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## Antibody responses to *Brugia malayi* antigens induced by DNA vaccination

Ben-Wen Li\*, Amy Rush, Shaorong R Zhang, Kurt C Curtis and Gary J Weil

Address: Infectious Diseases Division, Campus Box 8051, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA

Email: Ben-Wen Li\* - bli@im.wustl.edu; Amy Rush - arush@im.wustl.edu; Shaorong R Zhang - shaorong@yahoo.com; Kurt C Curtis - kcurtis@im.wustl.edu; Gary J Weil - gweil@im.wustl.edu

\* Corresponding author

Published: 22 January 2004

Received: 15 October 2003

*Filaria Journal* 2004, 3:1

Accepted: 22 January 2004

This article is available from: <http://www.filariajournal.com/content/3/1/1>

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### Abstract

**Background:** DNA vaccination is a convenient means of immunizing animals with recombinant parasite antigens. DNA delivery methods are believed to affect the qualitative nature of immune responses to DNA vaccines in ways that may affect their protective activity. However, relatively few studies have directly compared immune responses to plasmids encoding the same antigens after injection by different routes. Therefore, the purpose of this study was to explore the influence of the route of administration on antibody responses to plasmids encoding antigens from the filarial nematode parasite *Brugia malayi*.

**Methods:** Four *B. malayi* genes and partial genes encoding paramyosin (BM5), heat shock protein (BMHSP-70), intermediate filament (BMIF) and a serodiagnostic antigen (BM14) were inserted in eukaryotic expression vectors (pJW4303 and pCR<sup>TM</sup>3.1). BALB/c mice were immunized with individual recombinant plasmids or with a cocktail of all four plasmids by intramuscular injection (IM) or by gene gun-intradermal inoculation (GG). Antibody responses to recombinant antigens were measured by ELISA. Mean IgG1 to IgG2a antibody ratios were used as an indicator of Th1 or Th2 bias in immune responses induced with particular antigens by IM or GG immunization. The statistical significance of group differences in antibody responses was assessed by the non-parametric Kruskal-Wallis test.

**Results:** Mice produced antibody responses to all four filarial antigens after DNA vaccination by either the IM or GG route. Antibody responses to BM5 paramyosin were strongly biased toward IgG1 with lower levels of IgG2a after GG vaccination, while IM vaccination produced dominant IgG2a antibody responses. Antibody responses were biased toward IgG1 after both IM and GG immunization with BMIF, but antibodies were biased toward IgG2a after IM and GG vaccination with BMHSP-70 and BM14. Animals injected with a mixture of four recombinant plasmid DNAs produced antibodies to all four antigens.

**Conclusions:** Our results show that monovalent and polyvalent DNA vaccination successfully induced antibody responses to a variety of filarial antigens. However, antibody responses to different antigens varied in magnitude and with respect to isotype bias. The isotype bias of antibody responses following DNA vaccination can be affected by route of administration and by intrinsic characteristics of individual antigens.

## Background

*Brugia malayi* is a mosquito-borne nematode parasite and a cause of lymphatic filariasis in humans [1]. The parasite is transmitted when third stage larvae (L3) of *B. malayi* enter the human host following a bite by an infective mosquito. Control is based on treatment of microfilaria (MF) carriers and anti-mosquito measures that decrease transmission; no vaccines are available for prevention of infection.

Prior studies have shown that a degree of protective immunity to filariasis can be induced in animals by vaccination with irradiated L3 [2,3]. The potential for use of live filariasis vaccines in humans is limited because of safety issues and limited availability of larvae. Several laboratories are working to develop effective recombinant antigen-based vaccines that would be more practical and effective than live parasite vaccines.

DNA vaccination is a promising approach that may have advantages over vaccination with live parasites or protein antigens. DNA vaccines have been shown to be an effective means of generating cellular and humoral immune responses, and they have conferred protection against a wide range of infectious agents including viruses, parasites, and bacteria in animal models (reviewed in [4]).

We have previously reported that vaccination with recombinant *B. malayi* paramyosin (BM5) protein induced partial immunity to challenge infections in jirds [5]. More recently, we reported that mice injected IM with plasmid DNA encoding BM5 developed antigen-specific humoral and cellular immune responses [6]. However, this vaccination failed to protect jirds from challenge infections. These results raised the issue of how to optimize DNA vaccination to induce protective immunity.

Considerable effort has been expended toward improving the efficacy of DNA vaccines through vector design, optimization of immunization schedules, and by combining DNA vaccination with other vaccine types (reviewed in [7]). The most widely used DNA vaccination methods are intramuscular injection of plasmid DNA in an aqueous solution (IM) and ballistic intradermal injection of plasmid DNA-coated gold particles by gene gun (GG). Intramuscular injection and gene gun inoculation differ in the efficiency of DNA delivery [8,9]. Following IM injection, cells take up DNA from the extracellular space by poorly understood mechanisms [10,11], while the more efficient GG immunization directly transfects cells by depositing DNA-coated gold beads into the cytoplasm of antigen presenting cells [12,13]. Some reports have claimed that the route of DNA delivery has a major impact on the type of immune responses induced by DNA vaccines, with GG inducing Th-2 biased immune responses dominated by

IgG1 subclass antibodies and IM inducing Th-1 biased responses dominated by IgG2a antibodies (9, 14, 15). However, most studies of the effects of route of administration for DNA vaccination have been performed with viral antigens, and none have employed nematode antigens. Therefore, the present study was designed to examine how the route of DNA delivery affects antibody responses to filarial nematode antigens in mice. We also examined the issue of whether antibody responses to individual filarial antigens are affected when mice are injected with a cocktail of plasmids encoding several antigens (polyvalent DNA vaccination).

## Methods

### Selection of Recombinant Antigens and Preparation of Plasmid DNA for Immunization

Genes and partial genes for *B. malayi* paramyosin (BM5) [16], intermediate filament (BMIF) [17], a heat-shock protein (HSP-70) (GenBank number:AY383564), and an antigen used for immune diagnosis (BM14) [18] were chosen for immunization. Two eukaryotic expression vectors, pJW4303 and pCR<sup>TM</sup>3.1, were used for DNA vaccination. We have previously shown that both vectors were capable of inducing immune responses to BM5 in mice [6]. The same study showed that mice injected with these vectors with no inserted gene sequences did not produce antibodies to *B. malayi* antigens. For this reason, control vaccinations with vector alone were not repeated in the current study.

BM5, BMIF and HSP-70 were inserted into pJW4303 with methods previously described in detail [6]. Briefly, cDNAs were recovered from pBluescript by EcoRI digestion and directly ligated into the EcoRI site of pJW4303, downstream from a tissue plasminogen signal peptide sequence and upstream of a bovine growth hormone transcription termination sequence. The cDNA of BM14 was produced by PCR amplification with specific primers and directly ligated into pCR<sup>TM</sup>3.1 creating BM14/pCR3 recombinant plasmid by methods previously described in detail [6]. The orientation and reading frame of all recombinant plasmids were confirmed by DNA sequencing.

Plasmid DNA for injection was prepared using the Qiagen Plasmid Maxikit (Hilden, Germany) according to the manufacturer's instructions. The quantity and purity of isolated plasmid DNA was assessed spectrophotometrically, and the ratio of OD 260 to OD 280 of DNA preparations used for immunization was > 1.8. Purified plasmid DNA was dissolved in 10 mM Tris EDTA, pH 8.0, and diluted in PBS to obtain a final concentration of 1 mg/ml for injection.

### Intramuscular and intradermal inoculation of DNA

For IM injection, a 1 cc insulin syringe with a 28 1/2-gauge needle was used to inject mice with 50  $\mu$ l of a 1 mg/ml solution of plasmid DNA in PBS into quadriceps muscles in both rear legs. For GG inoculation, DNA was precipitated onto gold beads (1.0  $\mu$ m) with a DNA loading ratio of 10  $\mu$ g/mg gold according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). A helium pulse gun (Bio-Rad) was used with discharge pressure of 250 psi to deliver 5  $\mu$ g of plasmid DNA in a single shot into freshly shaven abdominal skin for single antigens. For some experiments, all four plasmids were combined, and four nonoverlapping shots were administered with the gene gun to deliver a total of 5  $\mu$ g of each plasmid in the cocktail.

### Vaccination Protocol

Four-week-old female BALB/c mice were vaccinated with 100  $\mu$ g plasmid DNA in PBS by IM injection or 5  $\mu$ g plasmid DNA by GG at 0 and 4 wk. Sera were collected at 2, 6, and 8 wk after the first immunization.

### Antibody Assays

The proteins encoded by cDNAs used for DNA vaccination have been previously expressed in various expression vectors and purified [5,16-18]. Antibody responses to purified recombinant antigens (BM5/MBP, BMIF/GST, BMHSP/His, BM14/His) were assessed in mice by ELISA. Microtiter plates (Dynatech Technologies, Chantilly, VA) coated with purified recombinant filarial antigens (2  $\mu$ g/ml in carbonate buffer, pH 9.6) were incubated with mouse sera diluted 1/100 in PBS/T/FCS in duplicate wells for 2 hr at 37°C. Bound antibody was detected after incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a antibodies (Southern Biotechnology Associates, Birmingham, AL) for 1 hr at 37°C. Optical density at 490 nm (OD) was read versus a PBS blank at 490 nm with a Biotek 312e Micro plate ELISA reader. Sera that produced net OD values greater than the mean OD plus 3 SD obtained with a panel of normal mouse sera were considered to have significant antibody responses.

IgG subclass ELISAs were normalized as previously described (18). Briefly, microtiter plates were coated with 10  $\mu$ g/ml of either purified IgG, IgG1 and IgG2a mouse proteins (Southern Biotechnology Associates) overnight at 4°C, and then incubated with serial dilution of goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies to total IgG, IgG1 or IgG2a. The dilutions of each anti-mouse IgG subclass antibody conjugates that gave identical OD values in the ELISA were chosen for use in assays of sera from vaccinated mice. Since IgG2a and IgG1 have been used as indicators of the induction of Th1 and Th2 responses, respectively, the IgG1/IgG2a ratio can help to define the T-cell phenotype induced by vaccina-

tion [19]. Thus, IgG1/IgG2a ratios were used as indicators of Th1 or Th2 biased responses induced by plasmid DNA after immunization by different routes (GG or IM).

### Statistical analysis

The statistical significance of group differences in antibody responses was determined by the non-parametric Kruskal-Wallis test. Differences were considered to be statistically significant when  $P < 0.05$ .

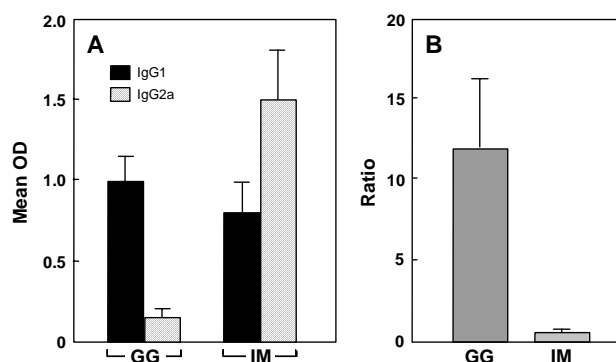
## Results

### Effect of Route of Immunization on Immune Responses in Mice

Mice produced IgG antibodies to all four of the recombinant antigens studied after DNA immunization by either route. Intramuscular immunizations induced stronger antibody responses in most mice, but GG immunization induced more consistent antibody responses. Results obtained with each antigen are presented below.

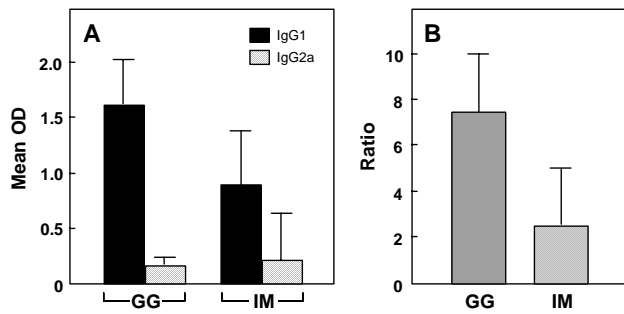
### BM5 DNA Vaccination

Gene Gun DNA vaccination with BM5 yielded a mean IgG1 to IgG2a ratio of  $> 10$ , signifying relatively more IgG1 than IgG2a (Figure 1B). In contrast, IgG1 to IgG2a ratios were  $< 1$  in IM vaccinated mice, signifying more IgG2a than IgG1. Overall, GG immunization with BM5 plasmid DNA produced a greater degree of isotype skewing than IM vaccination (Figure 1A)



**Figure 1**

This figure shows serum IgG isotype antibody responses in mice vaccinated with BM5 by intramuscular (IM)  $N = 7$  and Gene Gun (GG),  $N = 8$  immunization. Antigen-specific ELISAs were performed with sera collected 8 wk after the first immunization. **A:** IgG1 and IgG2a results shown are mean OD + SE for IM and GG mice; **B:** IgG1 to IgG2a ratios are shown for mice immunized with BM5 by IM and GG routes. Values shown are mean PD +SE. The ratios in the two groups were significantly different ( $p < 0.05$ ).



**Figure 2**  
This figure shows serum IgG isotype antibody responses in mice vaccinated with BMIF by IM (N = 12) and GG (N = 9). Antigen-specific ELISAs were performed with sera collected 8 wk after immunization. **A:** IgG1 and IgG2a antibodies shown are mean OD + SE; **B:** IgG1 to IgG2a ratios are shown for mice immunized with BMIF by IM and GG routes. Values shown are means + SE. The ratios were not significantly different.

**BMIF DNA Vaccination**

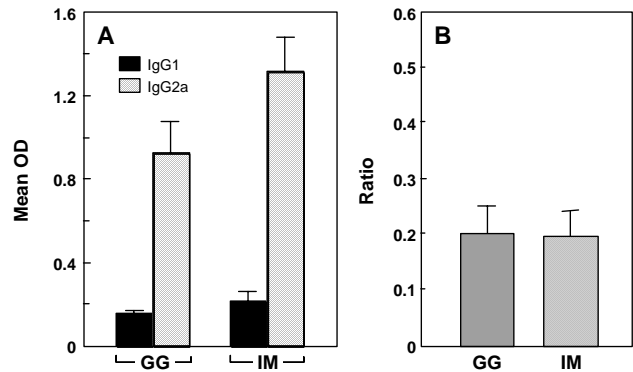
Mice immunized with BMIF antigen by either IM or GG produced very similar levels of total IgG and IgG2a antibodies to BMIF. Higher IgG1 antibody responses to BMIF were observed in mice immunized by GG (Figure 2A), but the ratios of IgG1 to IgG2a in GG and IM vaccinated mice were not significantly different. The ratios of IgG1 to IgG2a from IM and GG immunizations were > 1 (GG = 7.5, IM = 2.5) (Figure 2B), signifying more IgG1 than IgG2 in both groups. As observed after BM5 immunization, GG immunization with BMIF DNA produced more pronounced isotype skewing than IM vaccination (Figure 2A).

**BMHP DNA Vaccination**

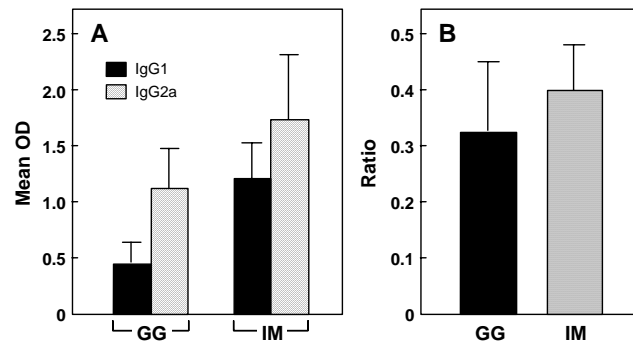
Gene gun and IM DNA immunization with BMHSP induced very similar IgG antibody response to BMHSP with strong IgG2a responses and very weak IgG1 responses (Figure 3A). The ratios of IgG1 to IgG2a antibodies were very low (< 0.3) after both GG and IM immunization with DNA encoding this antigen (Figure 3B).

**BM14 DNA Vaccination**

Intramuscular and GG DNA immunization with BM14 antigen produced antibody isotype responses similar to those observed after BMHSP vaccination, with stronger IgG2a than IgG1 (IgG1/IgG2a < 0.5) (Figure 4B). However, IM vaccination induced stronger antibody responses, with more total IgG and IgG1 antibodies to BM14 antigen, than those produced by GG immunization (Figure 4A).



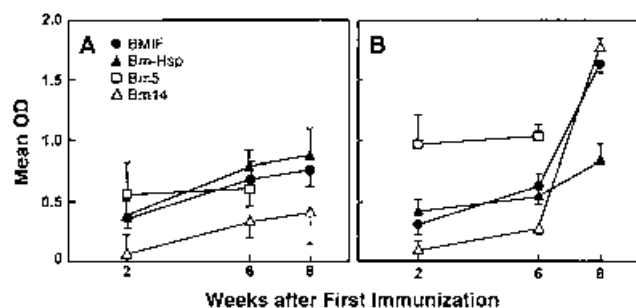
**Figure 3**  
This figure shows serum IgG isotype antibody responses in mice vaccinated with BMHSP by IM (N = 12) and GG (N = 9) route. Antigen-specific ELISAs were performed with sera collected 8 wk after immunization. **A:** IgG1 and IgG2a results shown are mean OD + SE; **B:** IgG1 to IgG2a ratios are shown for mice immunized with BMHSP by IM and GG route. Values shown are means + SE. The ratios were not significantly different.



**Figure 4**  
This figure shows serum IgG isotype antibody responses in mice vaccinated with BM14 by IM (N = 12) and GG (N = 9). Antigen-specific ELISAs were performed with sera collected 8 wk after immunization. **A:** IgG, IgG1 and IgG2a antibody results shown are mean OD + SE; **B:** IgG1 to IgG2a antibody ratios are shown for mice immunized with BMHSP by IM and GG routes. Values shown are means + SE. The ratios were not significantly different.

**Antibody Responses to Plasmid Mixtures**

The effect of mixing plasmids on DNA vaccination was determined by examining antibody responses to antigens encoded by plasmids combined in a four antigen DNA cocktail vaccine. Most mice vaccinated with the plasmid mixture produced IgG antibodies to each of the antigens



**Figure 5**

Time course of total IgG antibodies to individual recombinant *B. malayi* antigens after polyvalent and monovalent DNA vaccination by IM injection. ELISAs were performed with sera collected at different times after immunization. Results shown are mean OD + SE. **A:** IgG antibody responses to individual antigens after polyvalent DNA vaccination (5 mice). **B:** IgG antibody responses to individual antigens after monovalent DNA vaccination (7 mice for BM5, 12 mice for each of the other antigens).

encoded by plasmids in the cocktail. Polyvalent vaccination did not significantly alter the isotype bias of antibody responses to individual antigens (data not shown). Specific antibodies were detectable 2 wk after the first IM injection in most cases, and antibody levels increased after booster injections of plasmid DNA (Figure 5). Responses to polyvalent vaccination by GG were similar, but these took longer to develop (data not shown).

IgG antibody responses induced by the combination vaccine were compared to those obtained after injection with single plasmid components of the combination vaccine. However, polyvalent vaccination did not alter the isotype bias of antibody responses to individual antigens (data not shown). Antibody responses to three of the antigens (BM5, BMIF and BM14) were weaker following cocktail vaccination than after vaccination with single plasmids. However, antibody responses to BMHSP were the same after monovalent and polyvalent vaccination. Antibody responses to BM14 were lower than those to other antigens in the cocktail.

## Discussion

### Effect of Route of DNA Delivery on Antibody Responses to *B. malayi* Antigen(s) in Mice

One of the objectives of this work was to compare antibody responses induced by IM or GG immunization with plasmids encoding several *B. malayi* antigens. Several factors have been reported to influence the strength and nature of immune responses in mice after DNA vaccination [20,21]. Prior reports have emphasized the

effects of routes and methods of DNA delivery as factors that affect antibody responses because of differences in efficiency of gene transfer and types of cells transfected [9,15,22,23]. Early studies showed that IM injection results in low-level transfection of myocytes [24], whereas intradermal injection is believed to directly transfect antigen-presenting cells [25,26].

Our results showed that mice produced antibody responses to target filarial antigens after DNA vaccination by either IM or GG intradermal injection. Intramuscular injection tended to induce stronger antibody responses. However, the plasmid DNA dose was 20 times higher in i.m. immunized mice. Therefore, GG induced higher antibody responses per unit plasmid DNA. Similar results have been reported in mice immunized with other antigens [15,25].

Apart from antibody levels, several studies have reported that the route of DNA delivery can affect the protective efficacy of DNA vaccines, which does not always correlate with antibody titers. For example, DNA encoding a *Plasmodium yoelii* antigen (HEP17) protected mice equally well when given by the GG or IM routes, while DNA encoding two other antigens (CSP and SSP2) induced better protection by the IM route than by the GG route [27]. On the other hand, Leitner et al., reported that DNA encoding CSP was protective in a different malaria model only after GG vaccination [28]. Thus, neither route was conclusively favored in all cases; different injection methods may provide optimal immunogenicity for different antigens. Therefore, our results and the work of others suggest that it is not possible to reliably predict the optimal route for a given antigen to achieve desired immune responses. There is currently no substitute for empirical testing of each antigen.

Some prior studies have shown that GG vaccination tends to induce helper T-cell responses of the Th2 phenotype with a bias toward IgG1 antibody responses, while IM injection of DNA tends to produce a Th1-phenotype with a bias toward IgG2a [9,29]. In the present study, this pattern was observed for only one of four antigens tested (BM5). Other recombinant plasmids produced antibody responses biased toward IgG1 or IgG2a independent of route. These results suggest that the isotype profile of antibodies generated by DNA immunization depends not only on the route of DNA delivery but also on the intrinsic nature of antigens used for immunization.

### Antibody Responses to a Polyvalent DNA Vaccine

A second goal of our study was to compare antibody responses induced by monovalent and polyvalent DNA vaccines. One of the potential advantages of DNA vaccination is the ability to develop combination vaccines

consisting of multiple plasmids expressing different antigens. Polyvalent vaccines may be needed to achieve high levels of protection against complex pathogens like helminth parasites. It is important, therefore, to determine whether co-injection of multiple plasmids successfully induces antibody to each component antigen encoded by the plasmid mixture. We confirmed this to be the case in mice injected with a cocktail of plasmids encoding four *B. malayi* antigens. Total IgG antibody responses to three of these antigens (BMIF, BM14 and BMHP) tended to be weaker than those in mice injected with single plasmids.

In conclusion, we have studied murine antibody responses to four *B. malayi* antigens following DNA vaccination by the IM and GG routes. Our results show that antibody responses induced by DNA vaccines can be affected by the route of injection and also by the intrinsic nature of the antigens encoded. We also showed that a tetravalent DNA vaccine induced antibodies to all four *B. malayi* antigens encoded by the plasmids in the cocktail. Although these findings represent progress, we realize that much more work is needed before practical DNA vaccines for filariasis become a reality. Our next priority will be to determine whether the antigens employed in this study (alone or in combination) have protective activity against *B. malayi* in animals. In addition, more work is needed to optimize vaccination protocols, and many other candidate antigens need to be tested. We believe these results will be of interest to those working to develop DNA vaccines against complex pathogens such as filarial nematodes.

### Conclusions

The type of antibody responses induced by DNA vaccines can be affected by the route of injection and also by the intrinsic nature of antigens employed. Polyvalent DNA vaccines can induce antibodies to each component antigen.

### List of abbreviations

IM intramuscular injection

GG Gene gun-intradermal inoculation

MF Microfilaria

BM5 *Brugia malayi* paramyosin

BMHSP-70 *Brugia malayi* 70 kDa heat shock protein

BMIF *Brugia malayi* intermediate filament

BM14 *Brugia malayi* serodiagnostic antigen

### Competing interests

None declared.

### Authors' contributions

Benwen Li had primary responsibility for the study (planning, data analysis, and writing); Amy Rush cloned the recombinant sequences into vaccine plasmids and performed ELISA assays; Shaorong Zhang performed immunizations and some of the data analysis; Kurt Curtis performed immunizations and prepared recombinant antigens for ELISA assays; Gary Weil helped to plan the study, analyze results, and write the manuscript.

### Acknowledgements

We thank Dr J. Mullins, University of Washington, Seattle, WA, for his generous gift of plasmid pJW4303.

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