Tumor-reactive T Helper Lymphocytes Recognize a Promiscuous MAGE-A3 Epitope Presented by Various Major Histocompatibility Complex Class II Alleles

Hiroya Kobayashi, Yongsheng Song, Dave S. B. Hoon, Ettore Appella, and Esteban Celis

ABSTRACT

The development of effective T cell-based immunotherapy for cancer requires the identification of antigens capable of inducing both CTL and T helper immune responses. Although CTLs will participate in the antitumor response mainly by exerting their lytic activity on the tumor cells, helper T lymphocytes will be critical for the induction and maintenance of the CTLs. Thus, effective subunit therapeutic vaccines should include both CTL and T helper epitopes from antigens expressed on the tumor cells. The product of the MAGE-A3 gene is an attractive candidate for tumor immunotherapy because it is expressed in the majority of melanomas and in a great proportion of other solid tumors. Although numerous CTL epitopes for the MAGE-A3 antigen have been reported, only a few have been described for helper T cells. Here we show that a synthetic peptide derived from the MAGE-A3 sequence (MAGE-A3146–160) was effective in inducing in vitro T helper responses in the context of HLA-DR4 and HLA-DR7 alleles. Most significantly, the peptide-reactive helper T lymphocytes were capable of recognizing various forms of MAGE-A3 antigen (tumor cell lysates, dead/apoptotic tumor cells, or recombinant MAGE-A3 protein), indicating that the T-cell epitope represented by peptide MAGE-A3146–160 is naturally processed by antigen-presenting cells. These studies are relevant for the design of multi-epitope vaccines for treating MAGE-A3-expressing tumors through the simultaneous stimulation of CTL and T helper lymphocytes.

INTRODUCTION

The MAGE-A3 gene encodes for a protein antigen that is expressed in ~76% of melanomas and in a large proportion of other solid tumor types but is not detected in normal tissues, with the exception of the testicular germinal cells (1). Because the MAGE-A3 antigen can be recognized by tumor reactive CTLs, it is an ideal candidate for the development of antitumor therapeutic vaccines. Numerous CTL epitopes from the MAGE-A3 antigen have been identified, which were found to be restricted by commonly found MHC class I alleles such as HLA-A1 (2, 3), HLA-A2 (4, 5), HLA-A24 (6, 7), HLA-B37 (8), and HLA-B44 (9). As a result of these studies, clinical trials have been initiated in cancer patients using vaccines prepared with synthetic peptides corresponding to some of the CTL epitopes (10–14). Although promising, but nevertheless anecdotal, clinical responses have been reported in some of these studies, there is agreement that the current vaccination strategies need to be optimized to attain the desired therapeutic effects. One approach that is being given serious consideration is the design of vaccines capable of stimulating both tumor-reactive CTLs and HTLs.

Because HTLs play an important role both in the induction and maintenance of CTL responses, vaccines that activate both CTLs and HTLs should be more effective than vaccines that only target CTL responses (15). Experiments in animal models have demonstrated the importance of antigen-specific HTLs in the elimination of tumors by CTLs (16, 17). Thus, one obvious way to improve MAGE-A3 vaccines that induce CTLs would be to include HTL epitopes for this tumor antigen. Recently, three peptides from MAGE-A3 were described as HTL epitopes, which were restricted by the HLA-DR13 (18), HLA-DR11 (19), and HLA-DP4 alleles (20). Our laboratory focuses in the identification of “promiscuous” HTL epitopes, which can be recognized by HTLs in the context of more than one MHC class II allele. Using a computer-based algorithm (21), we have been successful in identifying promiscuous HTL epitopes from other tumor antigens such as HER2/neu (22), gp100, and carcinoembryonic antigen (23). Here we report that by using this approach, we have now identified a peptide from MAGE-A3 capable of triggering HTL responses restricted by HLA-DR4 and HLA-DR7, which are frequently found alleles in melanoma patients. Both HLA-DR4- and HLA-DR7-restricted HTLs, which were generated with peptide MAGE-A3146–160, were effective in recognizing naturally processed antigen in the form of melanoma tumor lysates, apoptotic melanoma cells, or recombinant MAGE-A3 protein. The HTL epitope described here, together with the three previously reported ones (18–20) should offer ample coverage to the melanoma (Caucasian) patient population.

MATERIALS AND METHODS

Cell Lines. Melanoma cell lines 697mel and 624mel were provided by S. Rosenberg (Surgery Branch, National Cancer Institute, NIH, Bethesda, MD). The melanoma cell lines HT-144 and SK-MEL-28 were obtained from the American Type Culture Collection (Manassas, VA). EBV-transformed lymphoblastoid cells (EBV-LCL) were produced from peripheral blood mononuclear cells of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line. Mouse fibroblast cell lines (L-cells) transfected and expressing individual human MHC class II molecules were kindly provided by R. W. Karr (Park-Davis, Ann Arbor, MI).

Synthetic Peptides and Recombinant Antigens. Potential HLA-DR promiscuous CD4+ T-cell epitopes were selected from the amino acid sequence of the MAGE-A3 antigen using the algorithm tables for three HLA-DR alleles (DRB1*0101, DRB1*0401, and DRB1*0701) described by Southwood et al. (21). Peptides that displayed high algorithm scores were synthesized and purified as described (22). The purity (>95%) and identity of peptides were determined by mass spectrometry. Recombinant MAGE-A3 and gp100 proteins were produced and purified as described (23–25). Recombinant HER2/neu protein was kindly provided by Corixa Corp. (Seattle, WA).

In Vitro Induction of Antigen-specific HTLs with Synthetic Peptides. The procedure selected for the generation of tumor antigen-reactive HTL lines and clones using peptide-stimulated PBMCs has been described in detail (22). Briefly, DCs were produced in tissue culture from adherent monocytes, cultured for 7 days at 37°C in a humidified CO2 (5%) incubator in the presence of cytokines: APC, antigen-presenting cell; TAA, tumor-associated antigen; TCR, T-cell receptor.

1 The abbreviations used are: HTL, helper T lymphocyte; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; GM-CSF, granulocyte/macrophage-colony-stimu-
of 50 ng/ml GM-CSF and 1000 IU/ml interleukin 4. Peptide-pulsed DCs (3 μg/ml for 2 h at room temperature) were irradiated (4000 rads) and cocultured with autologous purified CD4+ T cells (using magnetic microbeads from Miltenyi Biotec) in 96-well, round-bottomed culture plates. One week later, the CD4+ T cells were restimulated with peptide-pulsed irradiated autologous PBMCs, and 2 days later, human recombinant interleukin 2 was added at a final concentration of 10 IU/ml. One week later, the T cells were tested for their proliferative responses to peptide as described below. Those cultures exhibiting a proliferative response to peptide (at least 2.5-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous APCs. In some instances, T cell lines were cloned by limiting dilution and used for further characterization. Culture medium for all procedures consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers.

**Measurement of Antigen-specific Responses.** CD4+ T cells (3 × 10^6/ml) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides, tumors, or tumor lysates) in 96-well culture plates. APCs consisted of either PBMCs (1 × 10^6/well), HLA-DR-expressing L-cells (3 × 10^5/well), EBV-LCL or melanoma cells (3 × 10^5/well) that were treated with IFN-γ (500 units/ml) for 48 h to enhance MHC antigen expression. Tumor cell lysates were prepared by three freeze-thaw cycles of 10^6 tumor cells, resuspended in 1 ml of serum-free RPMI 1640. Lysates were used as a source of antigen at 5 × 10^3 cell equivalents/ml. Cell proliferation assays were incubated at 37°C for 72 h, and during the final 16 h, each well was pulsed with 0.5 μCi/well of [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto glass fiber filters. In some cases, culture supernatants were collected before the addition of [3H]thymidine for measuring antigen-induced lymphokine production by the HTLs using ELISA kits (PharMingen, San Diego, CA). To identify the MHC restriction molecules involved in antigen presentation, blocking of the antigen-induced proliferative response was investigated using anti-MHC class II antibodies. Anti-HLA-DR mononclonal antibody L243 (IgG2a) was prepared from supernatants of the hybridoma HB-55 obtained from the American Type Culture Collection (26), and anti-HLA-DQ mononclonal antibody SPV-L3 (IgG2a) was kindly provided by Dr. Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY; Ref. 27). Both mononclonal antibodies were used at a final concentration of 10 μg/ml throughout the 72-h assay, which in our hands is optimal for T-cell inhibition studies (22). All assessments of proliferative responses were carried out at least in triplicate, and results correspond to the mean cpm with the SD.

**RESULTS**

Selection of Potential Promiscuous HTL Epitopes for MAGE-A3. Because our original goal was to identify promiscuous MHC class II HTL epitopes for the MAGE-A3 antigen, we first examined the amino acid sequence of this molecule for the presence of peptide sequences containing binding motifs for HLA-DR*0101, HLA-DR*0401, and HLA-DR*0701. The binding motifs that we used are based on the algorithm values described by Southwood et al. (21), which calculate the potential (predicted) binding interactions of primary and secondary anchors of a 9-amino acid “core region” for each of the three MHC class II alleles. This approach has been very successful in our hands, allowing us to identify several promiscuous HTL epitopes from TAAs such as HER2/neu (22), gp100, and carcinoembryonic antigen.4 The two highest-ranking potentially promiscuous core sequences that were identified using this method were MAGE-A3_25-33 and MAGE-A3_151-159 (data not presented). Interestingly, MAGE-A3_151-159 is contained within the sequence of a peptide (MAGE-A3_146-160) that was reported recently by Manici et al. (19) to bind to several HLA-DR alleles including DR1, DR4, and DR7 (19). However, in the hands of these investigators peptide MAGE-A3_146-160 was not very effective at inducing significant T-cell responses. On the other hand, another peptide, MAGE-A3_281-295 did generate strong HTL responses that were restricted by HLA-DR11 (19). Because MHC class II molecules bind preferentially peptides of ~15 residues, we extended the size of the two candidate promiscuous epitopes and prepared synthetic peptides MAGE-A3_22-36, and MAGE-A3_146-160 which were used for subsequent in vitro T-cell immunization studies.

**T-Cell Responses to Peptides from MAGE-A3.** The two peptides selected from the promiscuous algorithm analysis (MAGE-A3_22-36, and MAGE-A3_146-160) were tested for their ability to stimulate T-cell responses using cells isolated from three healthy, MHC-typed individuals (HLA-DR1/13, HLA-DR4/15, and HLA-DR7/17). Purified CD4+ T cells were stimulated in primary cultures using peptide-pulsed autologous DCs as APCs as described in “Materials and Methods.” After three cycles of peptide restimulation using autologous PBMCs as APCs, the lymphocyte cultures were tested for their capacity to respond to the peptides presented by HLA-DR-transfected L-cells as APCs. Those cultures that exhibited at least a 3-fold increase of proliferative response to peptide were selected, expanded, and cloned by limiting dilution. Three CD4+ T-cell clones were successfully isolated, which proliferated to peptide MAGE-A3_146-160. Two of the T-cell clones recognized peptide MAGE-A3_146-160 in the context of HLA-DR4, and the third one was restricted by the HLA-DR7 allele (Fig. 1A). Furthermore, antibodies specific for HLA-DR, but not antibodies directed to HLA-DQ, inhibited the capacity of the peptides to trigger the proliferative responses of the T-cell clones (Fig. 1B). The anti-HLA-DQ monoclonal antibodies used here (SPV-L3) are potent inhibitors of the response to antigen of an HLA-DQw6-restricted HTL clone specific for the melanoma antigen gp100.4 Although the two HLA-DR4-restricted HTL clones were derived from the same blood donor, it is unlikely that they represent the same clonotype because these were derived from different initial cultures. Several lymphocyte cultures were obtained, which proliferated to peptide MAGE-A3_22-36 presented by L-DR4 cells (data not shown). However, the cells did not expand sufficiently to allow us to conduct further studies. Lastly, neither peptide was able to trigger T-cell responses in the HLA-DR/DR13 individual (results not presented).

To evaluate the avidity of the three T-cell clones for their ligands, peptide titration curves were performed using autologous APCs. These results show that all three T-cell clones displayed high avidity for peptide MAGE-A3_146-160 because <0.1 μg/ml was required to induce 50% of maximal proliferation (Fig. 2). However, it is evident that the HLA-DR7-restricted T-cell clone, 7A39, exhibited higher avidity than the two HLA-DR4-restricted clones, because only ~0.001 μg/ml of peptide was required to trigger 50% of the maximal response.

**Recognition of Tumor Antigen by MAGE-A3_146-160-reactive HTL Clones.** One of the critical attributes that we believe antitumor HTLs must possess is the capacity to recognize naturally processed antigen. Consequently, these HTLs should be able to react with APCs that take up and process the tumor antigen protein, tumor cell lysates, or apoptotic/dead tumor cells. Alternatively, antitumor HTLs may become stimulated by MHC class II+ tumor cells, which in some cases can present peptide epitopes derived from TAAs. Thus, we proceeded to test the capacity of the MAGE-A3_146-160-reactive HTL clones to become activated when cocultured with APCs in the presence of various sources of antigen which required processing. As shown in Fig. 3, the HLA-DR4-restricted HTL clone 8G9 was effective in recognizing purified recombinant MAGE-A3 protein as measured in T-cell proliferation assay (Fig. 3A) or by the capacity of the cells to produce lymphokines, as determined in ELISA (Fig. 3B). Furthermore, clone 8G9 was also effective in recognizing dead/apoptotic MAGE-A3+ melanoma cells when autologous DCs were used as APCs (Fig. 3C). On the other hand, this particular HTL clone was...
MAGE-A3 146–160 peptide antigen (3 μg/ml). B, monoclonal antibodies specific for HLA-DR (aDR) or HLA-DQ (aDQ) were tested at 10 μg/ml for their capacity to inhibit T-cell proliferation to 3 μg/ml of MAGE-A3 146–160 peptide antigen (Ag) in the presence of irradiated autologous APCs. For comparison, proliferative responses were observed in the absence of antibody (■, no Ab). No significant proliferation was observed in the absence of peptide (□). Values represent the means of triplicate determinations; bars, SD.

When the second HLA-DR4-restricted HTL clone (1F3) was examined for its capacity to recognize naturally processed antigen, we observed that in contrast to the results obtained with HTL clone 8G9, these cells did not react with APCs presenting recombinant MAGE-A3 protein or apoptotic/dead melanoma cells (data not shown). Nevertheless, HTL clone 1F3 was able to proliferate to some extent when freeze-thaw lysates from MAGE-A3 + melanoma cells (HT-144, 697mel, and SK-MEL-28) were used as a source of antigen (Fig. 4) but not to a lysate from a MAGE-A3-negative melanoma cell line 888mel. These results indicate that some degree of heterogeneity may exist in T cells recognizing the same epitope, presented by the same MHC molecule. Alternatively, it is possible that HTL clones recognize slightly different regions of peptide MAGE-A3 146–160 and that lysates, recombinant protein, and apoptotic/dead tumor cells may not be identically processed by APCs.

In similar experiments, the HLA-DR7-restricted HTL clone 7A39 was able to recognize recombinant MAGE-A3 protein and cell lysates from MAGE-A3 + tumors presented by autologous APCs (Fig. 5A). Moreover, these HTLs also had the capacity to produce IFN-γ when stimulated directly by MAGE-A3 +, HLA-DR7 + melanoma cells (Fig. 5B). These results indicate that the MAGE-A3 146–160 Peptide epitope may be able to associate with HLA-DR7 molecules on the melanoma cells themselves.

Fine Specificity Analysis of HTL Recognition of the MAGE-A3 146–160 Epitope. The results presented above indicate that both HLA-DR4 and HLA-DR7 alleles are able to present the MAGE-A3 146–160 epitope to HTLs. Nevertheless, it appears that the three HTL clones that we studied may recognize this epitope differently from each other. It is possible that the TCR of these clones may interact with different portions of the HTL epitope. To study the fine specificity of the three HTL clones, we prepared a set of truncated peptides from the MAGE-A3 146–160 epitope. A total of 12 truncated peptides were compared with the intact MAGE-A3 146–160 epitope for their capacity to stimulate proliferative responses of the three HTL clones described above. The data presented in Fig. 6 indicate that the smallest peptide recognized by all three HTL clones was the 11-mer MAGE-A3 150–160 (IFSKASSSLQL). However, it appears that the L at position 160 does not play a major role in stimulating the HTLs because peptide MAGE-A3 146–159 exhibited a near-identical level of activity than peptide MAGE-A3 146–160. Thus, these results suggest that amino acids 150–159 of MAGE-A3 comprise most of the residues necessary for binding to both HLA-DR4 and HLA-DR7 and for contacting the TCRs of the three HTL clones studied here.

DISCUSSION

We believe that effective antitumor immune responses will require the participation of both CTLs and HTLs capable of recognizing TAAs. It is evident that CTLs will play a dominant role in these responses because of their capacity to kill tumor cells expressing MHC class I peptide epitopes derived from TAAs. HTLs are likely to participate in antitumor responses through several mechanisms, such as by enhancing the induction and maintenance of CTL responses and by producing lymphokines that may have a direct effect on tumor cells. We have observed that HTLs are capable of providing strong costimulatory signals to CTLs during their interaction with tumor target cells, resulting in an enhanced survival and proliferation of the CTL. An additional and obvious role of tumor-reactive HTLs is to provide the necessary signals to B lymphocytes for the production of
antitumor antibodies, which may also have a therapeutic effect when these react with cell surface molecules.

Our laboratory has used the “reverse immunology” approach to identify both CTL and HTL epitopes from TAAs with the goal of developing therapeutic antitumor vaccines. The amino acid sequences of known TAAs were examined for the presence of peptides containing MHC class I and class II binding motifs using computer-based algorithms. Synthetic peptides corresponding to the highest ranking, motif-containing sequences were then prepared and tested in vitro for their capacity to induce T-cell responses against the potential peptide epitope. However, before selecting an epitope for vaccine development, it was critical to demonstrate that peptide-generated T cells were also capable of recognizing naturally processed antigens from the tumor cells. Following this approach, we have been successful in identifying several peptide epitopes recognized by tumor-reactive CTLs and HTLs (3, 5, 22, 28).

To produce CTL-based vaccines that can be used in the majority of patients, it is important to identify T-cell epitopes restricted by the most commonly found MHC alleles such as HLA-A1, A2, A3, A24, B7, and B44. In many cases, peptides that bind to these alleles can also bind to closely related members of their respective HLA-supertype families (29). Interestingly, CTL epitopes for MAGE-A3 have been described for the majority of these alleles with the exception of HLA-A3 and HLA-B7 (2–7, 9). Following the same rationale, the identification of HTL epitopes for vaccine development should focus on peptides that can be presented by frequently found MHC class II alleles or preferably on “promiscuous” peptides that have the capacity to bind to several MHC class II alleles (21). Three MAGE-A3 HTL epitopes, which are presented by frequent MHC class II alleles, were described previously (18–20). Here, we report that peptide MAGE-A3146–160 represents a “promiscuous” HTL epitope that can be presented to HTLs by both MHC class II molecules.

Fig. 3. HLA-DR4-restricted HTL clone 8G9 recognizes naturally processed MAGE-A3 antigen. A, proliferative T-cell responses induced by MAGE-A3146–160 (Peptide) recombinant MAGE-A3 protein (rMAGE-A3) or recombinant gp100 (rgp100, included as negative control), all tested at 10 μg/ml using irradiated autologous APCs. B, tissue culture supernatants from the experiment described in A were collected after 48 h, and the concentration of GM-CSF was measured by ELISA. C, T-cell clone 8G9 can recognize UV-irradiated melanoma cells, the express MAGE-A3 (+) via antigen cross-presentation by autologous DCs. In contrast, DCs incubated with the MAGE-A3 negative melanoma cell line 888mel (−) were not able to stimulate the T-cell clone. Autologous DCs were prepared as described in “Materials and Methods” and were incubated with irradiated melanoma cells at a 1:1 ratio for 48 h. The antigen-pulsed DCs were then mixed with HTLs (at a 1:20 ratio), and 2 days later culture, supernatants were collected and assayed for the presence of GM-CSF. Values shown are the means of triplicate determinations; bars, SD.

Fig. 4. HLA-DR4-restricted HTL clone 1F3 recognizes MAGE-A3 antigen in tumor cell lysates. Freeze-thaw lysates (equivalent to 1 × 10^6 cells) of MAGE-A3-positive melanoma cell lines, HT-144, 697mel, and SK-MEL-28 and the MAGE-A3-negative melanoma cell line 888mel were used as the source of antigen to determine the capacity of irradiated autologous APCs (PBMCs) to stimulate HTL clone 1F3. After 72 h in culture, proliferation was measured by the incorporation of [3H]thymidine. Values shown are the means of triplicate determinations; bars, SD.
The capacity of a peptide-reactive HTLs to directly recognize tumor cells or alternatively, APCs that have processed antigens derived from tumor cells, constitutes proof that the epitope represented by the corresponding peptide is naturally processed through the MHC class II pathway. It is obvious that the only way that a HTL will become activated in vivo and subsequently provide “help” to CTLs (and perform other functions) is if its epitope is expressed on MHC class II tumor cells or on APCs that have captured and processed the tumor antigen. In the absence of this evidence, the therapeutic value of a peptide shown to induce T-cell responses in vitro is questionable.

Our results show that the HTL epitopes represented by peptide MAGE-A3146-160 in the context of the HLA-DR4 or DR7 alleles are present on APCs that have processed MAGE-A3 protein or dead tumor cells/lysates (Figs. 3–5). However, major differences in the pattern of antigen recognition by the HTL clones were observed. For example, only the HLA-DR7-restricted HTLs recognized antigen directly on tumor cells, whereas the HLA-DR4-restricted clones did not. Similarly, two of the HTL clones (one HLA-DR4 restricted and the HLA-DR7 restricted) recognized recombinant MAGE-A3 protein presented by APCs, whereas the third one did not. These differences point to the possibility that various sources of antigen (lysates, apoptotic cells, and protein) may be processed in a different manner, generating slightly different variants of peptide MAGE-A3146-160. For example, melanoma cells may preferentially produce surface MHC class II complexes with a peptide similar to MAGE-A3146-157 (FPPVIFSKASSS), which is presented better by DR7 than by DR4 (Fig. 6). However, another possible explanation for these results could be that the TCR of HLA-DR4-restricted HTL clones focuses more on the three COOH-end residues of peptide MAGE-A3146-160 than the HLA-DR7-restricted clone, which tolerated their deletion (Fig. 6). Our results also show that HTL clones 1F3 (HLA-DR4 restricted) and 7A39 (HLA-DR7 restricted) were effective in recognizing tumor cell
lysates, but clone 8G9 (HLA-DR4 restricted) was not. One possible explanation could be whether lysate-pulsed APCs preferentially produced a peptide similar to MAGE-A3 150–160, which is more effectively recognized by HTL clones 1F3 and 7A39 than by clone 8G9 (Fig. 6). Regardless, whether these speculations turn out to be correct or not, it is evident that HTL epitopes can be processed in different manners, depending on the source of antigen and on the type of APC. Furthermore, epitopes on the same antigen may be processed differently by the same APC. For example, it was reported that MAGE-A3 HTL epitopes MAGE-A3 281–295 (HLA-DR11 restricted) and MAGE-A3 247–258 (HLA-DP4 restricted) but not MAGE-A3 114–127 and MAGE-A3 121–134 (both HLA-DR13 restricted) can be recognized directly on MHC class II melanoma cells by HTLs (18–20).

In conclusion, peptide vaccination to elicit antitumor immunity remains one of the most promising and attractive means to treat cancer patients. Because MAGE-A3 CTL epitopes that are restricted by common HLA-alleles (A1, A2, A24, B37, and B44) are available, vaccines that would cover the majority of the patient population could be produced. The recent identification of MHC class II-restricted epitopes should facilitate the development of improved vaccines that potentiate the induction and maintenance of CTL responses via the stimulation of tumor-reactive HTLs. The remaining challenges will be the design and production of multi-epitope vaccines and their unbiased evaluation in the appropriate clinical setting.

REFERENCES

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