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Nucleic Acid Vaccines

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INTRODUCTION

Candidate vaccines now under development and testing for the prevention of human immunodeficiency virus type 1 (HIV-1) infection include recombinant virus subunits, synthetic peptides, viral and bacterial vector-based vaccines, and nonreplicating virus particles. To date, vaccines containing recombinant subunit antigens have shown only limited immunogenicity in preclinical studies and partial protection in monkeys when the simian immunodeficiency virus (SIV) model system was used (12). Clinical trial volunteers immunized with recombinant HIV-1 envelope subunit vaccines have developed antibody that can neutralize laboratory isolates of HIV-1 but have developed minimal cytotoxic T lymphocyte (CTL) responses (24).

A live, attenuated vaccine (4) elicited significant protection against SIV in rhesus macaques. In this case, genetic sequences (i.e., the *nef* gene) associated with SIV pathogenesis and disease progression in rhesus macaques were deleted from the viral genome. This resulted in virus replication (albeit somewhat compromised) without disease progression in most animals. However, concerns about reversion or recombination of these vaccines to a pathogenic form and the uncertain long-term safety of attenuated retroviruses may delay or even preclude the general use of live, attenuated vaccines for the prevention of HIV infection in humans.

The use of nucleic acid-based vectors (DNA or RNA) as an alternative to live-attenuated immunization is a novel strategy now under development and evaluation. DNA-based vaccines are composed of purified closed-circular plasmid DNA or nonreplicating viral vectors containing genes that encode viral antigens.

A typical vector consists of several genetic elements required to drive intracellular expression of the foreign gene insert (Fig. 1). These include (i) a transcriptional promoter, (ii) an optional enhancer element to augment gene expression, (iii) the foreign gene encoding an antigenic gene product (e.g., a viral protein), and (iv) RNA-processing elements, primarily a polyadenylation signal and an optional intron element. Frequently, a marker gene conferring resistance to an antibiotic (e.g., neomycin phosphoryltransferase, which confers resistance to geneticin [Neo^r]) is included for detection and positive selection.

In addition, the plasmid contains two bacterium-specific genetic sequences to allow large-scale production of the DNA: an antibiotic selectable marker to permit identification and isolation of bacterial cells successfully transduced with the gene of interest, and a bacterial origin of replication to facilitate large-scale amplification of the plasmid within this host cell. Once the DNA enters the mammalian cell, the encoded antigens are expressed through normal cellular transcription and translation mechanisms.

Immunization with DNA-based plasmids has been successfully attempted in several tissues by various routes of administration. Most experiments have been conducted with DNA delivered to skeletal muscle or the epidermis.

Antigen expressed by DNA-based immunization is displayed on the cell surface in association with major histocompatibility complex class I molecules via the cytosolic antigen presentation pathway, similar to that occurring during natural viral infection or in response to live, attenuated vaccines (9, 14). Antigens expressed by nucleic acid vaccines and presented in the context of major histocompatibility complex class I molecules can thereby efficiently induce CTL responses. In addition, glycoprotein antigens produced by host cells possess host glycosylation patterns. Other advantages of DNA-based vaccines are that DNA (i) is simpler to produce and purify than recombinant protein antigens, (ii) is highly stable, and (iii) could be used to produce combination vaccines with antigens that have incompatible formulations when used as traditional vaccines.

Safety issues specific for DNA vaccines that are presently being addressed include uptake into cells other than the intended target cells, the potential for oncogenic mutagenesis through integration of the plasmid DNA, anti-DNA immune responses, and uncertainty of the fate of the administered DNA that fails to enter the target cells.

NUCLEIC ACID VACCINE DEVELOPMENT

The development of nucleic acid vaccines began serendipitously in experiments investigating whether the direct injection of DNA or RNA expression vectors for gene therapy could abrogate the need for live-virus vectors (5, 30). Wolff et al. (30) found that intramuscular (i.m.) injection of nonreplicative DNA or RNA expression vectors in cationic lipid vesicles re-

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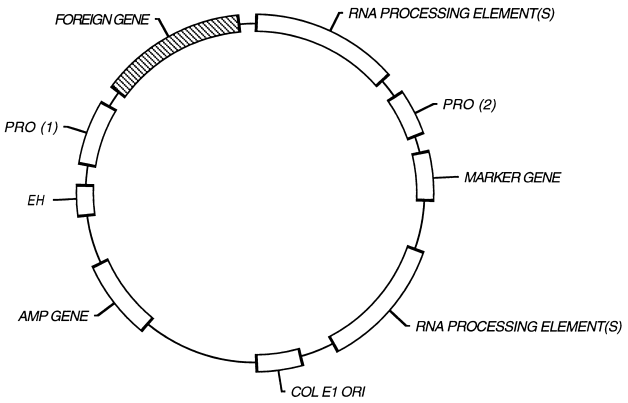

FIG. 1. A prototype plasmid vector for nucleic acid immunization. Starting at 9 o'clock and moving in a clockwise direction, the genetic elements are as follows. First is a transcriptional enhancer (EH) element (optional) appended to a transcriptional promoter positioned upstream of the foreign gene. The transcriptional cassette terminates with RNA-processing elements, including a polyadenylation signal and an intron sequence (optional). An optional transcriptional cassette for marker gene expression may also be included with its transcriptional promoter, the marker gene, and RNA-processing elements. The bacterial origin of replication (ColE1) and a gene conferring antibiotic resistance (in this case ampicillin, or AMP) are also included. Refer to the text for details.

sulted in the expression of gene products in muscle cells. Surprisingly, they found that for plasmid DNA vectors this occurred even without the lipid delivery system. In these studies, reporter genes such as the bacterial chloramphenicol acetyltransferase, firefly luciferase (*luc*), and bacterial β-galactosidase genes were used to assess gene expression. In situ cytochemical staining localized β-galactosidase enzymatic activity to muscle cells. The longevity of the gene expression was shown with a plasmid expressing luciferase. In these studies, luciferase was detected in skeletal muscle of mice for 19 months (29).

Davis et al. (5) compared the efficiency of gene transfer into mature (mitotically inactive) mouse muscle and into regenerating (mitotically active) muscle by using recombinant plasmid DNA, adenovirus, and retroviral vectors. Expression of the *luc* and β-galactosidase reporter genes in mice was shown to be more efficient in regenerating muscle than in mature muscle. These studies also showed that in regenerating muscle, recombinant plasmid DNA and adenovirus vectors were equally efficient in expressing the reporter genes and were superior to a retroviral vector.

These investigators had previously demonstrated that the variability of gene transfer in normal muscle was reduced by preinjection of a hypertonic sucrose solution. It was proposed that the improved efficiency of gene transfer was due to the effect of the hypertonic sucrose in shrinking muscle fibers or forcing them apart, thereby improving the distribution of plasmid DNA (7).

In similar studies, Williams et al. (28) showed that a plasmid vector containing the *luc* gene controlled by the human β-actin promoter could be introduced into the liver and skin of mice. The DNA was administered via a "biolistic" device that accelerates DNA-coated gold microprojectiles into the tissues. Luciferase activity was detected for 14 days.

Using the same biolistic device, Tang et al. (22) demonstrated that "genetic immunization" was possible by introducing into a mouse genes that induced an immune response to the encoded antigen. In these studies, mice were inoculated in the skin of the ear with gold microprojectiles coated with human growth hormone (hGH) plasmid vectors. hGH was expressed under the control of the human β-actin promoter or the cytomegalovirus promoter. Antibodies to hGH were demonstrated by precipitation of ^{125}I-hGH. Animals inoculated with control (non-hGH) plasmid had no detectable hGH antibody.

In subsequent experiments, these investigators demonstrated that mice could be immunized with plasmid vectors encoding human α_1-antitrypsin transcribed from the cytomegalovirus promoter and that animals coimmunized with both the hGH and the human α_1-antitrypsin plasmids produced antibodies to both proteins.

On the basis of earlier studies (30) of direct gene transfer into mouse muscle, Ulmer et al. demonstrated that i.m. injection of plasmid DNA encoding influenza A virus (A/PR/8/34) nucleoprotein (NP) elicited protective immune responses in BALB/c mice against a heterologous strain of influenza virus (A/HK/68) (23). Immunized animals had reduced virus titers in their lungs, decreased weight loss, and increased survival compared with challenged control mice. Both NP-specific CTL and NP antibody were generated. The latter was shown to be ineffective at conferring protection. In contrast, CTLs, including primary CTLs (not requiring restimulation with antigen in vitro), were demonstrated to kill virus-infected cells and cells pulsed with the appropriate major histocompatibility complex class I-restricted peptide epitope. It was further demonstrated that i.m. injection of plasmid DNA encoding influenza virus A/PR/8/34 hemagglutinin (HA) resulted in the generation of neutralizing antibodies that protected mice against a homologous lethal influenza virus challenge (18). In a ferret model, immunization with HA DNA likewise resulted in neutralizing antibody and protection from a homologous influenza virus infection as measured by decreased nasal virus shedding following challenge with influenza virus A/PR/8/34 (27).

In studies conducted in chickens, Robinson et al. showed that the direct inoculation of chickens with a defective avian leukosis virus-based plasmid vector (p118) encoding influenza virus HA H7 protected chickens from 100 lethal doses of H7N7 influenza virus (20). In these studies, the DNA was administered simultaneously via the intraperitoneal, intravenous, and subcutaneous routes. Each chicken received 100 μg of DNA by each route of injection and was reimmunized via the respective routes 1 month later. In a series of four experiments, 50% (28 of 56) of the immunized chickens were protected while only 2% of the control chickens survived the challenge. Very low to undetectable levels of H7-specific antibodies were present postimmunization and after reimmunization. However, H7-specific antibody appeared within 1 week of challenge, suggesting that the observed protection was due to priming of an immune response by the nucleic acid vaccine.

This group extended these results in further studies comparing the protective effect of DNA-based immunization by various routes of administration (8). Mice and chickens were immunized with 50 to 300 μg of plasmid DNA encoding influenza virus HA. In these studies, 67 to 95% of mice and 25 to 63% of chickens were protected from lethal homologous influenza virus challenge. Significant protection was observed in animals immunized parenterally by the i.m. and intravenous routes. Mucosal immunization via the nares or trachea was also successful. Highly efficient immunizations were also achieved by using a gene gun to deliver DNA-coated gold beads into the epidermis. In these studies, two immunizations with 0.4 μg of plasmid DNA encoding HA protected 95% of mice from death following lethal influenza virus challenge (8).

DNA-based vaccines have been shown to generate immune responses against various pathogens in diverse animal species. Antibodies against bovine herpesvirus 1 glycoproteins were

demonstrated in mice and cattle (3). Moreover, protective neutralizing antibody responses, as measured by a decrease in nasal viral shedding, were demonstrated in cattle immunized with DNA encoding bovine herpesvirus 1 gIV glycoprotein and subsequently challenged with live virus (3). Experimental nucleic acid vaccines against a wide variety of infectious diseases, including leishmaniasis (31), tuberculosis (15), malaria (10), and hepatitis B (6), are under development.

DNA VACCINES AGAINST RETROVIRUSES

Wang et al. (26) immunized BALB/c mice with four biweekly injections of a plasmid vector (pM160) encoding HIV-1 HXB2 gp160. The plasmid comprised two eukaryotic transcriptional cassettes: the first expressed the HIV-1 *env*, *tat*, and *rev* genes, all driven by the mouse mammary tumor virus long terminal repeat promoter and the Rous sarcoma virus transcriptional enhancer. The second cassette expressed the neomycin phosphoryltransferase marker gene, to allow positive selection with the antibiotic geneticin (G418) as well as molecular detection (e.g., via PCR and in situ analyses) of HIV genetic elements in target cells. In addition, the plasmid contained a bacterial origin of replication and an ampicillin antibiotic marker gene to allow propagation of the DNA plasmid in a bacterial host and selection of positive clones, respectively. DNA immunization was enhanced by preinjection (day −1) of 100 μl of 0.5% bupivacaine hydrochloride. Antibody responses were observed against recombinant gp160 (rgp160) and gp160-derived antigens of several virus isolates including HXB2 gp120 V3 loop peptide, HXB2 CD4-binding-site peptide, HIV-1 BRU V3 peptide, HIV-1 MN V3 peptide, HIV-1_{Z6} (a Zairian HIV-1 isolate) V3 peptide, gp41 fusogenic region peptide (F560), BRU gp41 peptide, and HXB2 N-terminal peptide. Control mice were immunized with HIV-1_{IIIB} rgp160 (MicroGeneSys, Meridian, Conn.) in complete Freund's adjuvant followed 2 weeks later by two secondary immunizations spaced 2 weeks apart. The CFA-pM160 induced higher antibody levels than did the plasmid vector only to the whole rgp120 and to the N-terminal peptide. Antibodies generated in pM160 mice also neutralized cell-free HIV-1_{IIIB} virus in vitro, inhibited syncytium formation, and blocked gp120 binding to CD4.

Taken together, these results indicate that gp160 antigen expressed in vivo through DNA immunization in mice is highly immunogenic and may be effective in inducing broadly cross-reactive antibodies, an observation of particular relevance to protection in HIV infection. Of note is the additional demonstration that lymphocytes from pM160-immunized mice proliferated in vitro in response to recombinant gp120 glycoprotein (rgp120).

In a parallel study, this group evaluated DNA immunization against HIV in nonhuman primates (25) by using a construct similar to that described above but containing the *env* gene of an HIV-1_{Z6} isolate. Cynomolgus macaques received a total of three biweekly i.m. injections of 100 μg of the plasmid DNA (pM160-Z6) in addition to the envelope region of HIV-1_{Z6}. The pM160-Z6 plasmid also contained the coding regions for HIV-1 *tat* and *rev* genes. Sera from an immunized macaque, collected 2 weeks after the third immunization, neutralized cell-free HIV-1_{MN} incubated with MT-2 cells as scored by a syncytium inhibition assay.

RETROVIRUS-MEDIATED GENE TRANSFER

Retrovirus-mediated gene transfer is an efficient means of delivering genes encoding foreign proteins into mammalian cells. Indeed, retrovirus gene transfer is the most frequently used vector in clinical studies of human gene therapy. Immunization by direct in vivo injection of nonreplicating retroviral vector particles is also being used to elicit immune responses in HIV-seropositive individuals. Unlike the previously cited studies of DNA plasmid-mediated delivery, in which the delivered genes remain unintegrated, genes transferred via retroviral vectors are inserted into the host chromosome, thereby ensuring the perpetuity of the genetic information in the target cells.

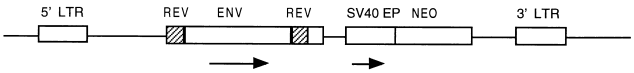

FIG. 2. Retrovirus-based vector for genetic immunization. The retroviral vector N2 IIIBenv was generated by inserting the *env* gene (including the two exons of the *rev* gene) from HIV-1_{IIIB} into the N2 amphotropic Moloney murine leukemia virus retrovirus vector. For selection purposes, the neomycin phosphoryltransferase (Neo^r) gene, conferring resistance to geneticin, was included. 5′ LTR, Moloney murine leukemia virus transcriptional promoter; REV, first and second exons of HIV-1 *rev* gene; ENV, HIV-1 *env* gene coding for gp160; SV40 EP, the early promoter region of simian virus 40 driving the *neo* gene; 3′ LTR, RNA-processing element (polyadenylation) of Moloney murine leukemia virus. Adapted from reference 1 with permission of the publisher.

However, this property carries some risks. A major concern associated with retrovirus gene delivery is the potential for insertional mutagenesis, the process of activating deleterious genes (or inactivating essential genes) as a result of chromosomal integration at inappropriate sites. The choice of plasmid versus retrovirus gene delivery is thus a compromise between risk factors and efficiency of transfer, issues that are still being sorted out.

Irwin et al. (11) recently reported on direct injection of the nonreplicating N2 IIIBenv retroviral vector containing HIV *env* and *rev* genes. The vector used (1) (Fig. 2) is the nonreplicating amphotropic Moloney murine leukemia virus (N2) backbone. HIV-1_{IIIB} *env* and *rev* genes were inserted between the 5′ and 3′ long terminal repeats, with the 5′ long terminal repeat providing the promoter function and the 3′ long terminal repeat providing the polyadenylation signal. For selection purposes, the neomycin phosphoryltransferase gene was included downstream of an internal simian virus 40 early promoter. Expression of gp160 and Rev proteins resulted in activation of class I MHC-restricted $CD8^+$ T-cell responses specific to these viral proteins in three animal models tested (mouse, rhesus macaque, and baboon). CTL responses have been observed for several months in these models, indicating induction of long-lived memory CTL activity. Furthermore, the CTL responses generated in some mice exhibit cross-reactivity on targets coated with an immunodominant Env peptide derived from the V3 loop of IIIB and MN Env proteins. The CTL response directed to Rev determinants is of particular importance because (i) Rev is an early viral protein, and elimination of infected cells prior to extensive virus release may affect virus spread, and (ii) *rev* is a highly conserved gene, suggesting that resulting immune responses may be cross-reactive with diverse viral isolates.

On the basis of these and additional data, clinical protocols using this modality of genetic immunization have been approved by the Food and Drug Administration and the first human clinical trials in seropositive individuals have been initiated. Data for this and subsequent studies are forthcoming.

PARAMETERS AFFECTING GENE EXPRESSION AND IMMUNOGENICITY OF DNA VACCINES

The efficiency of expression of antigen genes and the immunogenicity of DNA vaccines can be influenced by several pa-

rameters, including (i) the construction of the plasmid vector, in particular the choice of the promoter used to drive expression of the antigen gene; (ii) the route of administration; (iii) the tissue or organ in which the antigen is expressed; and (iv) the physical nature and properties of the expressed antigen that govern whether it is secreted by the cell, remains bound in the cell membrane, or remains sequestered within the cell.

Using gold particle bombardment to transfer the *luc* gene to cells of various tissues, Cheng et al. (2) investigated the effect of different promoters and target tissues on gene expression. Gold particles (diameter, 1 μm) were coated with DNA and then accelerated into the various tissues via the Accell gene delivery system (Agracetus, Middleton, Wis.). Several plasmid vectors and promoters were used to test the relative efficiency level and stability of gene expression: pNASSluc (no promoter), pADluc (adenovirus type 2 major late promoter), pCMVluc (cytomegalovirus immediate early gene enhancer/promoter), pRSVluc (Rous sarcoma virus long terminal repeat), pSVluc (simian virus 40 early enhancer/promoter), pMLVluc (murine leukemia virus long terminal repeat), pmMTluc (mouse metallothionein gene promoter), pPEPluc (mouse phosphoenolpyruvate carboxykinase gene promoter), pBLGluc (bovine β-lactoglobin gene promoter), pPLluc (bovine prolactin promoter), and pPGKluc (mouse phosphoglycerate kinase gene promoter). Gene expression was observed in all species tested including rats, rabbits, mice, and rhesus macaques. The organs and tissues evaluated for *luc* gene expression were epidermis, dermis, muscle, liver, and pancreas.

In the rat, the cytomegalovirus immediate-early promoter showed the highest activity in each tissue tested. The relative strengths of various other promoters showed tissue specificity. The pPEPluc gene promoter and the pmMTluc gene promoter were inducible in liver posttransfection at 1 and 5 days, respectively.

A second parameter affecting the nature of immune responses obtained with DNA immunization is the localization of the expressed antigen. Rhodes et al. (19) observed that DNA immunization evoked antibody responses in mice when the DNA vaccines, such as influenza virus NP or HIV gp120, expressed secreted forms of the antigen. For HIV gp120, antibody titers greater than 10,000 were observed. In contrast, immunization of mice with a plasmid vector containing the *luc* reporter gene (which codes for a nonsecreted gene product) resulted in undetectable antibody to luciferase, although it was estimated that luciferase was produced at a rate of 0.5 to 5 ng/day at the site of injection. From these observations, it was concluded that secreted antigens proceed to peripheral lymph nodes, where a humoral response is evoked. In this case, the muscle would act as a reservoir of antigen that would be released over an extended period.

SAFETY CONSIDERATIONS FOR NUCLEIC ACID VACCINES

In studies to date, plasmid DNA has been shown to exist only extrachromosomally without integration into the host cell chromosome (29). Myocytes are terminally differentiated and do not undergo further cell division. Thus, these muscle cells, which efficiently take up and express DNA delivered as plasmid vectors, would have a decreased probability of integrating the plasmid DNA into the host chromosome compared with actively dividing cells. Furthermore, PCR amplification of DNA recovered from vaccine-injected muscles as long as 19 months after administration has demonstrated retention of a bacterial methylation pattern, indicating that DNA replication did not occur in the mammalian host (29). Despite the low probability of integration of plasmid DNA, the low likelihood that such an event would result in activation or disruption of a gene, and despite existing clinical experience with immunization with live DNA viruses (smallpox and varicella-zoster viruses), the possibility of integration will have to be carefully evaluated. More definitive and sensitive evaluations of the fate of the injected DNA will have to be done to determine that no integration occurs and that other cells do not take up or integrate low levels of the injected DNA.

Another potential safety issue is whether the injected DNA will induce anti-DNA antibodies similar to those associated with autoimmune diseases. Double-stranded chromosomal DNA has been shown to be nonimmunogenic (16), despite the immunogenicity of denatured single-stranded DNA complexed to protein. The latter, however, generated antibodies that are specific to the protein in the complex and which do not recognize the mammalian chromosomal DNA. Importantly, studies in nonhuman primates have failed to demonstrate anti-DNA antibodies following immunization with plasmid DNA (13). These findings are in agreement with studies showing lack of immunogenicity of double-stranded DNA. Further studies are necessary to rule out the possibility that immunization with nucleic acid vaccines could evoke or exacerbate autoimmune reactions.

RNA VACCINES

Initially, mRNA was shown to be capable of inducing protein production in situ following i.m. injection (30). Recent studies have demonstrated that mRNA formulated in liposomes and administered subcutaneously or intravenously (17) effectively generated antibody and CTLs directed against the encoded protein. Yang et al. (32) have also reported that particle-mediated (biolistic) delivery of RNA encoding human α_1-antitrypsin into mouse epidermis elicited high antibody responses to human α_1-antitrypsin. The use of mRNA as a vaccine vector would obviate the potential safety issue of insertional mutagenesis related to DNA immunization, since RNA does not integrate into chromosomal DNA. However, the difficulty and expense of large-scale RNA production and the relative instability of mRNA compared with DNA might render RNA vaccines an impractical means of immunization.

FUTURE DIRECTIONS

Nucleic acid immunization is a novel and promising strategy for the development of vaccines against human diseases. Equally promising is the value of this technology as a research tool to study the basic immune mechanisms of vaccination, such as antigen processing and presentation, and the contribution of "professional" antigen-presenting cells (e.g., macrophages) in processing and in presenting antigen secreted from target cells (e.g., muscle cells). The ability to express discreet antigens similar to those expressed by cells infected with live, attenuated viruses may illuminate mechanisms involved in long-lasting protection afforded by live, attenuated virus immunization and ways to reproduce that response without the associated potential risks.

Development of nucleic acid-based immunogens as vaccines for human use requires further research and development to ensure the safety of these products. New assays specifically designed to carefully evaluate this class of vaccine for potency, general safety, purity, and identity should be developed in addition to tests addressing genetic toxicity (i.e., integration), tumorigenicity, and teratogenic toxicity (21).

Further product development may include the development

of methods to enhance the performance of nucleic acid vaccines through improved facilitators or other delivery vehicles designed to optimize uptake and gene expression in target tissues. In addition, formulation of nucleic acid vaccines with immunologic adjuvants designed to enhance and direct immune responses to expressed antigen may improve the immunogenicity of these products. The use of nucleic acid-based vaccines in multivalent vaccines and combination vaccines designed to immunize against multiple diseases is also a promising area of development.

At the level of the vaccine construct itself, nucleic acid vaccine design could be improved through better plasmid construction or through the use of tissue-specific promoters that target antigen expression to a specific site or cell type.

Because oral or nasal administration may ultimately reduce the cost of immunization, delivery of nucleic acid vaccines by other than the parenteral route should be explored. Oral or nasal delivery may further improve the performance of these vaccines through the induction of potent mucosal immune responses, particularly against diseases in which the pathogen enters via oral, respiratory, or intestinal routes of infection or via genitourinary surfaces.

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ADDENDUM IN PROOF

A clinical protocol for direct i.m. injection of a plasmid DNA construct expressing HIV-1 *env* and *rev* genes has been approved by the Food and Drug Administration. A study with HIV-infected patients is scheduled to begin in mid-1995.

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