Construction and Immunological Evaluation of Dual Cell Surface Display of HIV-1 Gag and Salmonella enterica Serovar Typhimurium FliC in Lactobacillus acidophilus for Vaccine Delivery

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Oral vaccines that elicit a mucosal immune response may be effective against human immunodeficiency virus type 1 (HIV-1) because its transmission occurs mainly at the mucosa. The aim of this study was to construct recombinant Lactobacillus for oral delivery of oral vaccines against HIV-1 and to evaluate their immunogenicity. A recombinant Lactobacillus acidophilus strain expressing the HIV-1 Gag on the bacterial cell surface was established by fusion with the signal peptide and anchor motif of a mucus binding protein (Mub) from L. acidophilus with or without coexpression of Salmonella enterica serovar Typhimurium flagellin (FliC) fused to a different Mub signal peptide and anchor. Using HEK293 cells engineered to express Toll-like receptor 5 (TLR5), the biological activity of FliC on the bacterial cell surfaces was determined. The surface-exposed flagellin retained its TLR5-stimulating activity, suggesting that the recombinant strain with Gag and FliC dual display might provide a different immunopotency than the strain expressing only Gag. The immunological properties of the recombinant strains were assessed by coculture with human myeloid dendritic cells (DCs). The heterologous antigens on the cell surface affected maturation and cytokine responses of DCs. Acquired immune responses were also investigated by intragastric immunization of mice. The enzyme-linked immunosorbent spot assay showed induction of gamma interferon-producing cells at local mucosa after immunization of mice with the Gag-producing strain. Meanwhile, the immunization with L. acidophilus displaying both Gag and FliC resulted in an increase of Gag-specific IgA-secreting cells. These results suggested that the Gag-displaying L. acidophilus elicited specific immune responses and the coexistence of FliC conferred an adjuvant effect on local IgA production.

The global epidemic of human immunodeficiency virus (HIV) infection has imposed a heavy burden on our society. In spite of intensive research, no fully effective vaccine has been discovered. The first and only successful phase 3 human trial using a combinational HIV vaccine was reported in 2009; however, the efficacy remains limited (38). Tremendous efforts on the vaccine development have been continuously made by many researchers using various approaches (12). Transmission of HIV usually occurs at mucosal surfaces, especially the rectal and genital mucosa, and mucosa-associated lymphoid tissues are primary sites for virus replication. Mucosal vaccines are potentially capable of stimulating the mucosal immune system and inducing local and systemic immune responses. Hence, mucosal vaccines that elicit HIV-specific immune responses at the local mucosa may be more effective than conventional parenteral vaccines, which usually provide only systemic immunity.

Lactic acid bacteria are potential delivery vehicles for mucosal immunization because they provide immunopotency and safety aspects, which are essential for practical vaccines. Many previous studies have demonstrated that commensal microbes carrying antigens can confer protective immunity (39, 40). For example, recombinant Lactococcus lactis expressing the HIV type 1 (HIV-1) V2-V4 loop of Env induced antigen-specific immune responses that protected mice from challenge with recombinant vaccinia virus carrying the HIV env gene (41). The usefulness of L. lactis as an HIV vaccine, however, remains unclear because cholera toxin, which is a strong but toxic mucosal adjuvant, was required for protection. It is likely that vaccines based on lactic acid bacteria in combination with adjuvant molecules could offer better efficacy (5, 23, 34). In this regard, coexpression of safe adjuvant molecules may be needed for effective immunization when using lactic acid bacteria as delivery vehicles.

Flagellin, the major subcomponent of bacterial flagella, is the ligand of Toll-like receptor 5 (TLR5) and is also recognized by one of the cytosolic nucleotide binding oligomerization domain-like receptors (NLRs), NLRC4/IPAF. The flagellar antigen initiates innate immune responses by activating NF-κB via TLR5 and the caspase-1 inflammasome through NLRC4 (11, 15, 26, 27). Since antigens fused with flagellin have been shown to enhance immune responses, the use of flagellins as adjuvants is under consideration (4, 16, 17, 25). Lactobacillus strains are Gram-positive bacteria, which intrinsically present microbe-associated molecular patterns (MAMPs) such as lipoproteins and lipoteichoic acid (LTA) that are recognized by TLR2 and TLR2/6 (2, 6, 20, 24). Meanwhile, most lactobacilli, including Lactobacillus acidophilus, lack TLR5-stimulating activity due to the absence of flagella. In our previous study, two types of recombinant L. acidophilus displaying Salmo-
Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Plasmid</th>
<th>Description of bacterial strain and/or plasmid</th>
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<td>NCK2166</td>
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<td>Cell surface display of Gag</td>
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**MATERIALS AND METHODS**

Bacterial strains and growth conditions. Lactobacillus acidophilus NCFM (NCK56) and derivative recombinant strains were grown statically in MRS broth (Difco Laboratories, Detroit, MI) supplemented with or without 5 μg/ml of erythromycin (Em) at 37°C. MRS agar (1.5% agar) plates were incubated anaerobically. Escherichia coli DH5α and other recombinant strains were grown aerobically with shaking in LB medium (Difco) with or without 200 μg/ml of Em at 37°C. All bacterial strains used in this study are shown in Table 1.

Construction of plasmids. Regular and overlap PCRs were performed using the primers shown in Table 2. The design of an expression cassette is described in Fig. 1. Each subunit was generated by PCR and then combined with other recombinant components that were grown aerobiologically with shaking in LB medium (Difco) with or without 200 μg/ml of Em at 37°C. All bacterial strains used in this study are shown in Table 1.

**TABLE 1**

Bacterial strains and plasmids

<table>
<thead>
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**TABLE 2**

Primers used in this study

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<td>BglII, NcoI</td>
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Restricted sites are underlined.

For Western blotting, Detection of cell wall-associated proteins via Western blotting was described previously (19). In brief, bacterial cells collected from exponential-phase cultures were treated with mutanolysin and homogenized by bead beating. Protein inhibitor cocktail for bacterial cells (Sigma-Aldrich, St. Louis, MO) was supplemented to all buffers used in the preparation. The cell extracts were applied to SDS-polyacrylamide gels using 7.5% TGE precast gel (Bio-Rad, Hercules, CA) and then transferred to a polyvinylidene difluoride membrane by semidry blotting. Precision Plus Protein WesternC protein standard (Bio-Rad) was also loaded as a standard marker. The blot was incubated with a monoclonal anti-p24 IgG antibody (clone 183-H12-5C; from Bruce Chesebro and Kathy Wehrly, NIH AIDS Research and Reference Reagent Program) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA). Chemiluminescence using Luminata Forte Western HRP substrate (Millipore) was visualized by use of a ChemiDoc XRS+ system (Bio-Rad).

TLR5 stimulation assay. The reporter assay using TLR5-expressing HEK293 cells was described previously (35). Freshly prepared bacteria were added to the culture of TLR5-expressing cells and incubated for 48 h. TLR5 activation was determined by NF-κB induction using the secreted embryonic alkaline phosphatase (SEAP) pNiFty reporter plasmid (InvivoGen, San Diego, CA). SEAP activity in the coculture supernatants was detected by luminometer after development with the SEAP reporter gene assay per the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN).
Isolation of human myeloid DCs and coculture assay. Untested human blood buffy coats were obtained from the American Red Cross (Durham, NC). Human myeloid dendritic cells (DCs) were isolated in accordance with the method described previously (35). In brief, lymphocytes were separated from whole blood using Ficoll-Plaque Plus (GE Healthcare) and centrifugation. Enrichment of CD1c were separated from whole blood using Ficoll-Plaque Plus (GE Healthcare, NC). Human myeloid dendritic cells (DCs) were isolated in accordance with the procedure described previously (35). The care and use of experimental animals complied with the guidelines of North Carolina State University.

ELISpot assay. Ninety-six-well MultiScreen-IP filter plates (Millipore) were pretreated with 70% ethanol and washed three times with 200 µl sterile distilled water, before being coated with 10 µg/ml anti-mouse IgA (Mabtech, Mariemont, OH). The plates were incubated overnight at 4°C. Before addition of cells, plates were washed five times in sterile PBS and blocked with cytotoxic T lymphocyte (CTL) medium (35) at 37°C for 2 h. Cells were added in duplicate to the wells at a concentration of 2.5 x 10^5 per well, and then plates were incubated for 16 h for total IgA and 20 h for Gag (p24)-specific IgA at 37°C. To detect spots, plates were initially washed in PBS containing 0.05% Tween 20 (PBST) to remove cells. For the total IgA enzyme-linked immunosorbent spot (ELISpot) assay, 1 µg/ml biotinylated polyclonal goat anti-mouse IgA (Mabtech) was added in blocking buffer (PBS containing 1% BSA). For the p24-specific IgA, 0.5 µg/ml biotinylated p24 (Prospect, East Brunswick, NJ) diluted in blocking buffer was added. Biotinylated antibodies were incubated in the plates for 2 h at room temperature. Plates were washed three times in PBST and three times in PBS before addition of 100 µl of filtered 3,5,5-tetramethylbenzidine (Mabtech). Plates were incubated for 10 min at room temperature and then washed 10 times in water and air dried. Spots were counted in an ImmunoSpot analyzer (Cellular Technology Ltd., Shaker Heights, OH). For detection of IFN-γ producing cells, isolated lymphocytes were stimulated with HIV-1 lysate at 10 ng/ml. The virus lysate was prepared from aldritol-2 (AT-2)-inactivated HIV-1 IIIB, including isotopic Gag, by sonication for 1 min. The protein concentration of the virus lysate was measured by enzyme-linked immunosorbent assay (ELISA). Plates were blocked with 200 µl CTL medium and then coated with 50 µl of IFN-γ capture antibody (Mabtech) at a concentration of 10 µg/ml overnight at 4°C. Control wells consisted of unstimulated cells or cells stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (300 ng/ml). Cells were added at a concentration of 2.5 x 10^5 per well and incubated for 48 h at 37°C in 5% CO2. Plates were then washed six times with PBST and incubated with biotinylated IFN-γ detection antibody (Mabtech) at 1 µg/ml for 3 h at room temperature. Plates were washed 6 times, and 100 µl/well streptavidin-HRP (1 µg/ml; Pierce Biochemical, Rockford, IL) was added for 1 h. Plates were washed with PBST and PBS and then developed with 3-amin-9-ethylcarbazole substrate (Sigma, St. Louis, MO). The reaction was stopped after 8 min by rinsing the plates with...
deionized water. The assay was run in duplicate, and spot-forming cells were counted and analyzed on the ImmunoSpot analyzer. To determine the number of spot-forming cells, the counts for duplicate wells were averaged, background counts from unstimulated wells were subtracted, and the total was normalized to 1/100,000 cells.

**Statistical analysis.** The Wilcoxon signed-rank test was applied to evaluate significant differences in assays using DCs. For multiple comparisons, the level of significance was adjusted by use of the Bonferroni correction. Tukey’s multiple-comparison test was used for in vivo experiments.

**RESULTS**

**Expression and cell surface display of Gag and FliC.** Three recombinant *L. acidophilus* strains, NCK2152, NCK2162, and NCK2166, were constructed in this study. NCK2152 and NCK2166 harboring pTRK1030 and pTRK1037, respectively, were expected to express either FliC or Gag on the cell surface, respectively. NCK2162 carrying pTRK1035 was engineered to display both FliC and Gag. Production of the Gag protein by these strains was confirmed by Western blotting. As shown in Fig. 2a, Gag-specific bands were detected in NCK2162 and NCK2166. The estimated molecular mass of the recombinant protein on the cell surface is approximately 71 kDa where the major bands appeared. The TLR5-stimulating activity of the FliC-producing strains was determined by a reporter gene assay. Mean values of the fold increase in NF-κB activation with standard errors are calculated from triplicate assays. (c) Cell surface display of the proteins was detected by flow cytometry. Bacterial cells stained with antigen-specific antibodies followed by fluorochrome-conjugated secondary antibodies were analyzed in two-dimensional dot plots.

**Maturation and cytokine responses of human myeloid DCs induced by recombinant *L. acidophilus.*** The immunological impact of stimulation with the *L. acidophilus* strains on human DCs was assessed in vitro. Freshly isolated CD1a⁺ myeloid DCs from human blood were incubated with the recombinant lactobacilli. Before incubation, the DCs expressed low levels of CD40, CD83, CD80, and CD86 (Fig. 3a). These maturation markers were upregulated by 24 h, regardless of the bacterial strain used. For CD40 and CD83, however, stimulation with *L. acidophilus* caused additional upregulation. In order to compare the expression levels of CD40 and CD83 induced by different *L. acidophilus* strains, the median fluorescence intensity of CD40 and the percentage of CD83⁺ cells were analyzed. As shown in Fig. 3b, the levels of those molecules were slightly different depending on the strain. The DCs stimulated with NCK2162 expressed higher levels of CD40 than those stimulated with NCK2152 in all donors. NCK2166 induced CD83⁺ cells more efficiently than NCK2162 or NCK1895. For cytokine re-

![FIG 2 Recombinant *L. acidophilus* strains producing/displaying Gag, FliC, or both. Production and cell surface display of Gag, FliC, or both were confirmed by combination of different techniques. (a) Gag-specific bands were detected by Western blotting. Cell extracts of recombinant *L. acidophilus* strains and purified Gag protein were analyzed. Molecular masses were estimated using protein standards and are shown on the left margin. (b) TLR5-stimulating activity was determined by a reporter gene assay. Mean values of the fold increase in NF-κB activation with standard errors are calculated from triplicate assays. (c) Cell surface display of the proteins was detected by flow cytometry. Bacterial cells stained with antigen-specific antibodies followed by fluorochrome-conjugated secondary antibodies were analyzed in two-dimensional dot plots.](http://cvl.asm.org/ on May 20, 2021 by guest)
responses, NCK2162-stimulated DCs secreted larger amounts of IL-1ß and IL-6 than NCK1895-stimulated cells (Fig. 4). IL-12 production by NCK2162 stimulation was higher than that by NCK2152 stimulation. TNF-α was induced more efficiently by NCK2166 than by NCK1895. Extremely high levels of IL-8 production and low levels of IFN-γ secretion were also observed. Overall, the immune responses of the DCs stimulated with the recombinant lactobacilli varied widely, depending on the donors. This may be due to dissimilar genetic and immunological backgrounds. Since each datum obtained using DCs from the same donor was correlated, a statistical analysis for dependent samples was applied (Wilcoxon signed-rank test).

Induction of antigen-specific IgA and IFN-γ-producing cells by oral immunization of mice. To investigate whether Gag-displaying recombinant L. acidophilus would elicit specific immune responses, mice were immunized through a mucosal route by oral gavage. After repeated doses, mice were sacrificed to collect lymphoid tissues/organs. For detection of IgA-producing cells at local mucosa, total lymphocytes from Peyer’s patches, large intestine, and female reproductive tract were prepared and incubated to perform ELISpot assay. Total IgA-producing cells were present at 1.0 × 10^4 to 1.7 × 10^4 cells/million cells in Peyer’s patches, 1.0 × 10^5 to 6.5 × 10^3 cells/million cells in female reproductive tract, and 3.0 × 10^4 to 6.3 × 10^4 cells/million cells in large intestine. No significant difference in total IgA-producing cells was observed except for cells from the female reproductive tract from the NCK1895-immunized group (Fig. 5a). The isolated lymphocytes secreting Gag-specific IgA were also counted. As shown in Fig. 5b, a significant increase in Gag-specific IgA-producing cells was present in the female reproductive tract and large intestine from the NCK2162-immunized group. Meanwhile, Gag-specific antibodies were not titrated by ELISA due to their weak or nonspecific signals (data not shown). IFN-γ-producing cells were counted after incubation with HIV-1 lysate. Relatively large numbers of IFN-γ-producing cells were detected in mucosal lymphoid tissues, while few such cells were found in spleen (Fig. 6). In Peyer’s patches and female reproductive tract, significantly higher frequencies of IFN-γ-producing cells were observed in the NCK2166-immunized group. Especially in Peyer’s patches, a remarkable difference between the NCK2166-stimulated group and the other groups was shown. A relatively high background of IFN-γ-producing cells was observed in the female reproductive tract.
In the current study, recombinant *L. acidophilus* strains displaying HIV-1 Gag with or without coexpression of FliC as an adjuvant on the cell surface were constructed and were tested for in vitro and in vivo immunogenicity. To date, many *Lactococcus lactis* and *Lactobacillus* strains have been engineered to be potential mucosal vac-

**FIG 4** Cytokine profiling in cultures of human myeloid DCs stimulated with or without the recombinant lactobacilli. Seven cytokines in culture supernatants of stimulated DCs (24 h) were detected by use of a Milliplex suspension bead assay system. The concentrations of each cytokine are plotted individually. The median fluorescence intensity values of IL-8 and IFN-α2 are shown only for reference because the values were out of the certified detection range. The statistical significance of differences between each group was analyzed by the Wilcoxon signed-rank test with the Bonferroni correction. A *P* value of <0.01 is considered a significant difference in this multiple comparison.

**FIG 5** Counts of lymphocytes secreting IgA (total or Gag specific). Lymphocytes were isolated from Peyer’s patches (PP), female reproductive tract (FRT), and large intestine (LI) and analyzed by ELISpot assay. (a) Total IgA-producing cells were counted after 48 h incubation without stimulation. (b) Lymphocytes secreting Gag-specific IgA were detected after stimulation with HIV-1 lysate. Each column in the charts represent lymphocytes prepared from non-immunized (gray open bars), NCK1895-immunized (gray filled bars), NCK2162-immunized (black open bars), and NCK2166-immunized (black filled bars) mice. Tukey’s multiple-comparison test was applied for statistical analysis. A *P* value of <0.05 is considered a significant difference.

**FIG 6** Detection of IFN-γ-producing lymphocytes in the immunized mice. Lymphocytes were isolated from spleen (SP), Peyer’s patches (PP), female reproductive tract (FRT), and large intestine (LI) and applied for ELISpot assay. IFN-γ-producing cells were counted after 48 h stimulation with HIV-1 lysate. Each column in the chart represents lymphocytes prepared from non-immunized (gray open bars), NCK1895-immunized (gray filled bars), NCK2162-immunized (black open bars), and NCK2166-immunized (black filled bars) mice. Tukey’s multiple-comparison test was applied for statistical analysis. A *P* value of <0.05 is considered a significant difference.

**DISCUSSION**

In the current study, recombinant *L. acidophilus* strains displaying HIV-1 Gag with or without coexpression of FliC as an adjuvant on the cell surface were constructed and were tested for in vitro and in vivo immunogenicity. To date, many *Lactococcus lactis* and *Lactobacillus* strains have been engineered to be potential mucosal vac-
cine vectors. Most of these studies investigated the immunological properties of recombinant lactic acid bacteria producing antigen alone. A few have demonstrated the effectiveness of providing additional adjuvanticity via genetic modification of the bacteria. Adjuvant has more often been provided in trans and rarely in cis. For example, single-chain mouse IL-12 was produced by L. lactis and administered to mice along with another recombinant L. lactis strain expressing E7 antigen of human papillomavirus type 16 (5). Similarly, L. casei engineered to secrete mouse IL-1β was mixed with heat-killed Salmonella Enteritidis and dosed orally (18). An alternative approach employed by Mohamadzadeh et al. was to construct lactobacilli expressing antigen fused with a dendritic cell-targeting peptide to enhance uptake of antigen by DCs. This approach successfully protected mice against Bacillus anthracis using L. acidophilus as a delivery vehicle (29). Another study tested recombinant L. lactis secreting either murine IL-2 or murine IL-6 with coexpression of tetanus toxin fragment C (34). All of these approaches improved the efficacy and expanded the potential of lactic acid bacteria as delivery agents.

The strategy employed here was based on two lines of study. The first was the clear demonstration that stimulation of multiple TLRs can greatly enhance immune responses (21, 32). The second was the growing evidence that flagellin can be a potent adjuvant (28). In both cases, it is also clear that antigen delivery and TLR activation must be targeted to the same antigen-presenting cell as a fusion product or coformulation (1, 16, 25, 31). We employed the unique approach of displaying both antigen and adjuvant simultaneously on the bacterial cell surface using FlIc as the adjuvant. The recombinant surface FlIc-induced NF-κB via TLR5 in a reporter assay, thereby proving that it was biologically active. Lactic acid bacteria produce lipoteichoic acid, diacylated peptidoglycans, and muramyl dipeptides that lead to activation of TLR2, TLR2/6, and NOD2, respectively (22). Expression of FlIc resulted in an expansion of the TLR activation profile, as L. acidophilus and the majority of other Lactobacillus strains are intrinsically devoid of flagella.

DCs are professional antigen-presenting cells and play a crucial role in initiating acquired immune responses. Myeloid DCs are especially important in relation to HIV-1 infection and host defense (8). In the current study, immune responses elicited in human myeloid DCs stimulated with the recombinant lactobacilli were analyzed. The bacterial stimulation caused maturation and cytokine production of the DCs, regardless of the recombinant cell surface antigens. Previous studies showed that maturation and the cytokine responses of mouse or human DCs were induced by lactobacilli in a species/strain-dependent manner (7, 9, 30, 42). This is apparently the result of variation of the MAMP composition and structure (13, 24, 33). In this study, additional effects on DC maturation and cytokine production resulted from expression of recombinant antigens/adjuvants on the cell surface. Although there was no drastic alteration in DC maturation or cytokine production, several responses were enhanced by the Gag- and FlIc-expressing recombinant L. acidophilus. The strain displaying Gag alone induced greater CD83 upregulation and TNF-α secretion in the DCs than the reference strains. Large relative amounts of IL-1β and IL-6 were produced in the DCs stimulated with the strain displaying both Gag and FlIc compared to the amounts produced in the DCs stimulated by the control strain. It is critical to keep in mind that the DC is not the only cell that will ultimately be influenced by an orally delivered recombinant lactobacillus vaccine vector and that other immune cells and, importantly, the mucosal epithelium may play a prominent role in the immune response.

To this end, in vivo evaluation of immunogenicity is essential, and therefore, recombinant L. acidophilus strains were assessed by intragastric immunization in a murine model. Gag-specific IgA-producing cells were found in the large intestines and female reproductive tract of mice immunized with the recombinant strain displaying Gag and FlIc but not those immunized with the recombinant strain displaying Gag alone. This result implies that the coexpression of FlIc on the bacterial cell surface promotes induction of Gag-specific IgA-producing cells at the local mucosa. The fact that Gag-specific IgA cells were found in the lamina propria and not in the Peyer’s patches is consistent with a TLR5-mediated mechanism of induction. As shown by Uematsu and colleagues, CD11c+CD11b+ TLR5-positive DCs reside in the lamina propria and induce T cell-independent antigen-specific IgA-class switching of resident naïve B cells, and this requires flagellin signaling through TLR5 (36, 37). In contrast to the specific IgA responses, the L. acidophilus strain expressing Gag alone induced specific IFN-γ-producing cells most efficiently at the local mucosa. This result suggests that this strain promotes cellular immunity rather than humoral immunity. Thus, we show here that two different Gag-displaying L. acidophilus strains have dissimilar immunogenicity depending on the coexpression of FlIc. Additional studies will be required to fully elucidate the induction pathways of the cellular versus humoral responses and to determine whether both responses can be induced either with a mixed inoculum containing both recombinant strains or through temporal separation of immunization with each individual recombinant strain.

The major routes of HIV-1 transmission are mucosal surfaces of the lower intestine and genital tracts. Thus, vaccines which can confer appropriate HIV-1-specific immunity at these sites may provide robust protection. This study provides proof of concept that recombinant L. acidophilus without external mucosal adjuvant could be applied for development of oral vaccines against HIV-1 as a delivery agent. The next step is to determine whether immunogenic HIV-1 envelope antigens can be expressed by recombinant lactobacilli and whether a combination of local mucosal IgA- and cell-mediated responses can provide protection against challenge.

ACKNOWLEDGMENTS

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REFERENCES