Gene Therapy Using Adenovirus-Mediated Full-length Anti-HER-2 Antibody for HER-2 Overexpression Cancers

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Abstract

Purpose: Therapeutic monoclonal antibody is increasingly applied in many clinical applications, although complicated technologies and high cost still limit their wide applications. To obtain the sustained serum antibody concentration with one single injection and lower the cost of antibody protein therapy, an adenovirus-mediated full-length antibody gene therapy was developed.

Experimental Design: Full-length antibody light-chain and heavy-chain sequences were linked with internal ribosome entry site and constructed into adenoviral vector under the control of cytomegalovirus promoter. Antibody expression in vitro and in vivo were tested with ELISA, and its antitumor efficacy was evaluated in SKOV-3-inoculated nude mice.

Results: Ad5-TAb-generates anti-HER-2 antibody presented the similar binding specificity with commercial trastuzumab. A single i.v. injection of $2 \times 10^5$ plaque-forming units of Ad5-TAb per mouse resulted in not only a sustained over $40 \mu$g/mL serum antibody level for at least 4 weeks but also significant tumor elimination in the ovarian cancer SKOV-3-inoculated nude mice.

Conclusions: An in vivo full-length antibody gene delivery system allows continuous production of a full-length antibody at high concentration after a single administration. Bioactive antibody macromolecules can be generated via gene transfer in vivo. All the data suggest that this novel adenovirus-mediated antibody gene delivery can be used for the exploitation of antibodies, without being hampered by the sophisticated antibody manufacture techniques and high cost, and, furthermore, can shorten the duration and reduce the expense of antibody developments.

The application of antibody therapy is increasing for its specificity and sufficient effect. Full-length antibody has two valences and binds to antigen with high specificity and affinity. It also induces cytotoxic effect via Fc domain, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (1–4). The barrier for full-length antibody, however, is its high molecular weight, rendering it difficult to penetrate tumor tissue, which in turn limits its binding to the tumor cells. Based on these facts, single-chain antibody, Fab antibody, hypervariable peptide, and multivalence microantibody are studied to keep the antigen binding activity of the antibody (3, 5–9). Although these kinds of antibody are of low molecular weight, high penetrability, and less immunogenicity, however, their short half-life, fast clearance, low affinity, the lack of Fc function domain, and inability to induce antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, all contributed to reduce their antitumor efficacy. Full-length antibody cannot penetrate into the inner part of the large solid tumor because of its high molecular weight.

Genetic engineering technologies promote the development of monoclonal antibodies (mAb), which cumulated in the commercial therapeutic antibodies approved by Food and Drug Administration in the past several years. The demand for mAbs in preclinical and clinical development are accelerating, which is restricted by the lack of highly efficient expressing system for manufacturing, such as bacteria, yeast, plant, insect, and mammalian cells (10–15). However, all of these require the high-cost manufacturing and sophisticated technology, especially associated with large-scale mammalian cell culture and column chromatography of antibody (16–20). Moreover, the requirement on technology and cost had limited the application of antibody therapy to countries and regions at the high end of economic development. It is conceivable that a means that not only greatly simplifies antibody manufacturing but also economizes the process will greatly facilitate the application of therapeutic mAb to benefit human kind.
Even when there is a breakthrough in the antibody synthesis technologies, antibody purification will be the next hurdle. The current technologies can provide sufficient purity but not 100% pure antibody for human therapy at the recommended concentration in the serum. The therapeutic effect of mAbs is dose dependent, but it is impossible to increase the serum antibody concentration without limits. Application of large amount of antibody is limited by (a) high cost of antibody, (b) toxicity from impurities in the purified antibody, and (c) clearance through the kidney. Therefore, antibody in vivo gene therapy is supposed to be one of the best candidates, which can provide high concentration and full pure antibody for a long period of time. Herein, we report the feasibility of this approach by full-length antibody gene transfer in nude mice.

The initial barrier for in vivo antibody therapy via gene transfer is whether in vivo generated antibody is biofunctionally functional. Until now, the functional biomacromolecules are hard to be prepared and expressed by gene transfer, such as antibody. Furthermore, especially for antibody, not only four-subunit structure, but also the fine conformational structure. A slight difference on structure may affect the bioactivity profoundly. Antibody production also involved the antibody editing, hyper-mutational mutation, and affinity maturation in vivo, and all of these characteristics can be represented only in B-derived cells, such as B cell, B hybridoma cells. Therefore, there is a conflict whether it is possible to obtain the functional full-length antibody via gene transfer in non-B cell. Another barrier to the application of therapeutic full-length antibody gene transfer is to ensure stoichiometric amounts of both heavy and light chains in the antibody-producing cells. An imbalance in heavy and light chains production could be toxic. 2A self-processing sequences are located in between P1 and P2 proteins in some members of picornavirus family (21, 22). Recently Fang et al. (23) employed a modification of the 2A self-processing sequence to express a full-length mAb from a single open reading frame driven by a single promoter in the AAV8 vector and achieved remarkably high levels (>1,000 µg/mL) and long-term expression (>140 days) of an anti–vascular endothelial growth factor receptor 2 mAb in mice and showed therapeutic efficacy against two tumor cell lines, showing that in vivo therapeutic antibody gene transfer was indeed possible. However, the high antibody concentration over a long period of time in the serum might have some other unpredictable adverse effects. Another issue of concern is distribution of antibody expression. There are reports indicating that transgene protein expressed in the liver cell is less immunogenic than other cells (24). Intravenous adenoaviruses administration is considered to result in the most adenoaviruses kept in liver and therefore displays the high expression in the hepatocytes.

In the present study, adenoaviruses was chosen as expression vector for its many advantages (25–28). Full-length anti-HER-2 antibody light-chain and heavy-chain sequences were synthesized (29–34). Light-chain and heavy-chain genes, with respective signal peptide, were linked together with internal ribosome entry site (IRES) from eencephalomyocarditis virus and under the control of mCMV promoter. Recombinant anti-HER-2 –expressing adenoavivirus (Ad5-TAb), with expression cassette inserted in E1 region of adenoavivirus 5, was prepared in HEK293 cell. Ad5-TAb in vivo produced high serum concentration of the full-length antibody that is the same bioactive as commercial trastuzumab. A single injection of Ad5-TAb resulted in effective therapeutic concentration lasting for 4 weeks and peak serum level >160 µg/mL as early as day 7 after injection. In HER-2+/SKOV-3–inoculated nude mice, antibody generated in vivo exerted anticancer efficacy not only in small-volume solid tumors (60% tumor complete clearance, P < 0.01) but also in the large-volume tumors (P < 0.05). This is the first time to report that the full-length antibody could be produced via adenoaviruses in non–B cells with biofunctional activity. Our study confirmed that gene therapy approach for stable expression of full-length antibody by adenoavirial vector in vivo is feasible, which will be more time saving and economical than traditional antibody therapy.

**Materials and Methods**

**Plasmids and reagents.** Full-length light-chain and heavy-chain sequences of trastuzumab were synthesized (Bocai, Shanghai, China) according to the published sequences (U.S. Patent 5,821,337). The clone vector (pcDNA12/IRES) was constructed in our lab (as seen in the supplementary files). pDC315 was purchased from Microbix Biosys- tems (Toronto, Ontario, Canada). Recombinant human Erb-2/Fc (HER-2) Chimera protein is from R&D Systems (Minneapolis, MN). Enhanced chemiluminescence kit is purchased from Pufei (Shanghai, China). Commercial trastuzumab is purchased from Roche (Indian- apolis, IN). All the antibodies, such as rabbit anti-human IgG1 (H + L), mouse anti-human IgG1, goat anti-rabbit IgG, rabbit anti-mouse IgG, are from Southern Biotech (Birmingham, AL).

**Recombinant adenoaviral vector Ad5-TAb preparation.** Briefly, HEK293 cells at the confluence of 40% to 80% were cotransfected with pDC315-TAb and pBGHE3 (Microbix Biosystems) by Lipofect- AMINE 2000 (Qiagen, Chatsworth, CA) according to the manual provided by the manufacturer. Recombinant adenoavivirus was confirmed by PCR with sense primer of light chain and antisense primer of heavy primer and named as Ad5-TAb. Adenoavivirus was amplified in HEK293 cells, purified with cesium chloride gradient density centrifugation, and titrated by TCID50 assay provided by Obiogene (Carlsbad, CA).

**Cell culture and infection.** Human embryonic kidney cell line HEK293 (Microbix Biosystems), human normal hepatocyte cell line L-02, human ovarian cancer SKOV-3 (HER-2+), and BT549 (HER-2−) were purchased from the American Type Culture Collection (Rock- ville, MD). L-02 was cultured in RPMI 1640 (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum, whereas the rest cell lines were grown in DMEM (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum. For antibody anti-HER-2 antibody expression in vitro, L-02 cell were infected with titrated Ad-TAb and incubated for different period of time. Briefly, cells at 1 × 10⁵ per well were plated on 24-well plates and incubated for 24 hours at 37°C in 5% CO₂ humidified incubator. Cells then were switched to serum-free medium and infected with Ad5-TAb at a multiplicity of infection of 10. After 2 hours of incubation with gently mixing, cells were switched to 5% fetal bovine serum culture medium, and cell culture supernatant were harvested at days 3 and 7, respectively, for bioactivity and quantitative analysis.

**ELISA and Western blot.** Antibodies produced by L-02 in vitro or in nude mice at different time points were determined by indirect and sandwich ELISA. ELISA plates were coated with mouse anti-human IgG1 mAb that is able to immobilize antibody contained in the cell culture supernatant from Ad5-TAb –infected L-02 cells and followed by incubation with mouse anti-human IgG1 mAb conjugated to horse- radish peroxidase. Commercial trastuzumab was applied for standard curve preparation. The plates were read in the microplate reader at the absorbance of 450 nm.

Anti-HER-2 antibody containing cell culture supernatant, mouse serum, and the commercial trastuzumab were resolved by 12% SDS-PAGE under reducing or nonreducing conditions. Proteins in the...
polyacrylamide gel were transferred to the nitrocellulose membrane and probed with the goat anti-human IgG1 (H + L) polyclonal antibody (Southern Biotech) followed by rabbit anti-goat IgG conjugated with horseradish peroxidase (Southern Biotech). Protein bands were visualized by exposure on X-ray film (Kodak, Rochester, NY) after the membranes were treated with enhanced chemiluminescent solution (Pierce, Rockford, IL).

**Indirect fluorescence assay for binding specificity.** SKOV-3 (HER-2⁺) and BT549 (HER-2⁺) cells were plated on 96-well plates. After 24 hours of incubation, cells were rinsed with PBS three times and then fixed with 4% paraformaldehyde for 5 minutes. Cells were then incubated with culture supernatant or the commercial trastuzumab, respectively, at 4°C for 2 hours. Rabbit anti-human IgG conjugated with FITC at a dilution of 1:100 was applied, and the labeled cells were observed under the fluorescent microscope (Olympus, Tokyo, Japan).

**Determination of affinity constant (Kd).** Briefly, 100 µL of 1.0 µg/mL Erb-2/HER-2 resolved in coating buffer were added in 96-well plate and incubated for 15 hours at 4°C. ELISA plates were blocked with 5% nonfat dry milk. Fixed amount of antibody (5 × 10⁻⁵ mol/L) was mixed with different amount of Erb-2/HER-2 protein in 1% bovine serum albumin in PBS and incubated for 1 hour at 20°C. The mixture was transferred to the ELISA plate. Horseradish peroxidase–labeled mouse anti-human IgG mAb was added, and the staining was revealed by peroxidase substrate 3,3'-diaminobenzidine. The plates were read in the microplate reader at the absorbance of 450 nm. Calculation of affinity and dissociate variable was according to the formula of

\[ \frac{A_0}{A} = 1 + (K_J/C_0) \]

where \( A_0 \) is the absorbance value without antigen, \( A \) is the absorbance value of free antibody after different antigen, and \( C_0 \) is the primary concentration of the antigen.

**Animal tumor model.** Female BALB/C nude mice at 4 to 6 weeks old with average weight of about 25 g were provided by and bred in Animal Developmental Center, Chinese Academy Institute in Shanghai. Animals were housed under specific pathogen-free conditions. SKOV-3 cells were injected s.c. at right back of nude mice. Tumor therapy in SKOV-3-inoculated nude mice is implemented as two groups: early stage and late stage. Early-stage mice were treated at day 3 after inoculation, and late-stage ones were treated (tumor size around 200 mm³) at day 16. All the experiments were done double-blindly. Experimental mice were injected with 2 × 10⁹ plaque-forming unit Ad5-TAb each via the tail vein, whereas control mice were injected with Ad5-LacZ at the same dose. Tumor volume was measured with calipers at days 3, 7, 10, 14, 21, 28, and 35 for length, width, and height, which should cross the central point, and then were calculated by the formula of \( [( \text{width} \times \text{length} )/2] \). All the animals were euthanized on day 56. and heart, liver, spleen, kidney, lung, stomach, and tumors were processed for fluorescence-activated cell sorting and histology analysis.

**Results**

**Construction of recombinant adenovirus Ad5-TAb.** Trastuzumab, a well-characterized anti-HER-2 humanized antibody via i.v. administration for its definite antitumor efficacy, was chosen as a model antibody to evaluate the in vivo expression of full-length antibody generated by recombinant adenovirus gene transfer and its antitumor efficacy in nude mice. As shown in the Fig. 1, the light-chain and heavy-chain genes with the separate signal peptide were linked to the IRES region. The light-chain and heavy-chain genes were expressed by the Ad5 vector pDC315 and then subcloned into the pclone 12/IRES vector. Thereafter, the heavy-chain and light-chain gene linked by the IRES were together subcloned into

![Fig. 1. Full-length trastuzumab antibody expression cassette using IRES.](Image)

**Fig. 2. In vitro antibody expression in Ad5-TAb–infected L-02 cells. The L-02 cells were infected with Ad5-TAb at a multiplicity of infection of 10. Cell culture supernatants were harvested at different time points after infection for protein analysis.** A, ELISA analysis of supernatants of Ad5-TAb–infected L-02 cells. Columns, mean; bars, SD Western blot analysis of commercial trastuzumab and supernatants from Ad5-TAb– or Ad5-LacZ–infected L-02 cells under nonreducing (B) and reducing (C) conditions.
adenoviral shuttle vector pDC315 to construct the full-length antibody expression cassette driven by mCMV and ended with poly(A) in the plasmid pDC315Tab. A LacZ-containing control plasmid was generated and termed as pDC315-LacZ. Recombinant adenovirus Ad5-TAb or Ad5-LacZ was prepared in HEK293. Viral plaque became visible at 9 to 11 days after transfection. After plaque purification, identification of wild-type or recombinant adenovirus and titration, high-efficiency packaging recombinant adenovirus was achieved up to $4.2 \times 10^{10}$ plaque-forming units/mL.

**In vitro expression and assembly of anti-HER-2 antibody heavy and light chain.** L-02 cells were infected with Ad5-TAb or Ad5-LacZ at a multiplicity of infection of 10, respectively, and antibody expression was estimated by indirect ELISA assay. Antibodies expressed by Ad5-TAb were able to bind to the immobilized HER-2 recombinant protein. As shown in Fig. 2A, there was time-dependent expression in vitro around 268.8 ± 31.1 and 467.1 ± 30.6 ng/mL at days 3 and 7, respectively after infection, whereas there was no anti-HER-2 antibody expression in Ad5-LacZ–infected L-02 cells during the whole course. To further characterize the anti-HER-2 antibody light and heavy chain expressed by Ad5-TAb in vitro, the proteins in culture supernatant were separated on SDS-PAGE under reducing or nonreducing conditions, then transferred onto the nitrocellular membrane and subjected to Western blot analysis. The membrane was probed with goat anti-human IgG antibody as primary antibody that recognized both antibody heavy and light chain and horseradish peroxidase–conjugated rabbit anti-goat IgG as secondary antibody. Under reducing conditions, only one single band at molecular weight of 150 kDa was detected in the culture supernatant from the Ad5-TAb–infected L-02 cells, which is the expected size of dimerized full-length antibody containing two heavy and two light chains. No additional bands, which are normally expected when the ratio of heavy and light chain is balanced, were detected under nonreducing conditions (Fig. 2B), suggesting antibodies produced in hepatocytes in vitro were properly dimerized, and neither heavy nor light chain was in excess. Under reducing conditions, two protein bands at molecular weight of 50 and 25 kDa were detected, corresponding to IgG heavy and light chain proteins of trastuzumab, respectively (Fig. 2C). Bands of same size were detected in commercial trastuzumab under reducing and nonreducing conditions (Fig. 2B and C).

**Binding specificity of antibody expressed from Ad5-TAb.** The binding activity and specificity of the antibodies expressed by Ad5-TAb was evaluated by indirect immunofluorescence. Anti-HER-2 antibody expressed from Ad5-TAb was able to recognize HER-2 expressed on the cell surface. As shown in Fig. 3, there is strong FITC fluorescence signal on the surface of HER-2+ SKOV-3 cells, indicating that expressed antibody binds to HER-2 on the cell surface. There is no FITC fluorescence on the surface of HER-2−/BT549 cell, revealing that no antibody binds to BT549 cell due to negative HER-2 expression. Commercial trastuzumab exhibited the same FITC fluorescence density on HER-2−expressing cells as antibody generated by Ad-TAb, denoting that antibody Ad-TAb–expressing antibody carries the same activity as commercial antibody.

**Affinity dissociation of Ad5-TAb–encoded antibody in vivo.** Affinity is the most important variable characterizing the antibody bioactivity. According to formula of the affinity constant, $A_0 / (A_0 - A) = 1 + K_D/a_0$ (35), the $K_D$ of serum anti-HER-2 antibody in nude mice and commercial trastuzumab were determined as $(1.11 \pm 0.02) \times 10^{-10}$ mol/L and $(1.41 \pm 0.04) \times 10^{-10}$ mol/L, respectively, which are accordant with the reported value (0.1 nmol/L) of commercial trastuzumab (ref. 36; Fig. 4). Thus, it suggests that Ad5-TAb produced antibody in nude mice may be the same bioactive as commercial antibody.

![Fig. 3. Specific binding of anti-HER-2 antibody expressed from Ad5-TAb.](image-url)
expressed antibody can produce antitumor effect in the first 4 weeks is >4.0. The overall serum antibody concentration declined gradually. The high serum levels of antibody expression over the time course and the peak serum concentration of 158.6 ± 38.5 μg/mL at day 7 and then it declined gradually. The overall serum antibody concentration in the first 4 weeks is >40 μg/mL.

**Inhibition of in vivo tumor growth.** Having documented the high and sustained antibody expression in nude mice after Ad5-TAb mediated gene transfer, we further investigated whether the expressed antibody can produce antitumor effect in vivo. Given high serum levels of antibody expression in vivo, we did two different therapeutic approaches. In early treatment group as shown in Fig. 5A, significant antitumor activity was observed in Ad5-TAb–treated group with almost no visible tumor nodules growing up to 35 days; s.c. tumor nodules regressed completely in 6 of 10 mice, tumors went down to 10 mm³ in another two mice, and the rest two mice’s tumor volumes were 100 mm³ or so. Compared with Ad-TAb–treated mice, Ad5-LacZ–injected control group carried much larger tumors with average size of 683.2 ± 220.9 mm³. There is a significant statistic difference between these two groups (P < 0.01). Our data suggest that a single administration of Ad5-TAb resulted in high and sustainable antibody serum level that is sufficiently able to control tumor burden.

Because a single administration of anti-HER-2–expressing Ad5-TAb resulted in high and stable antibody serum level, we further studied whether this Ad5-TAb could achieve the therapeutic effect on animals burdened large tumors (>200 mm³). As shown in Fig. 6B, tumor growth was greatly inhibited in the Ad5-TAb–treated mice; the average tumor size was 417.8 ± 83.8 mm³ compared with 1,005.9 ± 189.8 mm³ in the Ad5-LacZ–treated mice. There is a statistic significant difference between Ad-TAb–treated and nontreated group (P < 0.05). All of these data suggest not only high and stable level of therapeutic antibody is critical for antibody cancer therapy, but also early treatment is necessary to obtain the satisfactory effect. Thus, antibody expressed by the recombinant adenovirus system is an effective and low-cost method to control tumor growth.

**Discussion**

Trastuzumab, a commercially available antibody proved by Food and Drug Administration, has been widely used clinically as anticancer medicine and proven to be very efficient in tumor inhibition. We have successfully achieved in vivo biofunctional therapeutic full-length anti-HER-2 expression by adenovirus and showed efficient anticancer efficacy after a single administration via tail vein in nude mice.

Biomacromolecules expression via gene therapy has been considered very difficult to keep their bioactivity, which brings up the issue whether it is feasible to introduce antibody therapy via gene transfer. Our study presented here strongly proved that antibody-gene therapy is absolutely applicable. Antibody generated in mammalian human cells in vivo needs to be glycosylated, which might not be precisely modified in Chinese hamster ovary cells. The novel antibody-gene therapy strategy via gene transfer provides many advantages: (a) long-term sustained antibody expression, (b) high serum antibody concentration, and (c) lacking the sophisticated purification and preparation process. This strategy can also provide a fast evaluation of antibody on tumor...
its safety, effect, and de novo antibody kinetic study in vivo. This novel adenovirus-mediated antibody therapy approach in vivo was confirmed to be feasible and produced antibody function very properly.

Full-length antibody expression in a single vector can be achieved only on the premise of coexpression of antibody heavy and light chain. Recently, novel linking sequence 2A and CHYSEL have been introduced to obtain full-length antibody heavy-chain and light-chain expression by a single promoter (17, 37–40). Fang was able to engineer the mAb expression cassette that, in the context of AAV-mediated gene transfer, resulted in high levels of full-length, functional mAb in vitro and in vivo with equivalent amounts of heavy and light chain. However, the 23-amino-acid “tail” adjacent to COOH terminus of heavy chain may influence the antibody conformational structure and in turn adversely affects its biofunction. Furthermore, the 23-amino-acid 2A self-processing peptide, although cleaved, might be processed and presented by MHC classes I and II and therefore may be detrimental to the host.

Anti-HER2 full-length antibody produced in our system is biofunctionally active. Consistent with the similarity of affinity constant between gene transfer generated antibody and commercial trastuzumab in vitro and in vivo generated antibody showed the same level binding quantification and qualification to HER-2 either negative or positive cells. All of these data indicated that the expressed full-length antibodies via gene transfer in vitro or in vivo are biofunctional, suggesting that gene transfer providing biofunctional biomacromolecules, such as antibody, is possible and practical.

Because of the in vivo potential initiating immune response risk of 2A self-processing peptide, IRES was employed to construct bicistronic vector for antibody heavy-chain and light-chain expression. Due to that, IRES was described to lead to substantial lower expression of the second gene (17). And being more cautious about the possible toxic effects in vivo owing to the imbalance of heavy-chain and light-chain expressions, heavy-chain gene was cloned behind IRES to avoid renal toxicity induced by extra antibody heavy-chain molecules. However, there is no extra light-chain expression observed in our experiment. In addition, we proved that the well-balanced coexpression of heavy and light chain was gained by this strategy. Adenovirus has been used for in vivo full-length antibody gene transfer and induced mouse mAb serum concentration >200 μg/mL for >1 month in mice, although the antibody against adenoviral backbone was detected in the serum, indicating the possibility and rationality of adenoviral vector applied for delivering therapeutic antibody (41). However, mouse mAb expressed in mice activates much lighter and milder immune response than humanized mAb; moreover, there was no confirmation of antibody bioactivity in vitro and therapeutic effect in vivo reported in Noel’s article.

We have been dedicating to the full-length antibody therapy for cancer, which is one of the most threatening diseases. Our data clearly showed that early antibody therapy on cancer exerted the much better prognosis on tumor-burdened mice. Meanwhile, late antibody therapy in large tumor is still meaningful and is able to slow down the tumor growth, to reduce the metastasis, and to prolong the survival of experimental mice. In summary, recombinant adenovirus 5 may have broad application as an in vivo full-length antibody therapy for cancer and other diseases.

This system is more efficient than traditional antibody protein therapy. Avoiding the complicate antibody protein preparation and purification process, this system could be used for antibody assessment and development with short duration as well.

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![Fig. 6. Antitumor activity of Ad5-TAb -- generated anti-HER-2 antibody via gene transfer in tumor-burdened nude mice. A, early stage with smaller size (< 20 mm³). B, late stage with large size (> 200 mm³).](image-url)
References

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