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DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations

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ABSTRACT **Plasmid DNAs expressing influenza virus hemagglutinin glycoproteins have been tested for their ability to raise protective immunity against lethal influenza challenges of the same subtype. In trials using two inoculations of from 50 to 300 μg of purified DNA in saline, 67–95% of test mice and 25–63% of test chickens have been protected against a lethal influenza challenge. Parenteral routes of inoculation that achieved good protection included intramuscular and intravenous injections. Successful mucosal routes of vaccination included DNA drops administered to the nares or trachea. By far the most efficient DNA immunizations were achieved by using a gene gun to deliver DNA-coated gold beads to the epidermis. In mice, 95% protection was achieved by two immunizations with beads loaded with as little as 0.4 μg of DNA. The breadth of routes supporting successful DNA immunizations, coupled with the very small amounts of DNA required for gene-gun immunizations, highlight the potential of this remarkably simple technique for the development of subunit vaccines.**

Gene vaccines, or the use of antigen-encoding DNAs to vaccinate, represent a new approach to the development of subunit vaccines (1–4, 29). A subunit vaccine presents only selected components of a virus to the immune system. Prior methods of subunit vaccination have used purified proteins or viral vectors. Each of these methods has substantial limitations that would be overcome if the immunizing protein (and only the immunizing protein) could be expressed in host cells. Gene vaccines offer this opportunity, with immunization being accomplished by host cells taking up and expressing an inoculated DNA.

In this paper we evaluate how the route of DNA inoculation affects the ability to raise protective immunity. In undertaking the study, we assumed that the efficacy of different routes of DNA immunization would reflect both the efficiency of *in vivo* transfection (DNA uptake and expression) and the efficiency with which transfected cells presented proteins to the immune system. Studies in rodents on the transfection efficiency of injected DNA have demonstrated that muscle is 100–1000 times more permissive than other tissues for the uptake and expression of DNA (5–8). Tissues also differ in the efficiency with which they present antigens to the immune system. Tissues, such as the skin and the mucosal linings of the respiratory tract and the gut, that serve as barriers against the entry of pathogens have associated lymphoid tissues that provide high levels of local immune surveillance (9–15). Such tissues also contain cells that are specialized for major histocompatibility class II-restricted presentation of antigens to T-helper cells. T-helper cells produce the lymphokines that induce growth and differentiation of lymphoid cells. In view of the above, DNA inoculations were undertaken (*i*) by a route that supports unusually efficient transfection (muscle), (*ii*) by routes that support less efficient transfection but represent routes frequently used for the administration of an antigen to a test animal (subcutaneous, intraperitoneal), and (*iii*) by routes that support less efficient transfection but deliver DNA to tissues with high levels of local immune surveillance (skin and respiratory passages).

The effect of the route of inoculation on DNA vaccination was evaluated in murine and avian influenza virus models. In both models, the vaccine consisted of purified plasmid DNA that had been designed to express an influenza virus hemagglutinin glycoprotein. This glycoprotein mediates adsorption and penetration of virus and represents a major target for neutralizing antibody (16, 17). A number of antigenically distinct subtypes of hemagglutinin glycoproteins are found in naturally occurring influenza virus infections (18). In the murine model, plasmid DNA expressing the hemagglutinin subtype 1 (H1) protein was used to protect against a lethal challenge with a mouse-adapted influenza virus with an identical H1 gene. In the chicken model, DNA expressing the hemagglutinin subtype 7 (H7) protein was used to vaccinate against a lethal H7 virus with an antigenically distinct H7 glycoprotein (19).

MATERIALS AND METHODS

Vaccine DNAs. Plasmids pCMV/H1 and pCMV/H7 were constructed by substituting cDNAs for H1 (20) or H7 (21) for interleukin 2 (IL-2) sequences in the pBC12/CMV/IL-2 expression vector (22). Substitutions were accomplished by blunt-end ligations into the ≈4.0-kb *Hin*dIII–*Bam*HI fragment of pBC12/CMV/IL-2. Constructs in the correct orientation to express the H1 or the H7 gene under the control of the cytomegalovirus (CMV) immediate early promoter were identified by restriction endonuclease digestions. Expression of H1 or H7 was confirmed by indirect immunofluorescent staining of transiently transfected COS cells. The pCMV/control plasmid was generated by deleting a 0.7-kb DNA fragment containing the gene for IL-2 from pBC12/CMV/IL-2. p188 DNA represents a previously constructed DNA that uses retroviral transcriptional control elements to express H7 (4). pRCAS, a replication-competent retroviral vector from which the replication-defective p188 was derived, served as a control DNA (23). DNAs were grown in *Escherichia coli* DH5 bacteria and purified on cesium chloride density gradients by standard protocols. DNA concentration was determined by optical density at 260 nm and confirmed by comparing intensities of ethidium bromide-stained restriction endonuclease fragments with standards of

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Abbreviations: CMV, cytomegalovirus; IL-2, interleukin 2; H1 and H7, hemagglutinin subtypes 1 and 7.

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known concentration. DNA was stored in 1 mM Tris, pH 7.8/0.1 mM EDTA. For injections, DNA was diluted in saline (0.9% NaCl).

Vaccine Trials. Vaccine trials in mice were accomplished by the administration of DNA to 6- to 8-week-old BALB/c mice. Two DNA inoculations were given, one at time 0 and the second 4 weeks later. Lethal challenge was administered at 10 days after the second DNA inoculation by inhalation of virus into the lungs of Metofane (Pitman–Moore, Mundelein, IL)-anesthetized mice. The challenge consisted of 250 plaque-forming units [10–100 times the median lethal dose (LD_{50})] of mouse-adapted A/PR/8/34 (H1N1) influenza virus in 100 μl of saline supplemented with 0.1% bovine serum albumin. The challenge virus underwent localized replication in the respiratory tract causing death due to pneumonia within 1–2 weeks. Routes of DNA inoculation included the following: intravenous (tail vein), intraperitoneal; intramuscular (both quadriceps), intranasal (DNA drops administered to the nares of mice anesthetized with Metofane), intradermal (foot pad), and subcutaneous (scruff of the neck). In general, 100 μg of DNA was administered in 100 μl of saline per test site. For foot-pad inoculations, 50 μg of DNA was administered in 25 μl.

Vaccine trials in chickens were conducted in a U.S. Department of Agriculture-approved P3 facility. Three-week-old specific pathogen-free chickens (SPAFAS, Norwich, CT) received two DNA inoculations, one at time 0 and the second 4 weeks later. Lethal challenges were administered via the nares at 1 or 2 weeks after the second DNA inoculation. This challenge consisted of 10^4 egg infectious doses (100 LD_{50}) of A/Chicken/Victoria/1/85 (H7N7) influenza virus. The challenge infection spread rapidly throughout the internal organs and brain of chickens, causing death within 4–7 days. Routes of inoculation included the following: intravenous (wing vein), intramuscular (breast muscle); intratracheal (DNA drops administered to the trachea), subcutaneous (nape), intrabursal (injections just above the chicken's vent), and intraorbital (DNA drops administered to the eye). In general, 100 or 200 μg of DNA was administered in 200 μl of saline.

In both murine and avian trials, sera were collected immediately prior to each DNA inoculation, immediately prior to challenge, and at two times after challenge. Test animals were observed throughout the trials, and mice were weighed regularly beginning at the time of challenge.

Gene Gun-Delivered DNA. Plasmid DNA was affixed to gold particles by adding 10 mg of 0.95-μm gold powder (Degussa, South Plainfield, NJ) and an appropriate amount of plasmid DNA to a 1.5-ml centrifuge tube containing 50 μl of 0.1 M spermidine. Plasmid DNA and gold were coprecipitated by the addition of 50 μl of 2.5 M $CaCl_2$ during vortex mixing, after which the precipitate was allowed to settle and was washed with absolute ethanol and resuspended in 2.0 ml of ethanol. The gold/DNA suspension was transferred to a capped vial and immersed in a sonicating water bath for 2–5 sec to resolve clumps. Then 163 μl of the gold/DNA suspension was layered onto 1.8 cm × 1.8 cm Mylar sheets and allowed to settle for several minutes, after which the meniscus was broken and excess ethanol was removed by aspiration. Gold/DNA-coated mylar sheets were dried and stored under vacuum. The total amount of DNA per sheet was a function of the DNA/gold ratio and ranged from 0.2 to 0.0002 μg per sheet. Animals were anesthetized with 30 μl of Ketaset/Rompun (10:2). Abdominal target areas were shaved and treated with Nair (Carter–Wallace, New York) for 2 min to remove residual stubble and stratum corneum. Target areas were thoroughly rinsed with water prior to gene delivery. DNA-coated gold particles were delivered into abdominal skin with the Accell instrument (Agracetus, Middleton, WI), which employs an electric spark discharge as the motive force (24). Each animal received two nonoverlapping deliveries per immunization, at a discharge voltage of 17 kV.

Serology. Anesthetized mice were bled from the eye vein into 40-μl nonheparinized microhematocrit tubes. Sera for members within a group were pooled. Hemagglutination inhibition assays were performed with chicken red blood cells and mouse serum that had been pretreated with kaolin to remove background activity (25). Hemagglutination inhibition titers are the reciprocal of the highest serum dilution giving complete inhibition of hemagglutination. The isotypes of mouse antibodies were determined by enzyme-linked immunosorbent assays (ELISAs) using standard protocols and microwell plates coated with purified A/PR/8/34 (H1N1) influenza virus. These assays used 1:1000 dilutions of isotype-specific peroxidase-conjugated antibodies that had been provided at titers with similar activities (Sigma Immuno-Chemicals).

RESULTS

Vaccine DNAs. Vaccine DNAs were constructed by creating plasmids that would express H1 or H7 glycoproteins in transfected eukaryotic cells. Two constructs, pCMV/H1 and pCMV/H7, placed cDNAs for H1 or H7 under transcriptional control elements found in the CMV immediate early promoter and the rat preproinsulin gene (Fig. 1) (22). A third construct, p188, used retroviral transcriptional control elements to express H7 (4). Control DNAs for these constructs consisted of plasmid vectors with transcriptional control elements but without inserted cDNA sequences.

Inoculations of DNA in Saline. Intramuscular, intravenous, intranasal, intradermal, subcutaneous, and intraperitoneal routes of DNA administration were tested for their ability to raise protective immunity in mice. With the exception of the intraperitoneal injections, each of these routes of inoculation raised at least some protection (Table 1). The level of protection varied, with from 67% to 95% of test groups surviving. All of the survivors developed transient signs of influenza. Excellent survival occurred in groups receiving intramuscular inoculations, intravenous inoculations, or inoculations by each of three routes (intramuscular, intravenous, and intraperitoneal). The relatively mild influenza that developed in these groups was associated with ruffling of fur and transient weight loss. Good survival, but more severe influenza, occurred in mice receiving DNA intranasally. Yet poorer survival (67–75%) and more severe signs of influenza occurred in mice receiving intradermal and subcutaneous inoculations. These groups exhibited only marginal protection by the DNA inoculations. None of the mice receiving only intraperitoneal injections survived the lethal challenge. Control groups (inoculated with pCMV/control DNA or no DNA) developed severe signs of influenza with very few mice (13%) surviving the challenge. Thus, the intramuscular, intravenous, and intranasal routes of administration each provided good protection.

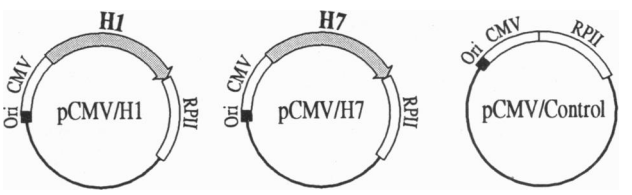

FIG. 1. Schematic of pCMV/H1, pCMV/H7, and pCMV/control DNAs used for immunizations. Ori, simian virus 40 origin of replication; CMV, CMV immediate early promoter; H1, hemagglutinin type 1 cDNA from A/PR/8/34 (H1N1) influenza virus (20); RPII, rat preproinsulin II sequences including an intron and a polyadenylylation site; H7, hemagglutinin type 7 cDNA from A/Seal/Mass/1/80 (21).

Table 1. Protection of mice against a lethal A/PR/8/34 (H1N1) influenza virus challenge by inoculation of pCMV/H1 DNA in saline

DNA	Route of inoculation*	Dose, μg	Signs of influenza	No. of survivors/ no. tested	% survival	Probability
pCMV/H1	i.v., i.p., i.m.	300	++	21/22	95	<0.0001
	i.m.	200	++	18/19	95	<0.0001
	i.v.	100	++	10/12	83	<0.0001
	i.n.	100	+++	13/17	76	<0.0001
	i.d.	50	++++	9/12	75	<0.001
	s.c.	100	++++	4/6	67	<0.02
	i.p.	100	+++++	0/6	0	
pCMV/control	Various	0–300	+++++	3/24	13	

See *Materials and Methods* for details on vaccination trials. Signs of influenza included weight loss, ruffled fur, and lethargy. These were scored as follows: +, transient weight loss but maintenance of smooth fur and normal levels of activity; ++, transient weight loss, some ruffling of fur and lethargy; +++, transient weight loss and more severe ruffling of fur and lethargy; ++++, more prolonged weight loss coupled with severe ruffling of fur and lethargy; +++++, weight loss and severe signs of influenza leading to death. Data are pooled from four independent trials. No data for the reported conditions have been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.
*i.v., Intravenous; i.p., intraperitoneal; i.m., intramuscular; i.n., intranasal; i.d., intradermal; s.c., subcutaneous.

The effect of the route of DNA inoculation on vaccination was further evaluated in the chicken influenza virus model. Again, good efficacy was demonstrated for intramuscular, intravenous, and mucosal administration of the vaccine DNA (Table 2). In this highly virulent model in which vaccine and challenge H7 glycoproteins were not identical [representing genes that had undergone a 15% drift in amino acid sequence (21, 25)], groups that received DNA by multiple routes showed the best survival (50–60%). About half this level of protection (24–30%) was achieved in groups receiving DNA by only the intravenous route, only the intramuscular route, or only the intratracheal route of inoculation. Much poorer (if any) protection was achieved by subcutaneous, intraperitoneal, intrabursal, and intraorbital inoculations. Chickens receiving control DNA developed lethal influenza, with very few chickens (≈2%) surviving the challenge. Within experimental groups, surviving chickens showed more variability in the severity of influenza-related illness than surviving mice. This may have reflected the outbred genetic background of the chickens.

Gene-Gun Delivery of DNA. Gene gun-based acceleration of DNA-coated gold beads into the epidermis proved to be by far the most efficient method of DNA immunization (Table 3). The beads deliver DNA into cells, where the DNA dissolves and can be expressed (24, 26). Expression is transient (24, 26), with most of the expression being lost within 2–3 days due to the normal sloughing of the epidermis (ref. 26 and unpublished observations). Tests of gun-delivered DNA in the murine model demonstrated that as little as 0.4 μg of DNA was sufficient to achieve 95% survival. These survivors developed very limited to no signs of postchallenge influenza. Mice receiving 0.04 μg of gun-delivered pCMV/H1 DNA had an ≈65% survival rate and suffered fairly severe signs of influenza. Mice that received 0.004 μg or 0.0004 μg of pCMV/H1 DNA succumbed to the challenge. As in tests of saline injections, mice receiving control DNA developed severe signs of influenza and had very limited survival (14%).

Antibody Responses in DNA-Vaccinated Mice. DNA vaccinations by the various routes appeared to prime antibody responses. Antibody responses were assayed using tests for hemagglutination-inhibiting activity and ELISA activity (for mouse data see Table 4; for representative chicken data see ref. 4). The DNA vaccinations and booster inoculations raised only low to undetectable titers of hemagglutination-inhibiting antibodies and ELISA activity. These low levels of activity underwent rapid increases after challenge. Protection occurred in both mice and chickens that did not have detectable levels of anti-influenza antibodies before challenge. However, the best protection occurred in groups in which the DNA inoculations had raised detectable titers of antibody (Tables 1, 3, and 4).

Use of ELISAs to score the isotypes of the anti-influenza virus antibodies demonstrated that the immunizations had

Table 2. Protection of chickens against a lethal A/Chicken/Victoria/1/85 (H7N7) influenza virus challenge by inoculation of H7-expressing DNAs in saline

DNA	Route of inoculation*	Dose, μg	No. of survivors/ no. tested	% survival	Probability
p188	i.v., i.p., s.c.†	300	28/56	50	<0.0001
	i.v.	100	8/33	24	<0.01
	i.p.	100	0/8	0	
	s.c.	100	0/2	0	
pRCAS	i.v., i.p., s.c.†	300	1/55	2	
pCMV/H7	i.v., i.p., i.m.	300	19/30	63	<0.0001
	i.m.	200	3/10	30	<0.02
	i.t.	200	3/10	30	<0.02
	i.b.	200	1/10	10	
	i.o.	200	1/10	10	
pCMV/control	Various	0–300	1/43	2	

See *Materials and Methods* for details of vaccination trials. Within experimental groups, survivors showed varying signs of influenza. Data for p188 and pRCAS DNA are pooled from five independent trials. Data for pCMV/H7 and pCMV/control DNA are pooled from seven independent trials. No data have been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.
*i.t., Intratracheal; i.b., intrabursal; i.o., intraorbital; for others, see footnote to Table 1.
†Data reported in ref. 4.

Table 3. Protection of mice against a lethal A/PR/8/34 (H1N1) influenza virus challenge by gene gun-delivered pCMV/H1 DNA

DNA	Dose, μg	Signs of influenza	No. of survivors/ no. tested	% survival	Probability
pCMV/H1	0.4	±	21/22	95	<0.0001
	0.04	+++	7/11	64	<0.01
	0.004	+++++	0/5	0	
	0.0004	+++++	0/4	0	
pCMV/control	0.4	+++++	3/22	14	

See *Materials and Methods* for details of vaccination trials and legend to Table 1 for description of signs of influenza. Data are pooled from four independent trials. No trial has been omitted. Probability was calculated by using Fisher's exact two-tailed test comparing the frequency of survival and mortality in vaccine versus control groups.

primed IgG responses. Low titers of anti-influenza IgG could be detected in the sera of mice vaccinated by gun delivery or intravenous or intramuscular inoculations of DNA. Borderline to undetectable titers of IgG were present in the sera of mice receiving DNA nose drops (consistent with the poorer protection provided by this route of DNA administration). By 4 days after challenge, increased levels of IgG were detected in mice undergoing the best protection. By contrast, mice receiving control DNA did not have detectable levels of anti-influenza virus IgG until the second serum collection after challenge. This was consistent with vaccinated, but not control, groups undergoing a secondary antibody response to the challenge.

Marginal to undetectable levels of IgM and IgA were detected in both prechallenge and postchallenge sera (Table 4). The low levels of these immunoglobulin isotypes throughout the trials indicated that none of the routes of DNA inoculation were effective at raising serum IgM or IgA.

DISCUSSION

The results of our vaccine trials demonstrate that epidermal, mucosal, intramuscular, and intravenous routes of administration can be used for DNA vaccines (Tables 1–3). Our results also demonstrate that gene-gun delivery of DNA into the epidermis is a very efficient method of inoculation, achieving protection with 250–2500 times less DNA than direct inoculations of purified DNA in saline (Tables 1 and 3).

Transfection Efficiency Versus Vaccination Efficiency. One of the striking results of our DNA vaccine trials is that the efficiency of transfection does not necessarily determine the efficiency of vaccination. The high ability of rodent muscle to take up and express DNA (5–7) did not correlate with an unusual efficiency of intramuscular vaccinations (Tables 1, 3, and 4). In the mouse trials, intramuscular inoculations worked well, but no better than intravenous inoculations, and only somewhat better than the administration of DNA nose drops (Tables 1 and 4). Similar results were obtained in the chicken trials, where intravenous and intratracheal inoculations achieved levels of protection comparable with those provided by intramuscular inoculations (Table 2).

The success of DNA immunizations by the intravenous and mucosal routes may reflect efficient antigen presentation and recognition compensating for inefficient transfection. Both blood and mucosal surfaces have associated lymphoid tissues that provide specialized and highly active immune surveillance. Thus, successful vaccination by these routes may reflect a highly efficient response to very low numbers of transfected cells.

High Efficiency of Gene-Gun Vaccinations. Highly efficient immunizations were achieved by gene-gun delivery of DNA to the epidermis of mice. This method of immunization

Table 4. Antibody responses in vaccine trials testing routes of inoculation in mice

DNA and route	Time of bleed	No. tested	Titers of antibody to A/PR/8/34 (H1N1) HI	ELISA value × 10^{-2} IgM	IgG	IgA
pCMV/H1 in saline						
i.v.	Prevac	2 (12)	<	<	<	<
	10 d PB	2 (12)	<	<	8	4
	4 d PC	1 (6)	20	<	128	4
	14–19 d PC	2 (10)	113	1	256	4
i.m.	Prevac	3 (19)	<	<	<	<
	10 d PB	3 (19)	<	<	3	<
	4 d PC	2 (13)	6	<	32	2
	14–19 d PC	3 (18)	127	<	406	2
i.n.	Prevac	3 (17)	<	<	<	<
	10 d PB	3 (17)	<	<	2	1
	4 d PC	2 (11)	<	1	2	1
	14–19 d PC	3 (17)	160	2	202	2
pCMV/control in saline						
Various	Prevac	3 (16)	<	<	<	<
	10 d PB	3 (16)	<	<	<	<
	4 d PC	2 (9)	<	<	<	<
	14–19 d PC	1 (2)	320	<	256	<
pCMV/H1, gene gun	Prevac	2 (10)	<	<	<	<
	10 d PB	3 (16)	10*	1	10	<
	4 d PC	3 (16)	20*	2	64	<
	14–19 d PC	3 (15)	160*	<	645	<
pCMV/control, gene gun	Prevac	2 (12)	<	<	<	<
	10 d PB	3 (16)	<	1	<	<
	4 d PC	3 (16)	<	2	<	<
	14–19 d PC	1 (3)	NT	4	512	<

Data are the geometric means of the reciprocal of the final dilutions of pooled sera that scored positive for a given condition. Prevac, bleed before DNA vaccinations; 10 d PB, sera harvested 10 days after the second DNA inoculation, immediately prior to challenge; 4 d PC, sera harvested at 4 days postchallenge. No. tested, no. of groups for which pooled sera were assayed (total no. of animals contributing sera to the pools); <, activity not detected in the lowest dilution of serum used in tests [1:10 for hemagglutinin inhibition (HI); 1:100 for ELISA]; NT, not tested.
*Only one of the three pools of sera was tested for HI activity.

required 250–2500 times less DNA than the saline inoculations (0.4–0.04 μg as opposed to 100–200 μg of DNA) (Tables 1 and 3). We think the remarkable success of gene-gun vaccinations reflects the combination of efficient transfection with efficient antigen presentation and recognition. The gene gun represents a very effective method of transfecting a tissue (24, 26). When the epidermis is transfected, DNA-expressed antigens are subject to immune surveillance by the skin-associated lymphoid tissue. This lymphoid tissue is rich in cells (such as epidermal Langerhans cells) that are capable of presenting transfected antigens to the T-helper component of the immune system (12–14, 27).

Induction of Memory by DNA Vaccinations. DNA immunizations rely on low numbers of transfected, antigen-expressing cells to raise immune responses. In our trials, these low numbers of antigen-expressing cells did not induce high-titer antibody responses (Table 4 and ref. 4). However, they did prime both T-helper and B-cell memory. This memory appeared to provide protection by supporting the mounting of secondary responses in challenged animals (Table 4). Evidence for the priming of memory is provided by the DNA inoculations raising antibodies belonging to the IgG

isotype. IgG is produced by differentiated plasma cells that have undergone immunoglobulin rearrangements in response to T-cell help (28). Evidence for the mobilization of memory in response to the challenge is found in the rapid increases in serum IgG after challenge (Table 4).

Summary. Our studies demonstrate that many routes of DNA inoculation can be used for raising protective immune responses. Two of these we consider particularly promising: (*i*) vaccination by gun delivery of DNA into the epidermis and (*ii*) vaccination by administration of DNA to mucosal surfaces. Both of these routes of administration should raise responses that will provide systemic immunity as well as specialized surveillance for major portals of pathogen entry.

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