergic sensitization and elicitation were assessed.

Block copolymer 304 was previously used for *in vivo* delivery of the EPO gene. In mice treated with DNA complexed with block copolymer 304, the hematocrit remained high for up to 70 days after the injection (15). Similar results were obtained with luciferase activity and again with EPO activity (11). Accordingly, we demonstrated that 6 days after immunization, block copolymer 304 enhanced up to 12-fold the expression of BLG compared with naked DNA. The homogeneity of this production was higher than with other block copolymers or naked DNA, which corroborated data obtained in cows with SP1017 (14). Interestingly, BLG expression was also detectable

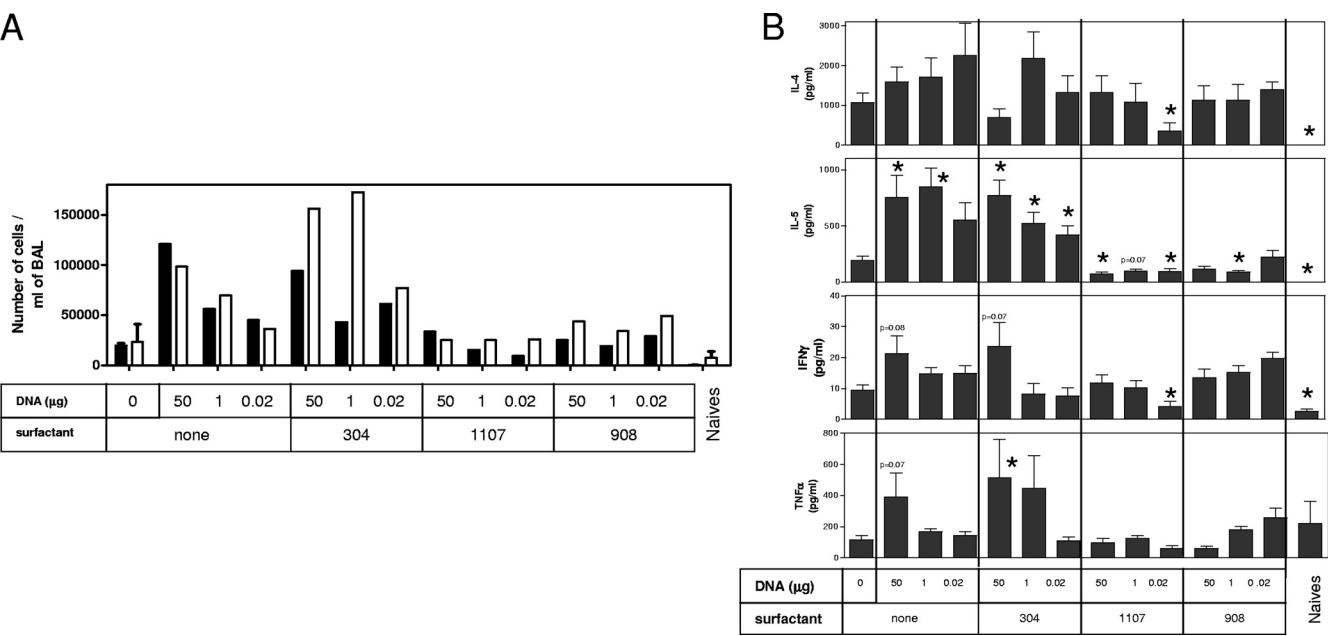


FIG. 6. Effect of block copolymer on local immune responses. Twenty-four hours after intranasal challenge, mice were anesthetized and BAL samples were collected. Eosinophil (black bars) and neutrophil (open bars) influx (A) and IL-4, IL-5, IFN- γ , and TNF- α secretion (B) were assayed in BAL fluid as described in Materials and Methods. The asterisks indicate statistical differences between the corresponding DNA-pretreated group and the group pretreated with saline ($P < 0.05$; Mann-Whitney nonparametric test). The error bars indicate standard deviations.

at the 1- μ g plasmid dose when block copolymer 304 was added. At later time points, i.e., 24 days after injection, block copolymer 1107 was the only one to induce significant expression of BLG at the 50- μ g plasmid dose. Conversely, block copolymer 908 did not significantly enhance BLG expression, whatever the conditions. In our study, the duration of expression was not linked to the highest expression at day 6 but might result from a more progressive release of plasmid DNA from copolymer and uptake by muscle cells.

Block copolymers have been used as adjuvants since 1981 (19). The adjuvant activities of block copolymers seem to be related to their structure and composition. Using 4 small non-ionic block copolymers, a correlation was found between the hydrophilic/lipophilic balance (HLB) and their adjuvant activities (19). The molecules that were strong adjuvants all had HLB values within a narrow range, which classified them as spreading agents. But the relationship between HLB and adjuvant activity is not as simple as it seems at first. Using ovalbumin as an antigen model, adjuvant activity is related to the size of the POP core block. Increasing the size of this block enhanced the adjuvant capacity of the copolymer, with a peak activity around 12 to 15 kDa (10, 21). However, adjuvant activity is also influenced by the amount of POE, with 5 to 10% being optimal (10, 21). All these values may differ slightly from one antigen to another (21). Here, we used block copolymers with a tetrafunctional structure consisting of four PEO/POP blocks centered on an ethylenediamine moiety (15). We tested their adjuvant capacities in DNA immunization using various criteria, such as primary specific antibody response, cytokine secretion after splenocyte reactivation (primary cellular response), and effects on further allergic sensitization and allergen challenge. To our knowledge, this is the first time that so

many parameters have been monitored in the same study. In rhesus macaques, the T-cell response induced by DNA immunization with the HIV *gag* gene was improved by the nonionic block copolymer CRL8623 (6). Although no differences in antibody responses were found when two different genes from herpes simplex virus type 1 (HSV-1) were administered to mice, with or without block copolymers, better protection against HSV-1 challenge was induced in mice pretreated with the nonionic block copolymers CRL 1029 and 1190 (4). The cytotoxic-T-lymphocyte (CTL) response against the HIV-1 *tat* protein was increased by the use of different block copolymers, even if no anti-*tat*-specific IgG was detectable (5). In dairy cows, the addition of block copolymer SP1017 to DNA immunization with factor A of *Staphylococcus aureus* did not enhance the antibody response but did increase the number of responsive cows (14). In our case, block copolymer 304 (mass, 1,650 Da; POE, 40%) induced strong cellular and humoral responses. As previously described (10, 21), the IgG response was not increased, or was even almost completely abolished, when the POP core block and the PEO proportion were enhanced, as demonstrated with block copolymers 908 (mass, 25,000 Da; POE, 80%) and 1107 (mass, 15,000 Da; POE, 70%). However, despite its effect on the IgG response, block copolymer 1107 elicited a strong cellular response, as demonstrated by the high IFN- γ secretion after splenocyte reactivation with BLG. The polymers used in this study elicited different types of primary immune responses: strong humoral and cellular responses with 304, only a cellular response with 1107, and weak cellular and humoral responses with 908. Our results are in agreement with those published previously (21), as the highest primary immune response was obtained with block copolymer 304, which has the lowest level of POE. Conversely,

block copolymer 1107 has a theoretically optimal size (11,000 Da) (21), but it seems that the high percentage of POE (70%) in its formula counterbalances this.

As for the effect of block copolymers on further allergic sensitization, we demonstrated that block copolymer 304 significantly and completely inhibited IgE production, whereas the other block copolymers were less efficient. This inhibition was accompanied by a strong Th1-specific immune response, at both the cellular and humoral levels, but also by a significant enhancement of the specific IgG1 level after sensitization. Although this may appear contradictory, it is worth noting that IgG1 and IgE can be independently modulated. As an example, IL-21 has been described as a multifunctional cytokine that inhibits IL-4-induced, Stat-6-dependent IgE production in B cells by the inhibition of germ line ϵ transcription. Conversely, IL-21 did not inhibit the generation of murine IgG1⁺ (mIgG1⁺) B cells by IL-4. Rather, IL-21 increased mIgG1⁺ cells in lipopolysaccharide (LPS)-stimulated B cells. Such a cytokine has been proposed to be involved in Th1-mediated downregulation of IgE production in immune responses (20). As switching to IgE occurs from IgM to IgG1 and then from IgG1 to IgE (24), it is possible that IL-21 and/or other cytokines increase IgG1-producing cells through the inhibition of switching to IgE, thus explaining the increased IgG1 and decreased IgE levels for some groups in our study.

An allergic response can be roughly separated into two steps: (i) sensitization and (ii) elicitation. If we consider the lowering of the IgE level in our murine model of sensitization, block copolymer 304 proved to be the most promising for further development of multigene DNA immunization to prevent or even cure polysensitization. However, this block copolymer elicited the highest cellular influx and secretion of Th1 and Th2 cytokines in BAL, showing that a strong local inflammatory response was induced after allergen challenge. Interestingly, this unexpected negative effect was not observed with block copolymer 1107. Block copolymer 1107 should be additionally tested, as it was shown in the present study to induce the longest BLG expression, a moderate Th1 cellular primary response, and, at low DNA doses, a decrease of cellular influx and Th2-type cytokines after allergen challenge, without inducing strong local Th1 response.

Here, we showed that the use of block copolymers as poloxamines has a direct influence on the immune response elicited by DNA immunization. We confirmed that increasing the percentage of POE diminishes the adjuvant capacity of the block copolymer. A good ratio between the size of the POP core block and the percentage of POE has to be found to optimize the antibody response. The use of block copolymers might orient the type of response, i.e., humoral, cellular, or both. However, here we show that a strong humoral response, as elicited with block copolymer 304, can be related to a strong local inflammatory response after allergen challenge. Thus, before the putative use of these block copolymers in humans, their various effects on the immune response have to be fully documented.

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