Large cutaneous rabbit papillomas that persist during cyclosporin A treatment can regress spontaneously after cessation of immunosuppression

Jiafen Hu,1,2 Xuwen Peng,3 Nancy M. Cladel,1,2 Martin D. Pickel1,2 and Neil D. Christensen1,2,4

The Jake Gittlen Cancer Research Institute1, Department of Pathology2, Department of Comparative Medicine3 and Department of Microbiology and Immunology4, Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

Cottontail rabbit papillomavirus (CRPV)-induced papillomas can progress into malignant carcinomas, remain persistent or regress. Both host immunity and virus genetic background play critical roles in these events. To test how host immunity influences CRPV-induced papilloma evolution, both EIII/JC (inbred) and New Zealand White (outbred) rabbits were treated with an immunosuppressive drug, cyclosporin A (CsA), for 80 days and the regression of three regressive constructs, H.CRPVr (a CRPV regressive strain), H.CRPVp-E6r (a progressive strain with regressive E6) and H.CRPVp-CE6rm (H.CRPVp with the carboxyl terminal of regressive E6, containing mutations at amino acid residues E252G, G258D and S259P) was checked. Papillomas induced by H.CRPVr and H.CRPVp-E6r on control inbred and outbred rabbits regressed totally around week 8, whereas papillomas on all CsA-treated rabbits grew progressively. After cessation of CsA treatment, papillomas began to regress in six outbred rabbits: 14 of 18 papillomas induced by CRPVr, 11 of 18 papillomas induced by H.CRPVp-E6r and eight of 10 papillomas induced by H.CRPVp-CE6rm regressed around week 21. In four CsA-treated inbred rabbits, two of 17 papillomas induced by H.CRPVr and one of 17 papillomas induced by H.CRPVp-E6r regressed. These data indicate that papillomas induced by a regressive CRPV strain can become persistent in the transiently immunosuppressed host. However, returning immunity can lead to regression and clearance of large papillomas (with increased antigenicity) in an outbred population, whilst these same antigenic papillomas persist in inbred rabbits.

INTRODUCTION

Papillomaviruses are small DNA tumour viruses that are correlated strongly with cervical carcinomas in human populations (Walboomers et al., 1999; Furumoto & Irahara, 2002). The cottontail rabbit papillomavirus (CRPV)/rabbit model is used widely to explore the interaction between host and viral infection and to develop vaccines against virus infections (Orth et al., 1980; Brandsma, 1994; Christensen et al., 1999). CRPV-induced skin lesions can regress spontaneously, remain persistent or become malignant. These characteristics resemble both high- and low-risk human papillomavirus (HPV) infections. Previous studies have shown that tumour progression depends on both the host genetic constitution and virus genetic variability (Han et al., 1992; Breitburd et al., 1996; Salmon et al., 1997, 2000; Hu et al., 2002a). Thus, investigations on the effect of the interaction between host immunity and virus genetic variability on tumour evolution are possible with the availability of different virus phenotypes and rabbits with different genetic backgrounds.

Our previous studies have demonstrated that CRPV containing the regressive E6 gene plays a dominant role in controlling the spontaneous regression of CRPV-induced papillomas (Hu et al., 2002a). However, 100 % papilloma regression was not always achieved, even by the regressive-strain infections (Christensen, N. D., Hu, J. & Cladel, N. M., unpublished observations). Incomplete wart regressions on the same host by a particular virus infection may be due to incomplete host immunity. This phenomenon has been well-characterized in vaccination studies (Sundaram et al., 1998; Han et al., 1999; Hu et al., 2002b). Different regression rates or phenotypes shown by the same virus infection in different hosts may result from inherent differences in host immunity (Salmon et al., 2000; Hu et al., 2002a). The link between papilloma regression and progression to the rabbit major histocompatibility complex (MHC) class II
alleles DRA and DQA was reported in a previous study (Han et al., 1992). Additional evidence demonstrated that rabbits homozygous for the DRA.D–DQA.B haplotype were preferentially associated with early regression, whilst those homozygous for the DRA.C–DQA.G haplotype were preferentially linked to wart persistence (Salmon et al., 2000). An association between HLA type and human papillomavirus-induced cervical cancer has been reported (Bavink et al., 1993; Davidson et al., 2003). In addition, malignant carcinomas associated with low-risk HPV types, such as HPV6 and HPV11, have been documented occasionally (Turazza et al., 1997).

Studies using different viral antigens as immunogens have demonstrated that enhanced host immunity increased virion- or viral DNA-induced papilloma regression rates (Furumoto & Irahara, 2002). Other studies have demonstrated an increase of different types of HPV infection in renal-transplant recipients (Tieben et al., 1994) and described the outgrowth of HPV-induced lesions in cyclosporin A (CsA)-treated transplant patients (Euvrard et al., 1981; Ali et al., 1982; Dupont et al., 1985). CsA has been widely used clinically to alleviate tissue allograft rejection (Jenkins et al., 1988).

To determine the impact of genetic differences in host and virus during evolution of papillomavirus-induced tumours in transiently immunosuppressed animals, we used CsA, an immuno-suppressant that has been shown to inhibit lymphokine-induced papilloma evolution, we used CsA, an immuno-suppressant that has been shown to inhibit lymphokine production by helper T cells in vitro and in vivo (Andrus & Lafferty, 1981; Ali et al., 1982; Dupont et al., 1985). CsA has been widely used clinically to alleviate tissue allograft rejection (Jenkins et al., 1988).

**METHODS**

**Preparation of plasmids containing mutant CRPV genomes.** H.CRPVp (Fig. 1a) and H.CRPVr (Fig. 1b) were cloned into pUC19 and utilized as described in a previous study (Hu et al., 2002a). H.CRPVp-Cr6rE252G258D259P was generated by mutagenesis based on the construct H.CRPVp-E6r (Fig. 1c) and was identified as H.CRPVp-Cr6erm (Fig. 1d). Several variants in the carboxy-terminal region of the hybrid CRPV E6 gene (Fig. 1c) were prepared, of which H.CRPVp-Cr6erm was one new construct that was chosen for this study, based on high levels of regression when this E6 mutation was placed into the H.CRPVp genome (Hu, J., unpublished data). All amino acid changes in this carboxy-terminal portion of E6 were designed as back mutations, representing amino acid mutations that were positionally present in the E6 gene from the progressive strain of CRPV (Fig. 1; Salmon et al., 2000; Hu et al., 2002a). The mutations were confirmed by DNA sequencing in the core facility of the College of Medicine, Pennsylvania State University, PA, USA. The constructs were prepared by using a Maxiprep kit (Qiagen), purified by using cesium chloride density-gradient ultracentrifugation and adjusted to a final concentration of 200 μg ml⁻¹ for rabbit skin inoculations.

**CsA treatment of both outbred and inbred rabbits.** Six outbred and four inbred rabbits were injected subcutaneously with CsA for 80 days, the time reported to be sufficient for the suppression of host immunity (Shah et al., 1992). The doses for CsA injection were 15 mg kg⁻¹ (days 1–6) daily, then 20 mg kg⁻¹ (days 7–29).
and 15 mg kg\(^{-1}\) (days 30–80) twice weekly. Rabbits were weighed monthly and weights (kg) were recorded.

**Inoculation of rabbit skin with plasmid viral DNA.** NZW outbred rabbits were purchased from Covance and EII/JC inbred rabbits were bred and maintained in the animal facilities of the Pennsylvania State University College of Medicine. The Institutional Animal Care and Use committee of the Pennsylvania State University, College of Medicine, approved all animal-care and handling procedures. Viral DNA constructs (10 \(\mu\)g per site) were placed onto scarified sites in 50 \(\mu\)l volumes as described previously (Hu et al., 2002a). For inbred rabbits, four left-side sites and four right-side sites were challenged for H.CRPVr and H.CRPVp-\textit{E6r}, respectively. For outbred rabbits, three of six CsA-treated rabbits were challenged at three sites each with H.CRPVr and H.CRPVp-\textit{E6r}, whereas the remaining three rabbits were challenged at four additional sites with H.CRPVp-\textit{CE6rm}.

**Flow-cytometry analysis.** Peripheral blood lymphocytes (PBLs) were isolated from 10 ml blood. In brief, blood was diluted 1:2 with RPMI 1640 medium buffered with 10 mM HEPES and then underlaid with 10 ml Lymphocyte-Rabbit (Cedarlane) and centrifuged at 1500 g at room temperature for 30 min. PBLs were collected at the interface and then diluted 1:2 with RPMI 1640 medium and centrifuged at 1500 g for 10 min. Contaminating red blood cells were lysed with ACK lysis buffer (Biofluids). PBLs were washed three times with RPMI 1640 medium and then counted. 1 \(\times\) 10\(^6\) PBLs were cultured in Eagle’s medium [10% fetal bovine serum (FBS), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 \(\mu\)M 2-mercaptoethanol, 100 U penicillin ml\(^{-1}\) and 100 \(\mu\)g streptomycin ml\(^{-1}\)] for about 3 h to allow time for optimal membrane-protein expression. After washing three times with PBS containing 2% FBS, 1 \(\times\) 10\(^6\) PBLs were suspended in 30 ml PBS and incubated with 1 ml mAbs: mouse anti-rabbit CD4\(^+\) conjugated with fluorescein isothiocyanate (FITC) (RDI-CBL1400FT; Research Diagnostics) and mouse anti-rabbit CD8\(^+\) conjugated with FITC (RDI-CBL1402FT; Research Diagnostics) T-cell antibodies. The populations of cell-membrane markers on PBLs were determined by one-colour FSCAN flow-cytometry analysis (Hershey Medical Center Core Facility).

**Confirmation of viral DNA in papillomas by DNA sequencing.** Biopsies of papillomas were collected monthly from rabbits. Total DNA was extracted by using a Qiagen DNeasy tissue kit. CRPV E6 plus E7 DNA fragments were amplified from each sample and partially purified by using a Qiagen PCR clean kit prior to sequencing. DNA sequencing was performed in the core facility of Pennsylvania State University College of Medicine. DNA alignment was analysed with DNAMAN software (version 5.2.9; Lynnon Biosoft).

**Papilloma size determination and statistical analysis.** Papillomas were measured in three dimensions (length \(\times\) width \(\times\) height) in mm, from which a geometric mean diameter (GMD) was calculated. Measurements were conducted weekly, beginning 3 weeks after initial viral DNA challenge. Data were represented as mean\(\pm\)SEM GMDs for papillomas per construct per group of animals. Statistical significance was determined by unpaired \(t\)-test comparisons. Regression rates occurring from H.CRPVr, H.CRPVp-\textit{E6r} and H.CRPVp-\textit{CE6rm} genomes on animals treated with CsA were compared with regression rates of papillomas from untreated animals by using Fisher’s exact probability test for small samples.

### RESULTS

**Weight gain of rabbits treated with CsA**

CsA is an immunosuppressant that is used in clinical organ transplantation. Some side effects have been noticed for this drug. To determine whether similar side effects occurred in CsA-treated rabbits, monthly records of body weight gain of these rabbits were obtained. Weight gain of CsA-treated rabbits was significantly slower than that of the control rabbits (\(P<0.03\), unpaired \(t\)-test; data not shown). After termination of CsA treatment, rabbits began to gain weight. No difference in mean body weight could be found between these two groups 5 months later.

**CsA decreased CD4\(^+\) and CD8\(^+\) T-cell levels in PBLs**

CD4\(^+\) and CD8\(^+\) T cells are important for host defence against viral infections. The levels of CD4\(^+\) and CD8\(^+\) T cells in PBLs reflect levels of host immune response to pathogen invasion. CsA had been demonstrated to delay the maturation of thymus T cells. In our current study, significantly lower levels of CD4\(^+\) and CD8\(^+\) T cells were found in CsA-treated rabbits than in control rabbits (Fig. 2; \(P<0.05\), \(t\)-test) 14 and 80 days after CsA injection. However, the levels of CD4\(^+\) and CD8\(^+\) T cells in CsA-treated rabbits returned to normal 2 months after CsA treatment was terminated (data not shown).

**Papilloma outgrowth in CsA-treated and non-treated outbred rabbits**

In experiment 1, three outbred rabbits were treated with CsA and challenged with H.CRPVr and H.CRPVp-\textit{E6r}. Two rabbits that were challenged with H.CRPVr and H.CRPVp-\textit{E6r}, but that received no CsA, were used as controls.
controls. Papillomas appeared at the same time point (around week 3 after challenge) on both CsA-treated and control rabbits. In the control group, papillomas induced by both H.CRPVr and H.CRPVp-E6r began to regress and disappeared at all challenge sites around week 10. In contrast, papillomas on CsA-treated rabbits continued to grow for the duration of CsA treatment. Stronger immune responses to H.CRPVr-induced papillomas versus H.CRPVp-E6r-induced papillomas were evident, based on papilloma size and regression rates. Papillomas induced by H.CRPVr were significantly smaller than those induced by H.CRPVp-E6r (Figs 3 and 4; \( P < 0.05 \), t-test). Papillomas induced by H.CRPVr at six sites on two rabbits regressed around week 16 and six papillomas induced by H.CRPVp-E6r on two rabbits regressed around week 21 (Fig. 5). One rabbit failed to eradicate any papillomas, although the mean size was significantly reduced when compared with papilloma size at the time of CsA treatment (Fig. 3; Table 1; \( P < 0.05 \), t-test).

In experiment 2, three additional outbred rabbits were treated with CsA using the same protocol. Similar patterns of growth and regression were found for papillomas induced by H.CRPVr and H.CRPVp-E6r (Fig. 3); however, all papillomas induced by H.CRPVr were able to regress and five of nine papillomas induced by H.CRPVp-E6r regressed at week 21. In this experiment, the CsA-treated rabbits were challenged with a third regressive construct (H.CRPVp-C6erm). Papillomas induced with this third construct did not show as vigorous a regression rate when compared with H.CRPVr and H.CRPVp-E6r (Table 1). However, after cessation of CsA treatment, papillomas induced by H.CRPVp-C6erm began to shrink and they disappeared around week 21. No significant difference in

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**Fig. 3.** Evolution of papillomas induced by H.CRPVr (black bars) and H.CRPVp-E6r (grey bars) in CsA-treated outbred rabbits. CsA treatment was terminated after 80 days. Six rabbits in two experiments were challenged with both H.CRPVr and H.CRPVp-E6r at left- and right-back sites, respectively. *\( P < 0.05 \) compared with H.CRPVr, unpaired t-test.

**Fig. 4.** Papilloma growth rates in outbred rabbits. Papillomas induced by both H.CRPVr and H.CRPVp-E6r appeared around week 3 in both the control and CsA-treated groups. After week 6, papillomas in the control group began to regress and they disappeared around week 8–10; papillomas in the CsA-treated rabbits, however, continued to grow and reached a peak around week 9. After week 9 (the time of cessation of CsA treatment), papillomas began to shrink and most papillomas regressed completely.
papilloma regression rate was found between H.CRPVp-CE6rm (eight of 10) and H.CRPVr (eight of nine) or H.CRPVp-E6r (five of nine) at week 21 (Table 1; \( P > 0.05 \), Fisher’s exact test). No significant difference in regression rate of papillomas induced by each construct was found between the CsA-treated group and control group (Table 1; \( P > 0.05 \), Fisher’s exact test).

**Papilloma outgrowth in CsA-treated and non-treated EIII/JC inbred rabbits**

To explore whether immunosuppression delayed regression of papillomas induced by H.CRPVr and H.CRPVp-E6r in EIII/JC inbred rabbits, eight rabbits were tested using the same protocol as described for the outbred rabbits. Four CsA-treated (17 challenge sites for each construct) and four control (16 challenge sites for each construct) rabbits were challenged with both H.CRPVr and H.CRPVp-E6r.

In the control group, papillomas induced by both H.CRPVr and H.CRPVp-E6r appeared around week 3 on all rabbits and began to regress and disappeared at all challenge sites around week 10. In contrast, papillomas on CsA-treated rabbits appeared at the same time and continued to grow. Although the growth rate was reduced after termination of CsA treatment, papillomas remained for several weeks. A significant difference was found for papilloma regression rates between CsA-treated rabbits and control rabbits induced by both constructs (Table 1; \( P < 0.05 \), Fisher’s exact test).

Papillomas induced by H.CRPVr were significantly smaller than those induced by H.CRPVp-E6r (Figs 6 and 7; \( P < 0.05 \), t-test) at most time points. One of the CsA-treated rabbits (C0364) developed significantly smaller papillomas compared with the others. At week 21 for rabbit C0364, two papillomas induced by H.CRPVr and one...
induced by H.CRPVp-E6r regressed and the remaining ones were very small in size. However, papillomas on the remaining three rabbits continued to grow and were comparable in size to those that were formed during CsA treatment (Fig. 6).

When compared with the CsA-treated outbred rabbits, significantly lower papilloma regression rates were found for papillomas induced by both H.CRPVr and H.CRPVp-E6r \((P = 0.015\) and \(0.017\), respectively; Table 1).

### Table 1. Papilloma outcome following CsA treatment and infection with various CRPV genomes (NZW outbred and EIII/JC inbred rabbits)

Regression rate was calculated as regression sites/papilloma sites.

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>CsA*</th>
<th>Regression rates (week 10)</th>
<th>Regression rates (week 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H.CRPVr</td>
<td>H.CRPVp-E6r</td>
<td>H.CRPVp-CE6rm</td>
</tr>
<tr>
<td>NZW outbred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 ((n=3))</td>
<td>+</td>
<td>5/9 (56%)</td>
<td>2/9 (22%)</td>
</tr>
<tr>
<td>Group 2 ((n=3))</td>
<td>+</td>
<td>0/9† (0%)</td>
<td>0/9† (0%)</td>
</tr>
<tr>
<td>Groups 1 and 2 ((n=6))</td>
<td>+</td>
<td>5/18 (28%)</td>
<td>2/18∥ (11%)</td>
</tr>
<tr>
<td>Group 3 ((n=2))</td>
<td>–</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>Group 4 ((n=3))</td>
<td>–</td>
<td>5/5 (100%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>EIII/JC inbred</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5 ((n=4))</td>
<td>+</td>
<td>0/17** (0%)</td>
<td>0/17** (0%)</td>
</tr>
<tr>
<td>Group 6 ((n=4))</td>
<td>–</td>
<td>16/16 (100%)</td>
<td>16/16 (100%)</td>
</tr>
</tbody>
</table>

*CsA treatment ended 80 days after initial administration.
†\(P=0.019\) vs group 3.
‡\(P=0.033\) vs group 4.
§\(P=0.025\) vs week 21.
∥\(P=0.030\) vs group 3.
¶\(P=0.015\) vs group 5.
#\(P=0.017\) vs group 5.
**\(P<0.01\) vs group 6.

**Viral DNA stability in papillomas induced by different viral constructs**

Papillomas induced by a regressive viral strain that becomes persistent might be a consequence of mutational changes in the viral genome, with selection of antigenic-escape variants. To determine whether any mutations had occurred in the persistent papillomas, we collected a biopsy of each papilloma from each CsA-treated rabbit (all the papillomas on three outbred and four inbred rabbits) weekly until week 10 and monthly from that time point on. The genetic sequence that we checked was E6 plus E7, which is the region that allows us to discriminate between the constructs that we used (H.CRPVr contains regressive E6 and E7, whereas H.CRPVp-E6r contains regressive E6 and progressive E7). All papillomas tested retained the original DNA sequences for the challenge constructs.

**DISCUSSION**

Our current study investigated the impact of transient immunosuppression by CsA treatment on infection by three CRPV regressive strains in both EIII/JC inbred and outbred rabbits. As expected, papillomas induced by both H.CRPVr and H.CRPVp-E6r became persistent in both inbred and outbred rabbits that were treated with CsA and regressed completely in non-treated control rabbits. Interestingly, after cessation of CsA treatment, five of the six CsA-treated outbred rabbits were able to completely or partially eliminate papillomas induced by both H.CRPVr and H.CRPVp-E6r, whereas papillomas on the remaining three inbred rabbits remained persistent. The most striking result is that, after termination of CsA treatment, returning host immunity elicited by infection of both constructs could eradicate not only small, but also large, tumours in outbred rabbits. For example, two CsA-treated outbred rabbits (C0078 and C0079) eliminated large tumours up to a GMD of 27 mm (Figs 3 and 5). This result is striking, as many immunotherapeutic studies have demonstrated that immune-mediated cure of tumours is difficult once they reach a large size. In support of this latter observation is the outcome of large papillomas in the inbred rabbits, which persisted after cessation of CsA treatment, despite papillomas that are induced by these...
CRPV genomes being strongly regressive upon initial infection and thus when small in size.

We previously demonstrated that regressive E6 played a dominant role in triggering papilloma regression in both inbred and outbred rabbits (Hu et al., 2002a). The current study further supports this finding. The two regressive constructs that were tested in this study (H.CRPVr and H.CRPVp-E6r) did not show any differences in tumor outgrowth and regression in either inbred or outbred rabbits with intact host immunity. This implied that only the regressive E6 was strong enough to induce immune responses leading to the elimination of papillomas in these rabbits. However, in CsA-treated (immunocompromised) animals, remaining host immunity against viral DNA infection generated by H.CRPVr was much stronger than that induced by H.CRPVp-E6r, as manifested in earlier regressions and smaller papillomas (Table 1, Figs 4 and 7). Therefore, other genes or regions in the H.CRPVr genome must have played an additional role in triggering regression in these rabbits.

CsA has been used clinically during transplantation. CsA is able to inhibit keratinocyte cytokine-gene expression and T-cell activation (Andrus & Lafferty, 1981; Won et al., 1994). We found that the levels of CD4+ and CD8+ T cells in PBLs decreased in rabbits during the 80 days of CsA treatment. However, these levels returned to normal by 2 months after the cessation of CsA treatment (data not shown). Some side effects that correlated with CsA treatment were recorded clinically. In our study, we also noticed that rabbits injected with CsA had a lower intake of food and less weight gain when compared with the controls. After termination of CsA treatment, no difference in weight gain could be found between CsA-treated and non-treated rabbits. CsA treatment effectively suppressed host immunity in both inbred and outbred rabbits. Collectively, because of the immunosuppressive effect of CsA administration, both inbred and outbred rabbits showed weaker immune responses to both H.CRPVr and H.CRPVp-E6r infection than did control rabbits. However, after CsA treatment was ended, both inbred and outbred rabbits regained partial immunity, leading to reduction in papilloma size. Although regression of H.CRPVr-
H.CRPVp-E6r-induced papillomas was delayed or prevented by CsA treatment, five of six outbred rabbits showed gradual recovery from this immunosuppressive effect and were subsequently able to eliminate both H.CRPVr- and H.CRPVp-E6r-induced papillomas. This finding implied that, once host immunity was activated appropriately, effective cure of large lesions was possible. In contrast, inbred rabbits showed much slower antipapilloma effects following recovery from CsA treatment. Only one of four rabbits achieved partial regression several weeks after cessation of CsA treatment. Papillomas on the other three inbred rabbits became slightly smaller, but then stabilized and persisted until the experiment was terminated. As discussed previously, we found that the MHC class II genetic constitution of the inbred rabbits was not commonly found in outbred populations. In this study, both inbred and outbred rabbits showed the same growth pattern to infection with both H.CRPVr and H.CRPVp-E6r. However, when host immunity was suppressed temporarily by CsA, the two strains of rabbits showed different responses to these regressive strains, by either a delay or a change in their regressive phenotypes. In addition, individual differences were noticed in the same strain of the animals. For example, the outcome of papillomas on outbred rabbit C0080 was quite different from those of the other outbred rabbits, but similar to that of inbred rabbit C0364. These data imply that there were differences between these two strains of rabbits and between individuals from the same strain following recovery from transient immunosuppression. These observations are relevant to patients undergoing different immunosuppressive regimes and the outcome of HPV infections following removal of immunosuppression, despite the relative antigenicity of the HPV infection.

In summary, short-term suppression of host immunity by CsA delayed papilloma regression in NWZ rabbits and changed the regression phenotype of regressive papillomavirus strains in EIII/JC inbred rabbits.

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REFERENCES


